General Laboratory Procedures and Practices: *Deepwater Horizon* Laboratory Toxicity Testing

Prepared for:

U.S. Department of Commerce
National Oceanic and Atmospheric Administration

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Contents

Chapter 3 Appendices

Chapter 4 Appendices

Chapter 5 Appendices

Chapter 7 Appendices

Chapter 9 Appendices

Chapter 13 Appendices

Chapter 15 Appendices

Chapter 16 Appendices

0. Overview

This *General Laboratory Procedures and Practices: Deepwater Horizon Laboratory Toxicity Testing*[1] document contains a collection of standard laboratory protocols, procedures, and practices used, under the guidance of Stratus Consulting (now Abt Associates),[2] by institutions conducting toxicity testing in support of the *Deepwater Horizon* (DWH) Natural Resource Damage Assessment (NRDA). The principal investigators (PIs) from each institution and their corresponding team members are listed in Table 0.1. Each chapter describes the protocols and procedures for a specific institution or institutions. With the exception of Pacific EcoRisk (Chapter 10) and the Exposure Characterization and Chemistry (ECC) test chapter (Chapter 17), the PI from each associated institution wrote the protocols and procedures, in close collaboration with Status Consulting. Stratus Consulting created the Pacific EcoRisk chapter based on Pacific EcoRisk reports. The ECC chapter provides protocols for tests that were conducted by numerous institutions to characterize the chemistry or exposure conditions of different bioassays. To develop the ECC chapter, Stratus Consulting used either data summary reports from the appropriate toxicity testing laboratory or the corresponding toxicity testing protocols listed in the appropriate laboratory's General Laboratory Procedures and Practices (GLPP) chapter.

All of the details provided in this overview and the accompanying documents pertain to definitive tests. In most cases, testing protocols and test conditions tables (TCTs) were developed before the initiation of any test and were approved by Stratus Consulting. Following the completion of a test, minor modifications may have been made to the testing protocol to accurately reflect actual testing procedures. This document contains the final/modified versions of protocols. Additional information regarding each test can be found in the DWH Trustee toxicity database generated in support of the DWH NRDA available through the National Oceanic and Atmospheric Administration (NOAA) Data Integration, Visualization, Exploration, and Reporting (DIVER) data repository (DIVER, 2015). Any minor in-test modifications to the test conditions (e.g., test duration, replicate numbers) are reflected in the definitive test conditions reported for each test. Below is a brief description of how each chapter is organized, as well as information regarding supporting documents.

1. The recommended citation for this document is: Krasnec, M.O., H.P. Forth, M.W. Carney, R. Takeshita, A.K. McFadden, I. Lipton, B. Wallace, K. Dean, C.R. Lay, D. Cacela, J.V. Holmes, J. Lipton, and J.M. Morris. 2016. General Laboratory Procedures and Practices: *Deepwater Horizon* Laboratory Toxicity Testing. DWH NRDA Toxicity Technical Working Group. Prepared for National Oceanic and Atmospheric Administration by Abt Associates, Boulder, CO.

2. Stratus Consulting merged with Abt Associates in 2015; since much of this work was conducted by Stratus Consulting prior to the merger, the names of both firms appear in this document.

- **Chapter**
  - **GLPP (main chapter text)**

    The main text in each chapter provides information regarding institution-specific GLPP. It also includes general standard operating procedures (SOPs) that were followed in day-to-day testing activity for most tests (e.g., measuring basic water quality parameters).

  - **Testing protocols (chapter appendices)**

    The appendices provide specific protocols for the various types of toxicity tests conducted at each institution.

- **Supporting documents**
  - **Quality Assurance Project Plan (QAPP): DWH Laboratory Toxicity Testing (Carney et al., 2016)**

    Stratus Consulting prepared this document to outline the procedures used to ensure that data were collected and analyzed to meet project requirements; the document also includes some project-wide SOPs, such as the preparation of water accommodated fractions (WAFs).

  - **TCTs**

    Each test that was conducted is accompanied by a test-specific TCT that describes the test conditions (e.g., species, life stage, contaminant tested, WAF preparation method, nominal exposure concentrations, test duration, temperature). Any modifications to the GLPP or testing protocols were captured in the test-specific TCT. TCTs will be available through the NOAA DIVER data repository (DIVER, 2015).

When stated, samples were sent to ALS Environmental, formerly Columbia Analytical Services (CAS). Water sampling series or schedules listed in a specific testing protocol may have been modified from test to test because of specific testing conditions or earlier test results.

Each chapter contains institution-specific information regarding toxicity testing and is split into two major sections. The first section contains the GLPP from a specific institution. The second section contains appendices that comprise laboratory-specific testing protocols for definitive tests. Stratus Consulting does not endorse or recommend any commercial products cited in these documents. The stated use of any commercial products by authors of this report may not be used for advertising or product endorsement purposes. All trademark and copyright symbols have been removed from this document to avoid inconsistent and/or inaccurate use.

Table 0.1. NRDA toxicity testing program PIs and their corresponding team members

Toxicity testing laboratory	Laboratory location	PI	PI team/contributors	
Auburn University	Auburn, AL	James Stoeckel	Adam Kelly Catlin Ames Ginger Stuckey	Ian Palmer Michael Hart
Florida Gulf Coast University and University of North Carolina, Wilmington	Fort Myers, FL and Wilmington, NC	Aswani Volety	Ai Ning Loh Andy Griffith Anne Rolton Ashley Demey Ben Woodall Brooke Denkert Cecile Jauzein Chelsea Miley Christophe Lambert David Segal Emily Nickols Fu-Lin Chu Hunter Cox Ian Campbell Jeff Devine John Roberts Josh Forbes	Julie Neurohr Julien Guyomarch Julien Vignier Kathleen McNatt Kelsey McEachern Kyle Chenevert Leslie Haynes Lindsay Castret Ludovic Donaghy Michael Parsons Molly Rybovich Myrina Boulais Nelly Legoic Nicole Martin Philippe Soudant Rheannon Ketover Thomas Dolan
Louisiana State University	Baton Rouge, LA	Fernando Galvez	Benjamin Dubansky Kali Holder Catherine Simoneaux Brittney Keosayasing Courtney Poulos Eben Smitherman	Gabi Borel Gregory Long Florence Louann Johnson Charles Brown Sydney Hebert Tiffany Simms Lindsey
Louisiana Universities Marine Consortium	Chauvin, LA	Edward Chesney	Evan Kwityn Kathryn O'Shaughnessy Sam Leberg	Sarah Webb Tara Duffy Taylor Alexander William Childress
Marin Biologic Laboratories, Inc.	Novato, CA	Peter Ralph	Erin Accurso	Tania Weiss
Miami University of Ohio	Oxford, OH	James Oris	Andrew Tucker Dale Coffey Marlo Jeffries Leah Thornton	Alison Willis Graham Hughes Nora Covy Lucas Smith

Table 0.1. NRDA toxicity testing program PIs and their corresponding team members (cont.)

Toxicity testing laboratory	Laboratory location	PI	PI team/contributors	
Mote Marine Laboratory	Sarasota, FL	Dana Wetzel	Carlos Yanes-Roca Erin Pulster Kevan Main Kylee Bowling Matthew Resley Michael Nystrom Mike Henry Nathan Brennan	Nicole Rhody Patricia Blum Paula Puiggerver-Caldentrey Rebecca Medvecky Richard Pierce Samantha Harlow
Northwest Fisheries Science Center: NOAA	Seattle, WA	Nathaniel Scholz John Incardona	Allisan Aquilana-Beck Barbara French Bernadita Anulacion Catherine Sloan Cathy Laetz Darlye Boyd David Baldwin Gina Ylitalo Heather Day	James Cameron Jana Labenia Jennie Bolton Mark Tagal Nathaniel Scholz Richard Edmunds Ron Pearce Tanya Brown Tiffany Linbo Tony Gill
Pacific EcoRisk	Fairfield, CA	Scott Ogle	Drew Gantner Krista Prosser	Padrick Anderson
Hopkins Marine Station of Stanford University	Stanford, CA	Barbara Block	Ben Machado Fabien Brette John Dale	Luke Gardner Robert Schallert
Stratus Consulting/Abt Associates[a]	Boulder, CO	Jeffrey Morris	Andrew McFadden Anthony Berenguel Chad Mansfield Claire Lay David Cacela Fiona Garvin Heather Forth Ian Lipton James Holmes Jeffrey Cegan Joshua Lipton Karen Dean	Liza Hernandez (NOAA) Mary Huisenga Michael Carney Michael Duckworth Michel Gielazyn (NOAA) Michelle Krasnec Robert Vega (TPWD) Ronald Hall Ryan Takeshita Shane Bonnot (TPWD) David Abrego (TPWD)
University of Maryland	Solomons, MD	Carys Mitchelmore	Christopher Rowe Eric Schott Gregory Danvers Hannah Pie Jum Sook Chung	Maureen Strauss Nicole Chigounis Sarah Funck Steve Suttles

Table 0.1. NRDA toxicity testing program PIs and their corresponding team members (cont.)

Toxicity testing laboratory	Laboratory location	PI	PI team/contributors	
University of Miami Rosenstiel School of Marine and Atmospheric Science	Miami, FL	Martin Grosell Daniel Benetti	Andre Faul Andrew Esbaugh Charlotte Bodinier Daniel Benetti Danielle McDonald Edward Mager Jennifer Panlilio Jessica Wingar	John Stieglitz Kathleen Munley Kevin Brix Maria Rodgers Ron Hoenig Sasa Miralao Theresa Mackey Zack Daugherty
University of North Texas	Denton, TX	Aaron Roberts	Alexis Wormington Brian Matherne Brianne Soulen Carmen Overturf Celeste Ortega-Rodriguez Charles Mansfield Erin Ussery James Smith Jason Magnuson	Jennifer Gnau Jessica Trevino Kristin Nielsen-Bridges Lauren Sweet Leigh Taylor Matt Alloy Morgan VanAken Thomas (Ty) Curran Thomas Garner
University of South Florida	Tampa, FL	Anna Pyayt	Surya Cheemalapati Hao Wang	Karthik Raj Konnaiyan
University of Southern Mississippi	Ocean Springs, MS	Robert (Joe) Griffitt	Arthur Karels Beth Jones Binnaz Bailey Bryan Hedgpeth Carly Somerset Danielle Simning Idrissa Boube Jay Grimes Jean Jovonovich	Jenish Kumar Jeremy Johnson Jeremy Lindsey Jessica Holland Kim Griffit Lyndsay Carrigee Nancy Brown-Peterson Natalie Ortell (Cumbaa) Ryan Gordon
U.S. Army Corps of Engineers Engineer Research and Development Center	Vicksburg, MS	Guilherme Lotufo	James Biedenbach Ashly Harmon Daniel Farrar Jacob Stanley	James Lindsay Jamma Williams Jen Chappel Jennifer Laird

a. Some individuals from Texas Parks and Wildlife Department (TPWD) and NOAA contributed to the testing conducted by Stratus Consulting/Abt Associates.

References

DIVER. 2015. Data Integration, Visualization, Exploration and Reporting Application. Web Application. *Deepwater Horizon* Natural Resource Assessment Data. National Oceanic and Atmospheric Administration. Available: https://dwhdiver.orr.noaa.gov/.

Carney, M.W., H.P. Forth, M.O. Krasnec, R. Takeshita, J.V. Holmes, and J.M. Morris. 2016. Quality Assurance Project Plan: *Deepwater Horizon* Laboratory Toxicity Testing. DWH NRDA Toxicity Technical Working Group. Prepared for National Oceanic and Atmospheric Administration by Abt Associates, Boulder, CO.

1. Auburn University General Laboratory Procedures and Practices

1.1 Test Organism Collections and Husbandry

1.1.1 Artificial seawater

Artificial seawater (ASW) was made by bringing reverse osmosis, deionized (RO/DI) water up to the appropriate salinity with Instant Ocean synthetic sea salt. RO/DI water was obtained by running city water through an AquaFX water treatment system purchased from Aquatic Ecosystems and housed in a secure laboratory setting.

1.1.2 Fiddler crab: *Uca minax*

U. minax were obtained from Weeks Bay, Alabama. Male and female crabs were transported back to Auburn University (Auburn) in two 40-gal coolers that each contained 5 gal of seawater from the collection site, as well as bioballs to decrease water sloshing and provide substrate to minimize antagonistic interactions between crabs. In the laboratory, males and females were held in separate coolers in the dark, with aeration, at room temperature (~ 24°C). Bioballs were left in the coolers to serve as biofilters and to minimize aggressive interactions between crabs. The water was changed twice weekly with 12 ppt ASW, which matched the salinity of the water at the crab collection site. Crabs were fed commercial shrimp pellets *ad libitum* three times weekly.

1.1.3 Fiddler crab: *Uca longisignalis*

U. longisignalis were collected at Point Aux Pines, Alabama [Alabama Department of Conservation and Natural Resources (ADCNR) Wildlife Management Area]. General culture procedures were the same as for *U. minax*, except that crabs were held outdoors in a secure area and exposed to ambient light and temperature regimes. Also, in addition to shrimp pellets, clumps of sediment from the collection site were added to the holding tanks to serve as an additional source of food. Water was changed three times per week with ~ 15 ppt ASW, which matched the salinity of the water at the crab collection site.

1.1.4 Marsh periwinkle: *Littoraria irrorata*

Marsh periwinkle snails (*L. irrorata*, ~ 560 individuals) were collected from the Alabama Coastline on Dauphin Island, Alabama, near Mobile Bay (~ 30°25’16.91”N, 88°08’27.46”W).

Simultaneously, *Spartina alterniflora* shoots were collected from the Alabama coastline southwest of Bayou La Batre near Sandy Bay (~ 30°22'48.93"N, 88°18'45.65"W) for use in snail toxicity studies. Snails and *Spartina* were transported from the collection site to the laboratory in a 5-gal cooler containing a small amount of seawater included for maintaining humidity levels. Upon arriving at the laboratory, and before the start of the experiment, the snails were placed in a 5-gal plastic bucket containing shoots of *Spartina* and ~ 15 ppt ASW with aeration. The *Spartina* shoots served as habitat and a food source for the snails.

1.2 Toxicity Test Exposure Media Preparations

Protocols for test solution preparations are found in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. The types of toxicity testing solutions follow.

1.2.1 Oil emulsions

Oil emulsions were prepared according to the procedure for preparing high-energy water accommodated fractions (HEWAFs), except that after blending, the resultant emulsion was used in its entirety rather than transferred to a separatory funnel for a 1-hour separation of layers. Oil emulsions were prepared using Slick A (CTC02404-02) oil obtained from Stratus Consulting.[1]

1.2.2 Sediments

Sediments were collected and oiled at appropriate levels according to more detailed procedures described in individual test protocols.

1.2.3 Seawater

All tests used ASW prepared with Instant Ocean and RO/DI water as described in the Fiddler Crab Culturing section.

1. Stratus Consulting merged with Abt Associates in 2015; since much of this work was conducted by Stratus Consulting prior to the merger, both firm names will appear.

1.2.4 Water accommodated fraction exposure media

Water exposures were conducted using HEWAFs prepared as outlined in *Protocols for Preparing Water Accommodated Fractions* found in the QAPP. Water accommodated fractions (WAFs) were prepared with ASW and Slick A (CTC02404-02) oil.

1.3 General Testing Standard Operating Procedures

1.3.1 Standard operating procedure: Water quality monitoring

Water quality measurements [temperature, pH, dissolved oxygen (DO), salinity, ammonia, alkalinity, and hardness] were taken from water sources and specific frequencies as described in specific test protocols.

Standard operating procedures

Meters were bench calibrated or their accuracy verified against standards or alternate methodologies prior to use. Bench calibration (i.e., verification) records specific to the National Oceanic and Atmospheric Administration (NOAA) *Deepwater Horizon* projects were maintained in a laboratory file specific to instrument type and traceable to individual units by serial number. Calibrations and calibration verifications were performed prior to sampling; calibration or verification protocols were based on the manufacturer's specifications and/or standard methods. For measurements using probes in test chambers or sumps, measurements began in the lowest concentration, and then proceeded to the next higher concentration until the highest concentration chamber was measured. Probes were rinsed with RO/DI water between measurements to reduce the risk of cross-contamination.

pH: HANNA HI 9813-6 meter

Record meter and probe serial numbers.

1. Turn the meter on.

2. Calibrate the meter prior to each sampling event.

3. Immerse the probe in the calibration solution. Use pH 7.01 calibration solution if you expect the sample to be near neutral, pH 4.01 if you are going to measure acidic samples, or pH 10.01 if you expect the samples to be alkaline (basic).

4. Press °C and record the calibration solution temperature.

5. Adjust the pH knob to display the solution value at the measured temperature (refer to table “pH vs. temperature” on the solution packet).

6. Take sample measurements.

7. Submerge the probe in the sample to be tested.

8. Push the pH button.

9. Record the pH measurement once it stabilizes.

DO: YSI ProODO

1. Record meter and probe serial numbers.

2. Calibrate the meter at the beginning of each day of use. Calibration will hold for the remainder of the day, but adjust salinity compensation if needed.

3. Moisten (do not saturate) the sponge in the calibration/transport sleeve with a small amount of clean water.

4. Turn the meter on and wait 5 minutes for the calibration/transport sleeve to become fully saturated.

5. Press the Calibration button, highlight DO, and press Enter.

6. Once DO and temperature readings are stable (wait at least 30 seconds), highlight Accept Calibration, and press Enter. The screen will indicate if the calibration has been accepted.

7. If taking measurements in saltwater, enter the salinity value of the water to be tested in the Probe menu. This will allow for the proper salinity compensation of the mg/L DO values.

8. Take sample measurements.

9. Turn the meter on.

10. Insert the probe into the sample. Give an initial “shake” of the probe to release any air bubbles. Note that continuous movement/stirring is not required.

11. Wait 25−30 seconds for the readings to stabilize.

12. Record the DO value.

ProOBOD probes when used with a ProODO meter have built-in temperature sensors. Temperature calibration is neither required nor available. Record temperature directly from the meter display as you read the DO measurement.

Ammonia: Tetra Easy Strips and YSI 9300 photometer

1. Calibration is not applicable for the Tetra Easy Strips. The YSI 9300 photometer has its own internal calibration curve that is not adjustable by the user.

2. Remove one test strip from the bottle and replace and tighten the cap.

3. Hold the strip at the end and dip it into the sampled water collected from the appropriate chamber, tank, or system, swishing the water back and forth for 10 seconds.

4. Remove and shake the excess water from the strip.

5. Compare the strip immediately to the color chart for saltwater.

Calcium hardness and alkalinity: YSI 9300 photometer

The YSI 9300 photometer has its own internal calibration curve that is not adjustable by the user.

General instructions

1. Do not pour out the samples or prepare the tests directly over the instrument.

2. Cap the test tubes after preparing the blank and test samples.

3. Wipe the test tubes with a clean Kimwipe and RO/DI water to remove dirt and residue, and dry with a clean Kimwipe to remove excess water, drips, and/or condensation immediately before placing in the photometer.

4. Remove tubes from the photometer immediately after each test.

5. Immediately wipe up any drips or spills on the instrument or in the test chamber with a clean tissue.

6. Clean the test chamber regularly using a Kimwipe or cotton ball moistened with RO/DI water.

7. Ensure that the carrying case is dry before the case is closed and the instrument is stored.

Calcium and alkalinity

1. See the manual for specific test instructions.

2. Follow the specific instructions for saltwater – reagents and protocols may vary between freshwater and saltwater.

3. Record the appropriate units.

Salinity: Pinpoint salinity meter or YSI Y30 SCT meter

Pinpoint salinity meter

1. Record the salinity meter serial number.

2. Calibrate the salinity meter before each sampling event.

3. Empty 53.0 mS standard calibration fluid into a small, clean cup and swirl the probe to dislodge air bubbles.

4. Wait 1–2 minutes for the proper temperature compensation.

5. Use a screwdriver to turn screw #3 (inside the battery cover) until the display reads 53.0 mS ± 0.5.

6. Do not turn screw #4 (also inside the battery cover).

Take sample measurement

1. Calibrate pinpoint salinity meter as described above or calibrate the YSI Y30 SCT meter according to manufactures directions.

2. Place the probe into the sample and swirl to dislodge air bubbles.

3. Record the salinity in mS and ppt units of measure, taking care to use the appropriate units (you will need to use the chart to convert salinity units of measure from mS to ppt).

4. Do not get the salinity meter wet as it is not waterproof.

1.3.2 Standard operating procedure: Analytical chemistry

The final confirmatory analytical chemistry was conducted offsite at ALS Environmental, as described in the QAPP. Offsite analytical testing was used to characterize oil emulsions, exposure sediments, and natural sediments. Samples were shipped to the offsite laboratory under strict chain-of-custody procedures in laboratory-provided sample containers and shipping coolers, as described in the QAPP.

A. Testing Protocol 1: Assessing the Effects of Maternal Oiled Sediment Exposure on Fiddler Crab Larval Production and Survival

A.1 Crab Collection and Maintenance

Fiddler crabs (*Uca longisignalis*) were collected from the Alabama coastline near Sandy Bay (~ 30°22'46.11"N, 88°18'23.27"W) and transported to Auburn, Alabama. Male and female crabs were housed outdoors in separate 30-gal coolers containing aerated ASW (~ 15 ppt; same salinity as the collection site) and bioballs prior to initiation of the experiments. Bioballs served as a biofilter, as well as a structure to reduce antagonistic interactions between crabs.

A.2 Sediment Collection

Sediments were collected from the same location as the fiddler crabs on the Alabama coastline near Sandy Bay (~ 30°22'46.11"N, 88°18'23.27"W).

A.3 Maternal Exposure Experimental Design

The maternal exposure consisted of 20 individual chambers (4 treatments plus a control, each with 4 replicates). Each chamber contained 5 male and 5 female crabs. The individual chambers were 18 in. in diameter and contained approximately 8 gal of non-oiled sediments obtained from the crab collection site. The sediment was sloped from 5 to 8 in. deep. Each chamber was housed inside a larger container that was connected to its own sump pump and water supply. ASW (~ 15 ppt to match the crab collection site) pumped between the sump and the outer container simulated low and high tides that were set to mimic ambient cycles near the collection site. Small holes drilled into the sides of the chamber allowed the water to move in and out of the sediments during tidal flow (see Figure A.1 for schematics). The chambers were kept outdoors so that the crabs were exposed to natural sun and lunar cycles.

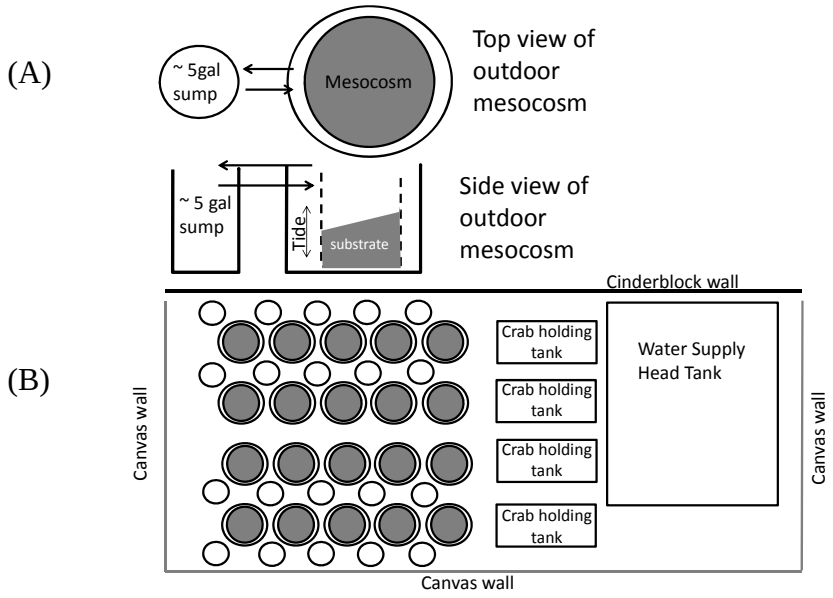

Figure A.1. Experimental design schematics. (A) Schematic of individual exposure chambers showing tidal flow between the chamber and a 5-gal sump. (B) Schematic of the layout of the replicate chambers.

A.4 Maternal Exposure Experimental Methods

At the initiation of the experiment (8/5/2012), 5 males and 5 females were placed in each of the 20 outdoor chambers (see Figure A.1). Each female crab was checked carefully for eggs before being placed in the chambers. If eggs were observed, that female was not used in the study.

After crabs were allowed to acclimate (~ 24 hours) to the chambers (8/6/2012), the chambers were dosed with oil. The general oiling procedures were as follows: the tidal water of the chambers was brought up to a level at least 2 cm above the surface of the sediments. An oil:ASW emulsion was then added to the top of each chamber and allowed to spread across the surface of the tidal water. For controls, the same volume of clean ASW was added to each chamber. After the addition of oil, the tidal water was slowly drained to at least 2 cm below the surface of the sediments, and sediments were allowed to drain for 30 minutes. A modified tidal flux (a 15-minute flooding of all sediments was followed by a 15-minute drain, until it reached 2 cm below the lowest sediment surface) was then repeated 3 times to allow oil to saturate the sediments. Note that the oil emulsion was only added during the initial tidal cycle. After the fourth tidal flux of the oiling process, the pumps were set so that normal tidal fluxes were resumed.

The oil:ASW emulsion that was added to the chambers was made using the procedure described in the Auburn Toxicity Testing General Laboratory Procedures and Practices (GLPP). Once prepared, the emulsion was immediately separated into beakers, each containing an appropriate volume to dose an individual chamber with the desired amount of oil. Once the emulsion had been added to each beaker, clean ASW was added to bring the total volume to 1,000 mL. These solutions were well mixed before being poured into their respective chambers using stir bars and a stir plate. To minimize residual oil in the beaker after the emulsion had been added to the chambers, the beakers were rinsed once with ~ 100 mL of clean ASW, and that rinse water was added to its respective chamber.

A.5 Chamber Observations

Once daily for 9 consecutive days, observations of mortality, number of females at the sediment surface, number of open burrows, and number of sealed burrows (i.e., a male burrow into which a female had entered and subsequently sealed) were recorded for each chamber. To help track the location and status of the burrows within a chamber, burrow locations were marked on a standardized diagram each day. Courtship behavior was recorded with 24-hour surveillance cameras above each chamber. If dead crabs were discovered, they were removed, given a unique identification (ID) number, wrapped in aluminum foil, and frozen at -20°C according to the archive protocols in the QAPP.

Crabs remained in oiled chambers for 9 days (~ 1.5 weeks), starting from the date of oiling, and removed on 8/16/2013. Any gravid females in the chamber were placed into individual Aquatic Habitat (AHAB) tanks (Figure A.2) containing ASW at ~ 20 ppt (salinity was increased to better match the higher salinities experienced by early life stage fiddler crabs in the environment). The chamber from which each female was taken was marked on the AHAB tanks. The females were kept in these tanks until their eggs hatched, or for no more than 2 weeks following removal from the chambers. Once hatched, zoeae were collected in filter cups placed at the outflow of each tank. Any larvae used in subsequent tests were removed before the zoeae were anesthetized in ethanol. The number of remaining zoeae was estimated for each female. To facilitate counting, the zoeae were sacrificed and preserved in ethanol. Preserved zoeae were diluted to 200 mL in tap water, and then mixed with a plunger to ensure an even distribution. Then 5-mL subsamples were removed and the zoeae were counted until either > 100 zoeae had been enumerated or > 60% of the sample had been examined.

Following these counts, the samples were retained in ethanol at 4°C for archiving. Females were put on ice to anesthetize, wrapped in aluminum foil, given a unique ID, and stored at -20°C.

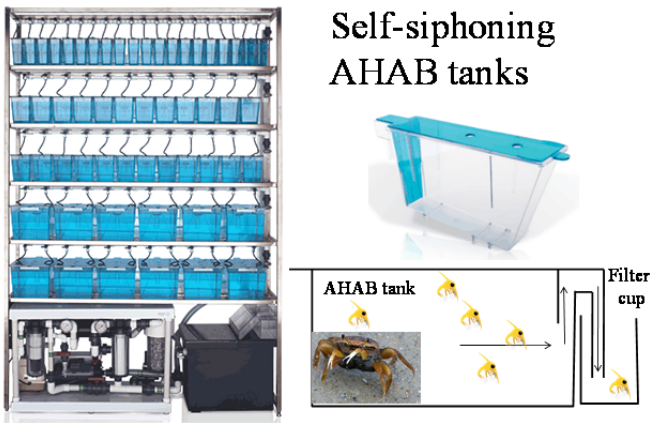

Figure A.2. Recirculating AHAB tank system used to hold gravid females and collect and enumerate fiddler crab larvae. Water first circulates through each tank, and then through biological and mechanical filters to maintain water quality. Each tank drains through a spigot under which collection filter cups can be placed. Gravid females were placed in individual tanks, and filter cups were checked daily for crab larvae.

Source: AHABs (photographic example from catalog; all tanks used during testing were the same size).

A.6 Larval Production

Gravid female crabs from the maternal exposure study were placed in individual AHAB tanks 7 days following their first observable eggs. The AHAB tanks contained ~ 20 ppt ASW. The aquarium from which each female came was marked on the appropriate AHAB tank. Gravid females were held until their eggs hatched or for no more than 2 weeks following removal from the chambers. Once hatched, zoeae were collected in filter cups placed at the outflow of each tank. Zoeae used in survivorship tests were removed immediately and transferred to appropriate experimental vessels. Remaining zoeae were sacrificed by chilling to -20°C. Frozen zoeae were thawed and then clean 20 ppt ASW added until the total liquid volume reached 200 mL. The sample was then mixed with a plunger to ensure an even distribution, 5-mL subsamples were removed, and zoeae were counted until either > 100 zoeae had been enumerated or > 60% of the sample had been examined. The number of zoeae per female was estimated as:

$$N = S/ss \times n + x$$

where:

N = # zoeae per female
S = sample volume (mL)

ss = cumulative subsample volume (mL)
n = cumulative count of larvae in subsamples
x = # live larvae removed before preservation.

Adult females were anesthetized on ice, wrapped in aluminum foil, given a unique ID, and stored at -20°C for archival purposes. After enumeration, larvae were re-frozen and shipped to the Roberts Lab at the University of North Texas (UNT) for storage and potential polycyclic aromatic hydrocarbon (PAH) analysis.

A.7 Water Quality

Three times weekly, pH, DO, salinity, and ammonia were monitored in the outer container tidal water. Temperature was measured hourly from each chamber with a data logger buried ~ 4 in. below the surface of the sediment in the center of the chamber. Temperature was also measured three times weekly in the water of the outer container during routine water-quality measurements. Water that evaporated from the system during the course of the experiment was replaced with RO/DI water to maintain consistent tidal cycles without affecting salinity levels.

A.8 Sediment, Water, and Tissue Sampling

Two 8-oz jar samples of the bulk sediment used to load the chambers were collected and analyzed for PAH, total extractable hydrocarbon (TEH), total organic carbon (TOC), metals, pesticides/polychlorinated biphenyls (PCBs), and grain size. The high concentration oil:ASW emulsion that was used to oil the chambers was also sampled for PAH analysis.

Water samples were collected from the sumps of each chamber every 3 to 4 days during the test. Replicate water samples within each treatment were composited and sent to ALS Environmental for PAH analysis.

A.9 Collection of Crabs, Sediment Samples, and Temperature Loggers

The collection of crabs, sediment samples, and temperature loggers was conducted according to the following protocol:

1. Move cameras off the pad.

2. Move sumps off the pad.

3. Arrange tubs by treatment type, with a workup space by each group of tubs.

4. Remove surface males from each chamber and freeze in tinfoil and Ziploc baggies. Males from the same chamber can be frozen together. Label bags with a unique ID number for that sampling event.

5. Remove surface females from each chamber.

    a. Freeze non-gravid females and archive in tinfoil and Ziploc baggies. Non-gravid females from the same chamber can be frozen together. Label the bags with a unique ID number for that sampling event.
    b. Place gravid surface females in a cup labeled with the appropriate chamber number (1 female per cup). Immediately bring the female(s) to the crab laboratory and place in the appropriate and labeled AHAB.

6. Score the sediment surface to a depth of 2 cm (using a spatula) in parallel lines across the supra-tidal zone (top one-third). Lines should be ~ 5 cm apart.

    a. Remove sediment to a depth of 2 cm and place on appropriate clean glass plate (each treatment/tidal zone gets its own plate), and mix with a spatula.
    b. Place 20 oz (one-quarter of the sample bottle) of sediment into the appropriate surface, supratidal, composite sample jar.
    c. Place 80 oz of sediment (the full sample bottle) into the appropriate surface, supratidal, individual chamber sample jar.
    d. Repeat Steps 6a–c for the remaining 3 chambers in the treatment. Rinse and wipe the spatula clean after each chamber workup.
    e. Repeat Steps 6a–d for the subtidal surface samples, scraping the lower half of the chamber surface to 2 cm.
    f. Place the excess sediment into 5-gal buckets with lids, labeled for waste pickup.
    g. Ensure that all surface sediment has been removed from the chambers.

7. Collect gravid females and data loggers.

    a. Carefully dig through the sediment to find burrowed males and females.
        i. Freeze burrowed males as in previous steps.
        ii. Freeze non-gravid females as in previous steps.
        iii. Clean each gravid female with an ASW squirt bottle. Quickly transport gravid females to the crab laboratory in labeled cups and place into the appropriate AHAB tank (as in previous steps).
    b. Collect data logger from each chamber. Ensure that each logger is correctly labeled as to the chamber it was in.

8. Collect subsurface sediments.

    a. Mix sediments in each chamber.
    b. Place 20 oz (one-quarter of the sample jar) from each chamber into the appropriate subsurface composite jar (one jar per treatment).
    c. Place 80 oz (full sample jar) of sediment into the appropriate subsurface chamber jar (one jar per chamber).

All collected samples were labeled, stored, and shipped according to the QAPP.

B. Testing Protocol 2: Larval Survival Studies Experimental Design

For this study, the survival of larvae hatched from all gravid females (from Auburn GLPP Testing Protocol 1) was assessed. The larval survival studies were done indoors as well as outdoors, with and without ultraviolet (UV) exposure. For the indoor study, four replicate dishes with 20 larvae each were assessed for each gravid female. For the outdoor study, three replicate dishes with UV exposure and three replicate dishes without UV exposure, each with 20 larvae, were assessed for every gravid female (see UNT GLPP Testing Protocol 5).

B.1 Testing Apparatus Design

Both the indoor and outdoor testing apparatuses consisted of a water table containing a small aquarium heater and/or chiller and a small recirculation pump to maintain an even temperature throughout the water bath. The water table acted simply as a warming/cooling bath, and organisms were never in contact with this water. Water was fed into the water table, and the rate of flow varied to maintain a constant temperature during the test. Test dishes were then placed in foam blueboard insulation floats (blueboard is available at Home Depot, Lowes, etc.) and floated in the water table.

For the outdoor tests, the water table was covered in either UV transparent or UV opaque clear plastic sheeting (Aclar and Cortgard are recommended). Plastic sheeting was attached to the system to allow some airflow over the table for cooling. The water table was placed in a location that was unshaded between 8:00 a.m. and 6:00 p.m. UV measurements were made either continuously [BioSpherical profiling ultraviolet (PUV)] or regularly at approximately 20-minute intervals (OceanOptics JAZ). Mortality was assessed twice daily (morning and evening) for up to 96 hours for the outdoor tests and up to 2 weeks for the indoor tests.

AHAB tanks were checked at least twice daily, in the morning and again in the evening, to determine if gravid females had hatched their eggs. For the outdoor tests, if larvae were found in the morning, they were collected from the filter cups and a subsample was immediately transferred to test dishes to be placed outdoors for the day (see steps 1–4 in Section B.2). However, if larvae were found in the evening, test dishes were prepared and kept in the dark until the following morning. For the indoor tests, newly hatched larvae were transferred to test dishes and held overnight on the table top (same conditions as for the outdoor tests) and tests were started the following day. Details are provided below.

B.2 Indoor Experimental Methods

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with the test treatment and tank # (replicate number).

2. Fill each dish with 200-mL ASW using a glass cylinder.

3. Place 20 organisms in each test dish and feed with 20 rotifers/mL every day. On days 2 and 3, supplement with a 100-µL shellfish diet (Reed Mariculture) per dish and then reduce to a 25-µL shellfish diet per dish for each subsequent day.

4. Complete test forms as described in the QAPP.

5. Turn the water table heaters on and heat to the desired temperature.

6. Once the water table has acclimated, randomly place test dishes in foam blueboard floats in the water bath.

7. Once daily, renew test dish water and assess organisms for mortality (see steps 8–10 below).

8. Place dish under a microscope and pipette all live larvae into a clean dish filled with new ASW.

9. Do not transfer or replace dead larvae. Remove all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Place “new” test dishes back into the water bath.

11. Repeat steps 7–10 until the last organism in the lowest WAF concentration has died or for a maximum of 2 weeks.

C. Testing Protocol 3: Assessing Burrowing Activity by Fiddler Crabs Following a Simulated Oil Slick

The indoor testing apparatus (see Figure C.1) consisted of a 26”L × 9”H × 0.75”W artificial burrowing chamber (ABC) modified from the design of Stoeckel et al. (2011), with the two large sides made from glass. Perforated polyvinyl chloride (PVC) formed the inner chamber that was filled with sediment, and a sump was located underneath the ABC. A timer was hooked up to a sump pump that pumped water into the ABC every 24 hours to simulate high tide. An adjustable standpipe allowed water to rise ~ 2 in. above the sediment surface before overflowing back into the sump. Low tide was simulated 12 hours later by manually lowering the standpipe to drain water ~ 5 in. below the sediment surface, back into the sump. High tide came in very rapidly, with water levels rising above the sediment surface and flooding burrows within 0.5 hour. Low tide occurred slowly as water gradually seeped out of the sediments and drained back into the sump over a period of several hours. The sump was filled with ~ 12 ppt salinity ASW, matching the salinity of the crab collection site for *U. minax*, the fiddler crab species used in this study. Sediment was obtained from the fiddler crab collection site at Weeks Bay, Alabama, where sediment primarily comprises organic material rather than sand.

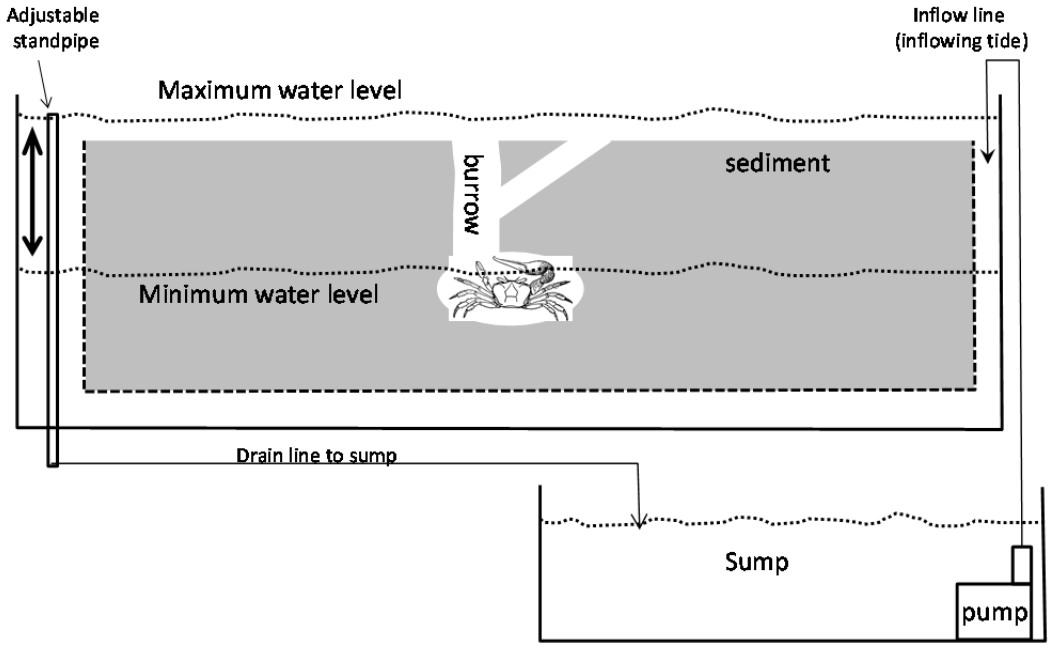

Figure C.1. Schematic of crab ABCs.

A total of 20 chambers were used for this experiment. Ten of these chambers (five randomly chosen chambers from each treatment) each had an infrared (IR)-capable, high-resolution surveillance camera mounted above it to monitor surface-burrowing activity, as well as another camera mounted near the side to monitor subsurface-burrowing activity (i.e., 2 cameras for each of the 10 chambers). In addition to the surveillance cameras, 2 high-definition IR camcorders were periodically used to record high-quality images of surface and subsurface activities in representative chambers for illustrative purposes (e.g., close-up shots of burrow excavation).

C.1 Test Procedure

C.1.1 Sample organisms

All crabs (*U. minax*) in this study were collected from Weeks Bay, Alabama. Male crabs were used in this experiment.

C.1.2 Acclimation

On day 0, one male fiddler crab was placed into each ABC and allowed to acclimate and burrow until all crabs had constructed a burrow and inhabited it for > 2 days. Tides were simulated during acclimation as previously described. Burrow construction and activity in 10 of the 20 chambers were filmed with surveillance cameras. Burrow outlines were traced immediately after initial burrow construction, as well as on the day before the experiment began.

C.1.3 Treatments

Upon initiation of the experiment, Auburn personnel subjected 10 of the 20 ABCs to a simulated oil spill, while the other 10 ABCs remained oil-free. A 2-L oil emulsion was created using ~ 20 g of Slick A oil blended into the ASW (see the Auburn GLPP). After initiation of the lowering of the water level to simulate low tide, approximately 200 mL of the oil emulsion was poured into the 10 “oil spill” ABCs when water levels were even with the surface. A sufficient volume of oil emulsion (~ 200 mL/chamber) was added such that there was a 2-cm deep layer of oil emulsion over the sediment. A bead of room-temperature, weathered oil was then applied on top of the oil emulsion using a syringe. The volume of oil emulsion and volume of oil applied to each chamber were recorded for each chamber. The oil emulsion and oil bead were allowed to coat sediment surfaces and the insides of burrows as water slowly receded during the low-tide drawdown. Any excess oil emulsion that drained into the sump was recirculated above the sediments during subsequent high tides. Five of the oiled and five of the non-oiled chambers were equipped with surveillance cameras as described previously.

C.1.4 Sample collection and endpoints

The experiment was run for 19 days post-oiling. Burrows were traced daily for the first 16 days in each chamber followed by a final tracing on the 19th day. Tracings were scanned and image analysis software was used to determine (1) total burrow area, (2) total burrow depth, (3) burrow area and depth below the minimum water line, (4) number of entrances/burrows, and (5) number of separate burrows.

Surface camera videos were utilized to determine (1) the proportion of time crabs spent on the surface during diurnal hours, and (2) the proportion of time crabs spent on the surface during nocturnal hours for 5 of the 10 crabs in each treatment. Side camera videos were analyzed to determine (1) the proportion of time crabs spent in the water while in their burrows, and (2) the proportion of time crabs spent actively burrowing.

On the final day of the experiment, 100 mL of "high tide" water was collected from each chamber and shipped to ALS Environmental for PAH analysis. Water was then drained from each chamber. Each chamber was then placed flat on its side and the side panel was removed. Crabs were then removed and held in individual cups for use in a subsequent escape-response study. Sediment was divided into three equal 2-in. layers (i.e., top, middle, and bottom), and each layer was homogenized with a stainless steel spatula. Individual ~ 8-oz samples were collected from each layer in each oiled chamber and placed into a glass 8-oz jar (3 layers × 10 oiled chambers = 30 samples). Soil from each layer was composited between control chambers (3 layers × 1 composite sample/layer = 3 samples) and ~ 8 oz of sediment from each composite sample placed into a glass 8-oz jar. Sample jars were stored at 4°C in darkness and shipped to ALS Environmental.

C.1.5 Escape-response experiment

The escape-response experiment followed the protocol of Culbertson et al. (2007) and Krebs and Valiela (1974). A single fiddler crab was placed underneath a plastic cup in the middle of a 44-cm diameter circle. At a prearranged signal, one person lifted the cup away from the crab and a second person immediately released a 5 × 5 cm weighted black square that swung on a pendulum directly above the crab. This process was repeated for each crab from all 20 burrowing chambers and the time to exit the circle was recorded for each crab.

Immediately following this experiment, each crab was individually wrapped in tinfoil, and frozen in individually labeled Ziploc bags.

D. Testing Protocol 4: Assessing the Effects of WAF Maternal Exposure on Fiddler Crab Larval Production

D.1 Crab Collection and Maintenance

Fiddler crabs (*U. longisignalis*) were collected from the Alabama coastline near Sandy Bay (~ 30°22'46.11"N 88°18'23.27"W) and transported to Auburn. Male and female crabs were housed outdoors in separate 30-gal coolers containing aerated ASW (Instant Ocean ~ 15 ppt; same salinity as collection site), and PVC ribbons prior to the initiation of experiments. PVC ribbons served as a biofilter while simultaneously serving as a structure to reduce antagonistic interactions between crabs. Auburn previously had good success housing crabs in this system for several months.

D.2 WAF Preparation

Exposures were conducted using Slick A HEWAF preparations (see the QAPP).

D.3 Maternal Exposure

The maternal exposure study occurred in 38-L glass aquaria containing 6 L of ASW (15 ppt) spiked with the appropriate amount of stock WAF. A PVC ribbon was added to each aquarium to serve as a structure. Previous pilot studies showed that fiddler crabs will develop broods in this setup, in the absence of sediment. At the initiation of this experiment, five males and five females were placed into each aquarium. Crabs were fed unlimited commercial shrimp feed. Each female crab was checked carefully for eggs before being placed into an aquarium. If eggs were observed, that female was not used in the study. Once added, crabs were allowed to acclimate for 12 hours before the aquaria were dosed with WAF.

Water in the aquaria was renewed every 48 hours. At each renewal approximately 80% of the water from each tank was siphoned out, and newly prepared WAF was diluted to the appropriate concentration before added to the aquaria.

Females were checked every 48 hours for the presence of newly fertilized eggs. If a female was found with eggs, that female was marked with a unique tag before being placed back into its respective aquarium. Each gravid female was held in an aquarium for an additional 7 days after the first observation of eggs. After 7 days, the gravid females were transferred to an individual

AHAB tank containing clean ~ 20 ppt ASW where they were kept until eggs hatched or for no more than 2 weeks. The larvae from each gravid female were collected to determine larval production (Section D.4) and to follow larval survival (described in the UNT GLPP Testing Protocol 5). The unique tags on females allowed them to be tracked individually during this process.

During the course of the test, additional females and males were added to the tanks to replace crabs that were removed. However, there were never more than five females and five males in a tank at one time.

D.4 Larval Production

Gravid female crabs from the WAF maternal exposure study were placed into individual AHAB tanks 7 days following their first observable eggs. The AHAB tanks contained ~ 20 ppt ASW. The aquarium from which each female came was marked on the appropriate AHAB tank. Gravid females were held until their eggs hatched or for no more than 2 weeks following removal from the chambers. Once hatched, zoeae were collected in filter cups placed at the outflow of each tank. Zoeae used in survivorship tests were removed immediately and transferred to appropriate experimental vessels. The remaining zoeae were sacrificed by chilling to -20°C. Frozen zoeae were thawed and clean 20-ppt ASW was added to bring the total volume to 200 mL. The sample was then mixed with a plunger to ensure an even distribution, 5-mL subsamples were removed, and zoeae counted until either > 100 zoeae had been enumerated or > 60% of the sample had been examined. The number of zoeae per female was estimated as:

$$N = S/ss \times n + x$$

where:

N = # zoeae per female
S = sample volume (mL)
ss = cumulative subsample volume (mL)
n = cumulative count of larvae in subsamples
x = # live larvae removed before preservation.

Adult females were anesthetized on ice, wrapped in aluminum foil, given a unique ID, and stored at -20°C for archival purposes. After enumeration, larvae were re-frozen and shipped to the Roberts Lab (UNT) for storage and potential PAH analysis.

D.5 Water Quality

Three times weekly, pH, temperature, DO, salinity, and ammonia measurements were taken for each tank. Water was renewed every 2 days. If ammonia levels rose above 20 mg total ammonia nitrogen (TAN)/L (~ 10% of the 24-hour LC50 for *Uca* sp. as reported by Azpeitia et al., 2013), additional water modifications would have been made; however, these modifications were not needed during this study. In addition, tanks were not initially aerated in order to minimize potential PAH loss; however, DO levels were monitored closely, and it was determined 2 days after initiation of the test that aeration was needed to maintain DO levels. Thus, all tanks were aerated using an airstone for the remainder of the test.

D.6 Water Sampling

Water samples were collected and analyzed for PAH levels. All collected samples were labeled, stored, and shipped according to the QAPP.

E. Testing Protocol 5: Assessing the Effect of Direct Exposure to Slick B Oil on Adult Periwinkle Movement and Survival

These tests were conducted in collaboration with the University of North Texas.

E.1 Direct Oil Exposure

For the direct oil exposures, 24-L (55.3 cm x 33.3 cm x 15.6 cm) plastic Sterilite storage containers were used as experimental chambers, with 5 replicates each of one oil treatment and one control treatment, for a total of 10 chambers. Containers were filled with roughly 5 gal of sand so that a baking pan could lay flush with the edges of the test chambers. Each baking pan was filled with 200 g of trimmed *Spartina alterniflora* shoots and leaves. Baking pans were then filled with 15 ppt ASW so that the *Spartina* could hydrate overnight. Before the snails were exposed to the oil or to the controls, baking pans were drained of water and placed inside experimental chambers to mimic "fallen" *Spartina* beds. In addition, 2 rows consisting of 8 shoots each of *Spartina* were inserted vertically into the sand at the rear of the exposure chamber to mimic unaffected *Spartina* beds (Figures E.1 and E.2).

In the oiled exposure chambers, the vertical grass shoots served as an unoiled habitat where the snails could go to escape the oil. Trimmed blades of *Spartina* were placed at the rear of the baking pans to bridge the horizontal and vertical vegetation. Once the vegetation was set up in each chamber, 1 L of Slick B oil was added to the horizontal vegetation in each of the 5 oiled exposure chambers and allowed to settle. Once the oil settled, the layer of oil across the exposure chambers was approximately 1-cm thick.

Twenty snails were counted for each exposure chamber, painted with Liquid Paper, and labeled according to treatment. This procedure improved the ability of laboratory personnel to see the snails, enabled the camera to take better images, and enabled personnel to identify any snails that escaped the exposure chambers. Once painted and labeled, snails were placed in the center of the baking pan, roughly 23 cm from the vertical vegetation. Exposure chambers were placed outdoors, on a tarp on top of gravel and under a tent at ambient temperature. See Figure E.2 for a depiction of the individual chamber design and the layout of the exposure chambers under the tent.

Snail movement was assessed and photographed at the following time points: 0, 0.8, 0.25, 0.42, 0.58, 0.75, 0.98, 1.17, 2.5, 4.25, 19.5, 24.25, 28, 44, 48, 52, 68, and 72 hours. At each assessment point, movement was documented by categorizing each individual snail into one of three groups:

on the horizontal vegetation, on the vertical vegetation, or exiting the exposure chamber. In addition, one control and one oiled replicate were video recorded for a cumulative total of 4 hours and 9 minutes in approximately 30-minute segments. The video camera only allowed for recording videos of this length, which provided approximately 9 segments before the battery ran out of power.

If snails exited the horizontal vegetation from a direction other than toward the vertical vegetation, or if they successfully traversed the horizontal vegetation into the vertical vegetation, laboratory personnel recorded this information and removed those snails from the exposure chambers and movement assay. Once the snails were removed from the exposure chamber, they were placed into individual scintillation vials using large forceps for additional monitoring (see Section E.2, Post-Exposure Monitoring). At the end of 72 hours, snails that remained in the horizontal vegetation were also transferred into individual scintillation vials for post-exposure monitoring. All bench sheets were completed as described in the QAPP.

Figure E.1. Control chamber, with horizontal and vertical stalks and no oil.

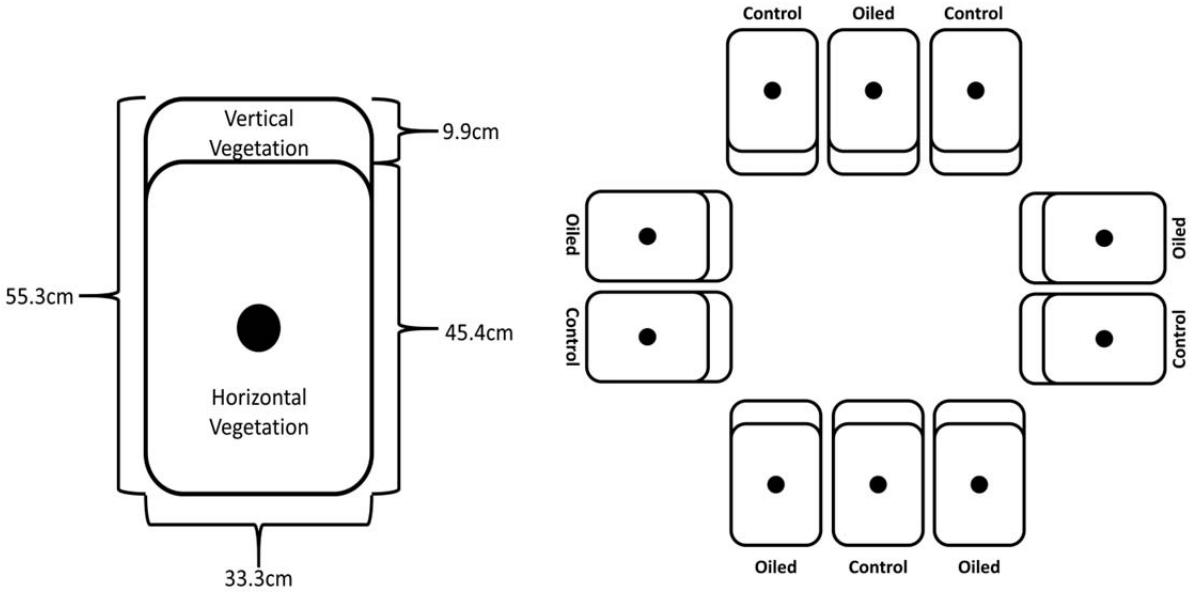

Figure E.2. Exposure chamber design and layout.

E.2 Post-Exposure Monitoring

After direct oil exposure, snails were monitored for 7 days to assess post-exposure mortality. All live snails were collected from the exposure chambers according to the procedures described above (Section E.1) and placed into individually labeled scintillation vials. Each vial contained a small volume of 15-ppt ASW to provide moisture, and a 2 in. stalk of *Spartina* to allow the snail to crawl up and out of the water. In addition, a small hole was drilled into the lid of the scintillation vial to allow for air movement. At the start of the 7-day monitoring period and every 24 hours afterward, snails were assessed for mortality. If a snail was attached to a surface using its foot, it was noted as alive, with no other assessment needed. Snails with no visible foot were removed and their operculum gently prodded with a bamboo skewer to elicit a response. If snails did not respond to the prodding, they were placed into a dish with 15-ppt ASW for 5–10 minutes; those that did not emerge out of their shells were noted as dead and archived according to the procedures described in the QAPP. Snails that did emerge were returned to their respective scintillation vial. At the end of 7 days, all living and dead snails were recorded, and then live snails were sacrificed and all dead snails archived according to the procedures described in the QAPP.

F. Testing Protocol 6: Assessing the Effect of Direct Exposure to Slick B Oil on Adult Periwinkle Survival at Cool Temperatures

These tests were conducted in collaboration with the University of North Texas.

F.1 Testing Apparatus Design

The direct exposure outdoor testing area consisted of a concrete foundation over which a shade tent was placed. The water bath (Figure F.1) consisted of two shallow fiberglass tanks placed on top of a metal frame with a sump below the tanks to allow for placement of heaters and a pump to circulate the heated water between the sump and tanks. Water never came into direct contact with the organisms. The exposure chambers were set into foam blueboard floats which maintained an even spacing between chambers and minimized heat escape from the water bath. The foam board kept chambers from resting on the bottom of the tanks, thus allowing the water to flow underneath and around each chamber for even heat distribution. Small pumps maintained an even flow of water within the tanks, and the water level was controlled through the use of standpipes emptying into the sump below.

The exposure chambers were moved indoors immediately following the 32-hour exposure time point because of a storm that blew the shade tent over and caused outdoor temperatures to drop. Thus, exposure periods for the 1- to 32-hour time points were done outdoors and exposure periods for the 32- to 72-hour time points were done indoors.

At a predetermined time point, individual snails were moved into glass jars for a 7-day mortality assessment [see test-specific test conditions tables (TCTs)]. During the time points that the outdoor water bath was running (1–32 hours), glass jars used in the seven-day, post-exposure, mortality assessment were held in the same water bath as the experimental chambers. The spaces in the foam board were labeled by treatment and replicate number to ensure that jars containing the surviving snails were placed back into the same space from which the matching exposure chambers had been removed. After the 32-hour time point, all glass jars were placed indoors on a tabletop for the remainder of the trial.

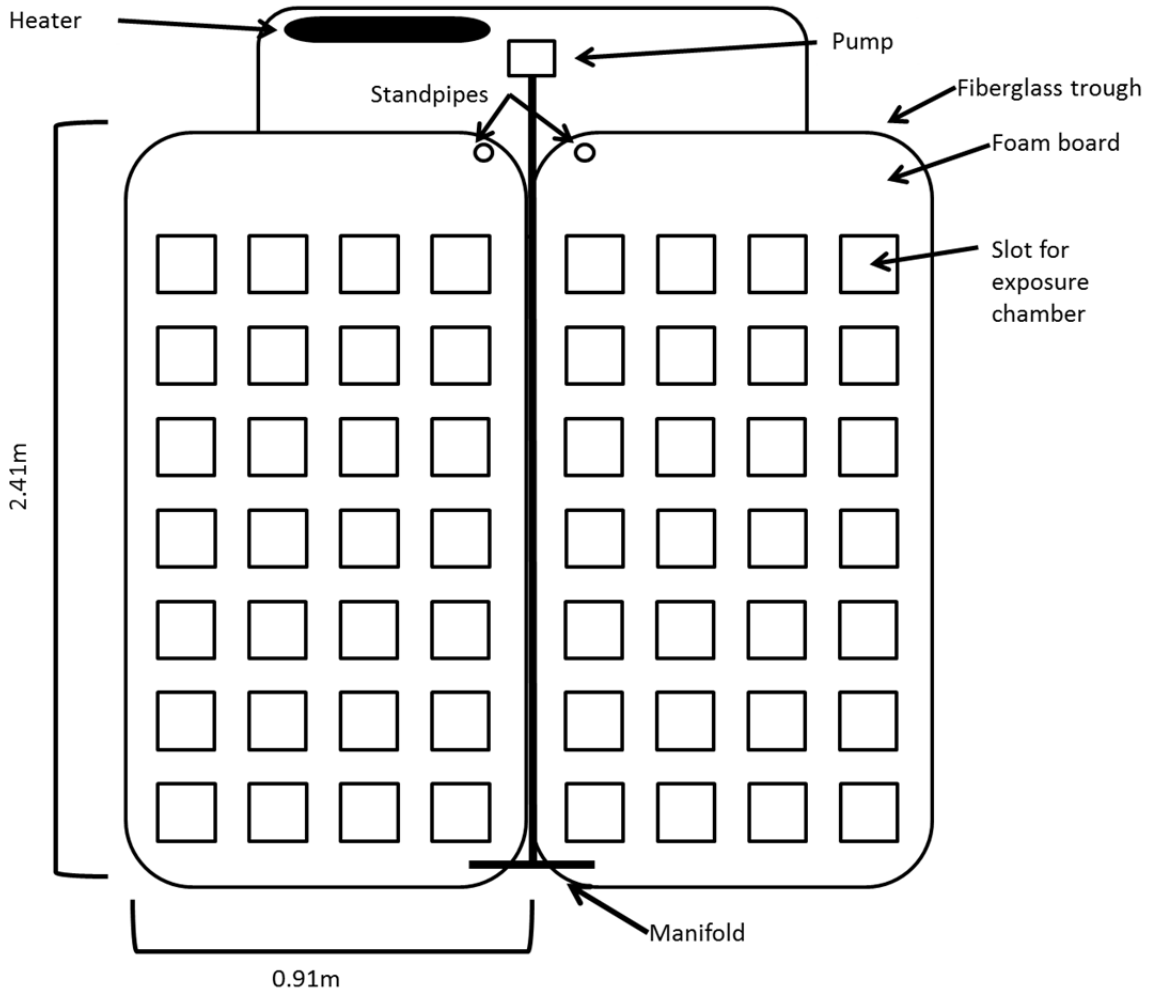

Figure F.1. Water bath design and layout.

F.2 Direct Oil Exposure

The direct exposure portion of this study used 0.9-L (0.15 m x 0.15 m x 0.045 m) plastic storage containers as experimental chambers (see Figure F.2). Each chamber was fitted with a stainless steel, wire-bristle brush around the container lip to prevent the snails from exiting the chamber. Each chamber contained a single layer of trimmed *Spartina alterniflora* and enough 15-ppt ASW to wet the *Spartina*. The *Spartina* soaked in the ASW overnight, and any excess water was removed the following morning. After the overnight soak, a 1-cm layer of Slick B oil (~ 200 g) was added to each oiled chamber. All chambers were placed in a shallow water bath. At the

32-hour time point, all chambers were brought indoors as described in Section F.1. The temperatures of the air, water bath, and chambers were monitored using daily minimum-maximum thermometers. At the start of the test, 10 snails were randomly assigned to each chamber. The treatments for these tests included 1, 2, 4, 8, 16, 32, and 72 hours of exposure in the oiled and control chambers (7 oil treatments plus 7 control treatments x 4 replicates each = 56 total chambers). At the end of each exposure period, snails from the appropriate control and oiled chambers were removed using large forceps, checked for mortality (see Section F.3), and placed into labeled quart jars corresponding to their treatment and replicate number for post-exposure monitoring.

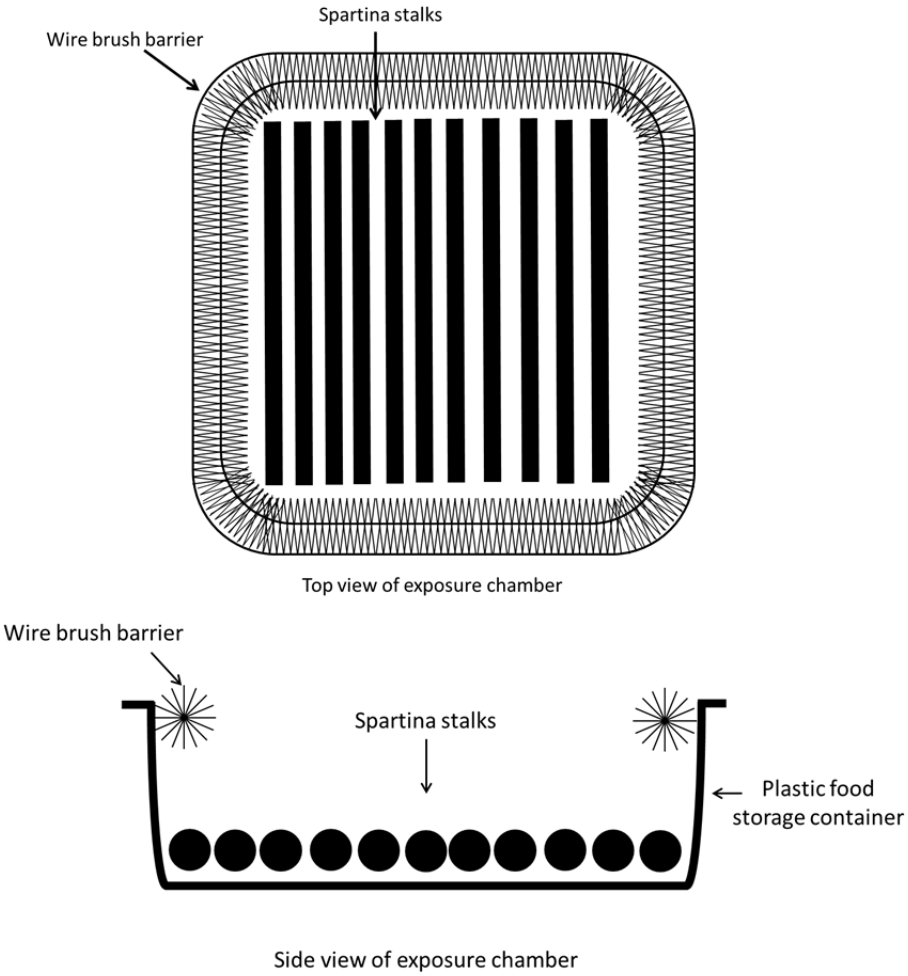

Figure F.2. Design of exposure chamber.

F.3 Post-Exposure Monitoring

After the direct oil exposure, snails were monitored for 7 days to assess post-exposure mortality. All live snails were collected from the exposure chambers and transferred to corresponding 1-qt jars (see Section F.2). Each jar contained a small volume of 15-ppt ASW to provide moisture and a small stalk of *Spartina* to allow the snails to crawl up and out of the water. In addition, the jar lid was screwed down over a piece of window screen to allow for air movement. At the start of the 7-day monitoring period and every 24 hours afterward, snails were assessed for mortality. If a snail was attached to a surface using its foot, it was noted as alive with no other assessment needed. Snails with no visible foot were removed and their operculum gently prodded with a bamboo skewer to elicit a response. Snails that did not respond to the prodding were placed into a dish with 15-ppt ASW for 5–10 minutes; snails that did not emerge out of their shells were noted as dead and archived according to the procedures described in the QAPP. If alive, they were returned to their respective jar. At the end of 7 days, all live and dead snails were recorded, and then archived according to the procedures described in the QAPP.

G. Testing Protocol 7: Assessing the Effect of Direct Exposure to Slick B Oil on Adult Periwinkle Survival at Warm Temperatures

These tests were conducted in collaboration with the University of North Texas.

G.1 Testing Apparatus Design

The direct exposure testing apparatus consisted of an indoor water bath in a room with temperature and photoperiod controls. The water bath (see Figure F.1 in the Auburn GLPP Testing Protocol 5) consisted of two shallow fiberglass tanks placed on top of a metal frame with a sump below the tanks to allow for placement of heaters and a pump to circulate the heated water between the sump and tanks. The water never came into direct contact with the organisms. The exposure chambers were set into foam blueboard floats that maintained an even spacing between chambers and minimized heat escape from the water bath. The foam board kept chambers from resting on the bottom of the tanks, thus allowing the water to flow underneath and around each chamber as well as even heat distribution. Small pumps maintained an even flow of water within the tanks, and water levels were controlled through the use of standpipes emptying into the sump below.

Glass jars used in the 7-day, post-exposure, mortality assessment were held in the same water bath as the experimental chambers. The spaces in the foam board were labeled by treatment and replicate number to ensure that jars containing the surviving snails were placed back into the same space from which the matching exposure chambers had been removed.

G.2 Direct Oil Exposure

The direct exposure portion of this study used 0.9-L (0.15 m x 0.15 m x 0.045 m) plastic storage containers as experimental chambers (see Figure F.2 in the Auburn GLPP Testing Protocol 5). Each chamber was fitted with a stainless steel, wire-bristle brush situated around the container lip to prevent experimental animals from exiting the chamber. Each chamber contained a single layer of trimmed *Spartina alterniflora* and enough 15-ppt ASW to wet the *Spartina*. The *Spartina* soaked in ASW overnight, and any excess water was removed the following morning. After the overnight soak, a 1-cm layer of Slick B oil (~ 200 g) was added to each oiled chamber. To maintain the appropriate temperature for the duration of the exposure, all chambers were placed into a shallow water bath. The temperatures of the air, water bath, and chambers were monitored using daily minimum-maximum thermometers. At the start of the test, 10 snails were

randomly assigned to each chamber. The treatments for these tests included 1, 2, 4, 8, 16, 32, and 72 hours of exposure in the oiled and control chambers (7 oil treatments plus 7 control treatments x 4 replicates each = 56 total chambers). At the end of each exposure period, snails from the appropriate control and oiled chambers were removed using large forceps, checked for mortality (see Section G.3), and placed into labeled 1-qt jars corresponding to their treatment and replicate number for post-exposure monitoring.

G.3 Post-Exposure Monitoring

After the direct oil exposure described above, snails were monitored for 7 days to assess post-exposure mortality. Live snails were collected from the exposure chambers and transferred to corresponding 1-qt jars (see Section G.2). Each jar contained a small volume of 15-ppt ASW to provide moisture and a small stalk of *Spartina* to allow the snails to crawl up and out of the water. In addition, the jar lid was screwed down over a piece of window screen to allow for air movement. At the start of the 7-day monitoring period and every 24 hours afterward, snails were assessed for mortality. If a snail was attached to a surface using its foot, it was noted as alive with no other assessment needed. Snails with no visible foot were removed and their operculum gently prodded with a bamboo skewer to elicit a response. Snails that did not respond to the prodding were placed into a dish with 15-ppt ASW for 5–10 minutes; snails that did not emerge from their shells were noted as dead and archived according to the procedures described in the QAPP. If a snail did emerge from its shell, it was considered alive and returned to its respective jar. At the end of 7 days, all live and dead snails were noted and archived according to the procedures described in the QAPP.

H. Testing Protocol 8: Effect of Oil Thickness on Adult Periwinkle (*Littoraria irrorata*) Movement Following Direct Exposure to Slick B Oil

This test was conducted in collaboration with the University of North Texas.

The movement of snails exposed to different thicknesses of oil was assessed. Some replicates were exposed to vegetative substrate covered by 0.1 cm of oil, and some replicates were exposed to vegetative substrate covered by 0.5 cm of oil. Control replicates were placed directly onto vegetative substrate. Each replicate contained 20 organisms, placed roughly 23 cm from the vertical structure, and movement into the vertical structure was monitored.

H.1 Testing Apparatus

The testing apparatus for snail movement tests at different oil depths consisted of mesocosms (2 oil treatments and 1 control treatment x 3 replicates each = 9 total chambers) placed inside a portable greenhouse dome (FlowerHouse). Mesocosms were fabricated to mimic marsh habitats impacted by oil. Metal trays were placed atop fiberboard, and each tray bottom was lined with a banana leaf to serve as a flat vegetative substrate. Wooden dowel rods (1.27-cm diameter) were inserted into fiberboard at the rear of the exposure chamber to mimic unaffected, standing *Spartina alterniflora*. A fiberboard ramp was fabricated and placed under the banana leaf at the rear of the exposure tray to facilitate movement into the vertical structure. Slick B oil was added to each of the 2 oiled treatments to depths of approximately 0.1 cm (~ 110 g of oil) and 0.5 cm (~ 560 g) to represent an oiled habitat.

H.2 Test Procedure

1. Label snails with Bic White-Out.

2. Label each clean mesocosm with the test treatment and replicate number, and secure the trimmed banana leaf to the metal tray using silicone or spray adhesive.

3. Loosen cap of bottled oil, warm in boiling water, and fill each mesocosm with oil to the corresponding treatment volume/depth (by mass) using a balance (see test-specific TCTs).

4. Place mesocosms in the greenhouse dome.

5. Place 20 organisms in each mesocosm in the center of the vegetative substrate.

6. Document the number of snails on the horizontal or vertical grass at time-points of 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, and 4 hours on the appropriate bench sheets.

7. Collect snails as they successfully migrate into the vertical structure.

8. At the test completion, collect all snails.

9. Retain test organisms according to the QAPP.

10. Complete bench sheets as described in the QAPP.

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2. Florida Gulf Coast University General Laboratory Procedures and Practices

During the *Deepwater Horizon* (DWH) Natural Resource Damage Assessment (NRDA) toxicity testing, Dr. Aswani Volety, the principal investigator (PI) at Florida Gulf Coast University (FGCU), transferred his laboratory to the University of North Carolina, Wilmington (UNCW). Most of the toxicity tests that are described in this document were conducted at FGCU. Testing Protocols 13 and 14 were conducted at UNCW.

2.1 Methods

These tests examined the response of eastern oysters to experimental exposure to oil constituents, dispersants, and contaminated sediments with regard to survival, reproduction, settlement, immune responses, and disease susceptibility. The oysters were exposed to different concentrations of polycyclic aromatic hydrocarbons (PAHs) and/or dispersants under laboratory conditions. Laboratory exposures involved both early lifestage (embryo, veliger, and early spat) and adult oysters, as well as direct exposure of gametes.

Upon exposure, the larval development, survival, and metamorphosis were used as end points for larval stages, while fecundity, gamete quality, immune system, and disease susceptibility to the oyster pathogen *Perkinsus marinus* were examined in adult oysters (Volety et al., 2009). Gonadal condition and histopathological alterations were examined using histological techniques (Volety, 2008; Volety et al., 2009). Total petroleum hydrocarbons (TPHs) were extracted using a modified Bligh-Dyer extraction (Kimbrough et al., 2006; Loh et al., 2008), followed by subsequent analysis using a gas chromatograph (GC) equipped with DB-5 fused silica capillary column interfaced with a flame-ionization detector (FID). Additional analytical analyses of hydrocarbon constituents were performed by ALS Environmental, and comparisons were made between exposed and control treatments. Methods and experimental protocols are described in detail below.

All organisms and tissues from organisms collected for and/or used during testing were archived according to the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3.

2.1.1 Source water

FGCU

The natural seawater used in these tests was pumped from the channel adjacent to the Vester Marine Field Station in Estero Bay, Florida. All of the water was filtered. Particles greater than 25 µm were removed by sand-and-bag filtration. The seawater was then purified by fiber cartridge and bag filters, ultraviolet (UV) sterilized, and then passed through a 0.1-µm filter before use in experiments. This seawater was adjusted to the desired test salinity via the addition of deionized water. At times when the salinity of the natural seawater in the bay was too low (< 20 ppt), artificial seawater was created by mixing Instant Ocean artificial salts with deionized water.

UNCW

The natural seawater used in these tests was pumped from Masonboro Sound outside of the Center for Marine Science in Wilmington, North Carolina. All of the water was filtered. Particles greater than 25 µm were removed by sand-and-bag filtration. The seawater was then purified by fiber cartridge and bag filters, UV sterilized, and then passed through a 0.1-µm filter before use in experiments. This seawater was adjusted to the desired test salinity via the addition of deionized water.

2.1.2 Experimental oyster sources and husbandry

FGCU

Healthy oysters were obtained from Estero Bay, Florida, which is Florida's first Aquatic Buffer Preserve and has extensive, healthy oyster reefs. Oysters were acclimated in 250-L tanks for up to 2 weeks at ambient salinity of collection site (~ 20–25 ppt) prior to using them in any experiments. During acclimation, water quality was maintained using flow-through conditions, and seawater from an onsite saltwater well at the field station was used for oysters and preparation of aqueous matrix of the toxicants. Oysters and larvae were fed a mixture of shellfish diet or freshly cultured algae (e.g., a mixture of *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., and *Thallasiosira weissflogii*). For some spat and juvenile oyster exposure experiments, oysters were obtained from the Auburn University Shellfish Hatchery (AUSH). Larvae and spat were shipped overnight from AUSH to the FGCU Vester Marine Field Station where they were placed in ambient seawater for acclimation and further experimentation.

UNCW

Healthy oysters were obtained from Masonboro Sound, North Carolina, and brought to UNCW's Shellfish Research Hatchery located at the Center for Marine Science in Wilmington, North Carolina. The oysters were acclimated inside the hatchery for at least 2 weeks at salinities that would be conducive to gonadal development and spawning (20–25 ppt) prior to strip spawning. During acclimation, the water quality was maintained using flow-through conditions, and the seawater was supplied by filtering the water from Masonboro Sound. The oysters were fed a mixture of live cultured algae. The tanks were maintained at a temperature of approximately 25°C.

Conditioning of broodstocks

Adult oysters were maintained at 20–24°C in 0.1-µm filtered seawater (FSW) and fed a daily ration of algae equal to approximately 3% of the oyster dry body weight. Oysters were artificially conditioned to the reproductively active stage (broodstock) by manipulating the culture temperature and feeding them a nutritionally enhanced diet (i.e., algae). Sub-samples of oysters were periodically shucked open and sampled to examine the maturity of gametes under a microscope. Spawning was induced in the laboratory and hatchery by thermal stimulation.

Spawning and larval culture

FGCU

Oyster gametes, embryos, and larvae were collected from the laboratory-conditioned broodstock. Experimental oysters were thermally induced to spawn at 17–18°C and 30°C in spawning flumes containing seawater. Once spawning occurred, spontaneously spawning oysters were separated and maintained in individual 1-L containers. Sperm and eggs were collected, and the number of eggs was counted using a hemacytometer or a Sedgwick-Rafter counter under a microscope. Sizes of eggs were measured at the same time. Eggs from individual oysters were fertilized with sperm pooled from two to three males of same treatment group. Fertilized eggs were rinsed with seawater to remove unfertilized sperm, then placed in 80-L larval tanks at a concentration of 25–35 fertilized eggs/mL. They developed to straight-hinge larvae (normally 18–24 hours after fertilization at 27–29°C). Straight hinge larvae were cultured to eyed larvae at a density of 5 larvae/mL in larval tanks. Larvae were fed daily with algae and water was changed every other day. Straight hinge, umbo, and eyed (pediveliger) larvae were used in the experiments. In cases where natural spawning did not occur after thermal stimulation, additional oysters were stripped to obtain gametes.

UNCW

Oyster embryos were generated after the adult oysters were strip spawned, which consisted of macerating the oyster gonad once it was fully developed and producing gametes. The female gametes were gathered from one individual, placed in fresh seawater, and fertilized within a 2-hour period after the eggs were stripped from the gonad. The male gametes were stripped from several males. The eggs were diluted by the addition of fresh, high-quality seawater and egg density was determined by counting the number of eggs in a 1-mL sample of the uniformly mixed dilution on a 1-mL rafter slide. Once the egg density was calculated, male gamete health and mobility and gamete density were assessed by taking a 10-µL sample of the male gamete dilution and placing it on a cleaned, glass cover slide. If the sperm were highly mobile and of high density, they were mixed with the egg dilution at a ratio of approximately 220 sperm/egg in a 2-L beaker. This was accomplished by adding a 1:20 volume ratio of sperm dilution to an egg dilution. The final egg density was approximately 25–35 eggs/mL. After 1 hour, fertilization success was evaluated by taking a 1-mL sample of mixed gamete dilution and counting the number of fertilized eggs on a 1-mL rafter slide. Hereafter, the embryos developed normally during testing conditions.

2.1.3 Exposure water and sediment preparations

- Three different water accommodated fraction (WAF) preparations: high energy (HEWAF), low energy (LEWAF), and chemically enhanced (CEWAF) WAFs; use of two oil types: Slick A (CTC02404-02) and weathered source oil (072610-W-A)
- Corexit only exposures – as a definitive test (dose-response)
- Control water – prepared similarly as WAFs but without the addition of oil
- Sediments – a range of contaminated and uncontaminated field-collected sediment brought back to the laboratory and/or uncontaminated field-collected sediment spiked with oil in the laboratory.

WAF preparation methods followed guidelines detailed in the QAPP. However, in some instances Slick A oil was added using a gas tight syringe.

Sediment-derived WAF

Sediment slurry method

Bioassays were conducted using decanted sediment and sediment elutriates according to Geffard et al. (2003). Decanted sediment WAFs were made by adding clean seawater (20–25 ppt) to contaminated sediment in a glass beaker, stirring for 10 seconds, and then allowing sediment to

settle for 2 hours. Supernatant solution of seawater was used in the test chambers. Exposure concentration of the sediment was determined in consultation with Stratus Consulting. Sediment from a known uncontaminated control location in Estero Bay, Florida (or reference sediment from Louisiana provided by Stratus Consulting) was used for controls.

For the preparation of sediment elutriates, contaminated sediment was mixed in artificial seawater at a ratio of 1:10 (sediment:water) and was mechanically shaken at 300 RPM for 6 hours and allowed to settle for another 12 hours. Supernatant was siphoned off and mixed with FSW.

2.1.4 Testing methods

Acute and chronic aqueous exposure toxicity tests

Methodology of the gamete, embryo, larval, and spat exposures are detailed in Testing Protocol 1 of the FGCU General Laboratory Procedures and Practices (GLPP).

C. virginica embryos, veligers or early spat, and adults were exposed to various concentrations of LEWAF, HEWAF, CEWAF, and dispersant in an aqueous matrix. Embryo and larval exposures generally followed the U.S. Environmental Protection Agency's Office of Prevention, Pesticides and Toxic Substances (OPPTS) protocol 850.1055 (U.S. EPA, 1996) for bivalve acute toxicity tests. Exposure and toxicity evaluation methods are described below.

Embryo/veliger exposures

The starting life stage of these exposures were embryos that, after approximately 24 hours, became veligers. Embryos that were at the 2–4 cell stage were exposed to HEWAF, LEWAF, CEWAF, and dispersant-only treatments for 96 hours. Control treatments were run concurrently for each toxicant. Control oysters were exposed to the same FSW used in exposure treatment, but without toxicants. Exposures were conducted in 400-mL glass jars, with 4 replicate jars for each treatment. Each replicate contained ~ 4,000 oyster embryos. Exposures were conducted under static conditions. Embryo survival and growth were assessed after 24 and 96 hours. Embryo development was assessed in morbid and surviving oysters at the end of the exposure (96 hours). These embryos were fixed with buffered 0.1% formalin and evaluated for developmental abnormalities using a microscope.

The developmental success of veligers was determined by their progress to the pediveliger stage, while the effect of various treatments on early spat and adult oysters was determined by examining the survival and growth (see above). In addition, histological sections of adult oysters were made (Volety, 2008; Volety et al., 2009) and any alterations in the somatic and/or gonadal

tissues observed under a microscope were compared with that of control oysters (see above). Concentrations of PAH in water and oyster tissues were analyzed.

Pediveliger exposure

The developmental success of pediveligers was determined by monitoring their progression to spat (settlement success). For these tests, pediveligers were exposed for 3 days to HEWAF, CEWAF, or sediment that had been spiked with oil. Each test chamber consisted of approximately 1,000 individuals. Survival and settlement success were assessed at the end of the exposure.

Early spat exposures

Early spat (5–15-mm length, ~ 30 days post-hatch) were exposed to either HEWAF, CEWAF, or dispersant for up to 14 days. Each test chamber consisted of 15–25 individuals. Exposures were conducted under static-renewal conditions. Exposure media were renewed every other day and water quality was monitored throughout the exposure period. Survival was assessed at each water change.

Adult exposures

Adult oysters were exposed to oil using algae as a carrier. Oil was sorbed onto the algae (shellfish diet) with or without dispersant and fed to adult oysters daily for 4 weeks, with gentle aeration to stimulate feeding. Individuals in control treatments were fed uncontaminated algae. Exposures were conducted in 40-L glass tanks with 20–25 individuals/tank and 3 replicates/treatment. For the duration of the experiment, water was changed thrice a week and oysters were fed a shellfish diet (5% wet weight) for maximal growth. Dissolved oxygen (DO), temperature, and salinity were measured just before water changes. Individuals were examined daily for obvious mortality, although mortality was at times difficult to ascertain from outside of the tank. Individuals were closely inspected for mortality during every water change. Mortality of test organisms was measured in the exposure chambers by evidence of lack of valve closure upon stimulation.

Fertilization success tests

Fertilizations between gametes were performed by mixing 10 mL of sperm with 200 mL of eggs in a 400-mL beaker. Aliquots of fertilized egg samples were fixed at 60-minutes post-insemination to assess fertilization success. Remaining embryos were followed for developmental abnormalities for 24 and 96 hours.

Samples were fixed in 3.2% paraformaldehyde in seawater. To label DNA, fixed samples were stained with 1-µg/mL Hoechst 33342 for 15 minutes, and then washed twice with seawater.

Fertilization success was determined by examining the formation of polar bodies and the cleavage of fertilized eggs into embryos. In addition, embryo growth and metamorphosis of embryos into veliger were followed over a span of 96 hours.

Acute effects on sperm and oocyte activity

Sperm activity after exposure to CEWAF, HEWAF, or dispersant was assessed by examining the viability, motility, mitochondrial membrane potential (MMP), and oxidative activity. Detailed procedures are described in Testing Protocols 1 (A.6) and 11 (K.1.1) of the FGCU GLPP. The cellular characteristics of developing oocytes were assessed following exposure to CEWAF and HEWAF by examining morphology, viability, and oxidative activity. Detailed procedures are described in Testing Protocol 11 (K.1.2) of the FGCU GLPP.

Adult dietary exposures, progeny effects assessments

HEWAF, CEWAF, and/or dispersant were added to the algal diet where the PAHs adsorb to the algal particles. Oysters were exposed to contaminant-spiked algae on a daily basis, during feeding, filtration, and ingestion of algal particles.

Acute suspended sediment exposure toxicity tests

Effects of sediment-associated contaminants on various life stages of oysters (Larvae–Adult) were assessed using decanted sediment suspension according to the protocol described above for HEWAF and CEWAF.

Adult immunological response effects assessments

Adult oysters collected from the field (e.g., Estero Bay, Florida) were exposed to similar concentrations of oil and/or dispersant for up to 30 days. Adult oysters were exposed to Slick A and/or weathered source oil using algae as a carrier. Oil was sorbed onto the algae (shellfish diet) with or without dispersant and fed to adult oysters daily for 4 weeks, with gentle aeration to stimulate feeding. Control treatments were fed algae only without oil and/or dispersant. Oysters from all treatments were sampled at the end of 2 weeks and 4 weeks, and analyzed for susceptibility to *P. marinus* (prevalence and intensity), inflammatory responses, and hemocyte immune responses (viability, phagocytosis, respiratory burst, and MMP). For more information, see Testing Protocol 1 of the FGCU GLPP under the section, *Hemocyte Populations, Concentration, and Viability*.

Acute aqueous exposure, salinity effects assessments

Oyster gametes, embryos, and larvae were exposed to multiple concentrations of oil and/or dispersant in an aqueous matrix. The aqueous matrix included two different salinities (5 ppt and

25 ppt) and two different ambient temperatures (25°C and 30°C). Tests were conducted in 400-mL exposure chambers for 4 days, with no water renewal (static exposure). Three replicates were used for each treatment; each replicate contained approximately 4,000 eggs and fertilized embryos, or 3,000 larvae. Survival, growth, and normal development were used as end points for larvae. Larvae were fed with cultured microalgae on day 1 and day 3 (for more details, see Testing Protocol 1 of FGCU GLPP).

Early spat of oysters were exposed to various concentrations of oil and/or dispersant in an aqueous matrix. The aqueous matrix included two different salinities (5 ppt and 25 ppt) and two different ambient temperatures (25°C and 30°C). Tests were conducted in 600-mL exposure chambers for 8–10 days. A minimum of 3–5 replicates were used for each treatment; each replicate contained 15 spat. Survival was used as an end point for early spat (10–15 mm). Clearance rates (i.e., the rate at which the oysters cleared the algae) were also measured. Spat were fed with a daily shellfish diet and water was changed every other day.

2.1.5 Water quality monitoring

See the QAPP and test conditions tables (TCTs) for required monitoring.

2.1.6 Histology

Gametogenic stage was identified under a microscope according to Fisher et al. (1996) and Goldberg (1980). This approach has been modified and successfully used by the PI's group (Volety et al., 2003, 2009; Volety, 2008).

For histologic sectioning, a 3–5 mm thick band of tissue was cut transversely with a razor blade in such a manner as to contain portions of mantle, gill, digestive tubule, and gonad (Figure 2.1). Dissected tissue was fixed for 1 week in Davidson's fixative, and stored in 70% ethanol for at least 24 hours before paraffin embedding. After embedding, sections were made with a microtome, and slides were stained with hematoxylin and eosin. Gonadal portions of the sections were observed by light microscopy to determine gender and gonadal condition (see Table 2.1).

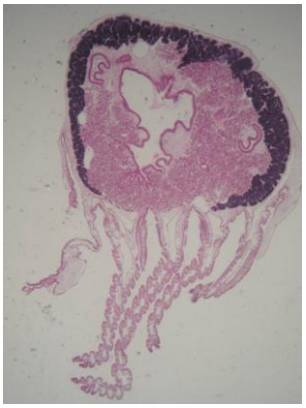

Figure 2.1. A cross-section of oyster tissue (histological section) showing mature gonadal tissue (stained dark). Histological sections are used to estimate the gonadal condition of oysters.

Table 2.1. Microscopic evaluation of gonadal condition in the eastern oyster

Value	Observations
0	Neuter or resting stage with no visible signs of gametes
1	Gametogenesis has begun with no mature gametes
2	First appearance of mature gametes to approximately one-third mature gametes in follicles
3	Follicles have approximately equal proportions of mature and developing gametes
4	Gametogenesis progressing, but follicles dominated by mature gametes
5	Follicles distended and filled with ripe gametes; limited gametogenesis; ova compacted into polygonal configurations, and sperm have visible tails
6	Active emission (spawning) occurring; general reduction in sperm density or morphological rounding of ova
7	Follicles one-half depleted of mature gametes
8	Gonadal area is reduced, follicles two-thirds depleted of mature gametes
9	Only residual gametes remain, some cytolysis evident
10	Gonads completely devoid of gametes, and cytolysis is ongoing

Hemolymph collection

Hemolymph was withdrawn from each oyster through a notch ground on the shell (Volety et al., 1999). Collected hemolymph was then immediately transferred into micro-tubes maintained on ice to minimize the cell clumping. All individual samples were microscopically checked for purity and then filtered through an 80-µm nylon mesh. Subsequent analyses were performed on individual samples (Donaghy et al., 2009a, 2009b, 2010; Haberkorn et al., 2010a).

Flow cytometry analyses

These were performed as previously developed by co-PIs P. Soudant and L. Donaghy (Donaghy et al., 2009b, 2010; Haberkorn et al., 2010a, 2010b). See the *Hemocyte Populations, Concentration, and Viability* section in Testing Protocol 2 of the FGCU GLPP.

2.1.7 Archived water and tissue samples

Archiving water and tissue samples (including any unused material) followed protocols outlined in the QAPP. During the acute toxicity testing, subsamples (~ 10 mL) of the solution containing gametes, embryos, or larvae were fixed using formalin for the analyses of fertilization success, gametogenic and larval development, developmental abnormalities, and survival. Any unused material from the formalin fixed samples was retained and stored at room temperature for further

analyses. Any unused tissue from the experiments that was not designated for any specific analyses was pooled from the same replicate tank/exposure chamber and frozen at 20°C.

2.1.8 Analytical chemistry sampling

Analytical chemistry of tissue or CEWAF, HEWAF, and LEWAF was conducted onsite at FGCU; the Centre of Documentation, Research and Experimentation on Accidental Water Pollution (CEDRE); France; and offsite at ALS Environmental. Oyster tissue sample analyses were conducted onsite and offsite at ALS Environmental, in addition to the water sample analyses. Generally, onsite analytical testing was conducted when quick turnaround results were needed. Offsite analytical testing was used to characterize the water chemistry of exposure treatments when conducting definitive testing. Samples were shipped to the offsite laboratory under strict chain-of-custody (COC) procedures in laboratory-provided sample containers and shipping coolers.

Concentrations of PAHs in oyster tissues and gametes were determined using gas chromatography-mass spectrometry (GC-MS) according to Munschy et al. (2005). When applicable, PAH concentrations in the water were determined according to Roy et al. (2005).

2.2 General Testing Standard Operating Procedures

2.2.1 Solutions preparation

Three or four treatments plus one control (below) were used in these studies. Dilutions were made from the stock solution to obtain appropriate concentrations for each treatment.

Final numbers and types of experimental treatments were provided in TCTs provided to Stratus Consulting during the test approval and test identification (ID) assignment process.

Oil and dispersant preparation

Preparation of LEWAFs, CEWAFs, and HEWAFs were carried out according to procedures outlined in the QAPP. Seawater at a salinity of ≈ 20 ppt was used as a control.

2.2.2 Collection and maintenance of the broodstock

Test oysters were collected from Estero Bay, Florida, and acclimated to test conditions (e.g., 25°C and ≈ 20 ppt) at the Vester Marine Station. They were thoroughly cleaned by hand using a brush to remove biofouling organisms and weighed; 50 adult oysters were stocked in a 250-L tank inside the hatchery. Oysters were maintained at 23°C ± 1°C in coarsely filtered

(20 µm) UV-treated seawater at ambient salinity (≈ 20 ppt). They were fed a mixture of freshly cultured microalgae (T-*Isochrysis*, *Tetraselmis* sp. and *Chaetoceros* sp.) at a daily ration of 3% of dry body weight for a period of 2 to 5 weeks to develop mature gametes at 23–25°C. Ten oysters were periodically sampled and examined for ripeness under a microscope.

2.2.3 Spawning method and gamete recovery

Mature oyster broodstocks were induced to spawn by thermal stimulation, alternating immersion in seawater at 17–18°C and 30°C every 30 minutes. Spawning females were isolated and placed in 1-L beakers filled with FSW for collection of oocytes, while spawning males were placed in about 200 mL of FSW to obtain a dense sperm solution. Oocytes and sperm were examined under a microscope for motility and viability and the best broodstocks were selected. After filtration through a 35-µm mesh to remove debris, sperm from several males were pooled in a 1-L sterile beaker. Eggs from several females, after successive sieving through 100-µm and 55-µm mesh to remove tissue and debris, were transferred into a 2-L sterile beaker filled with FSW. A total of 50 µL of eggs were taken from the 2-L stock and fixed and stained using Lugol for egg counts after continuous and gentle mixing (by means of a rod with attached perforated disc), using a Sedgwick-Rafter cell and a dissecting microscope.

2.2.4 Water quality analyses

Salinity, temperature, DO, and pH: Monitored daily in one replicate, and ammonia was tested from one replicate before each water change.

Temperature: Temperatures in the test chambers and stock solutions were obtained by a thermometer or alternatively using a YSI Pro ODO meter with an oxygen and temperature probe. When this probe was used, it was calibrated prior to every test.

Salinity: Salinity was obtained using a refractometer. This instrument is commonly used in the laboratory and field. Accuracy of the refractometer was checked once monthly using salinity standards.

DO: A YSI Pro series sonde with an optical DO probe was used to measure DO in all the stock solutions and test chambers. The probe was calibrated according to manufacturer's specifications prior to every experiment.

pH: pH was measured in a subset of exposures and litmus paper was used to determine any pH changes in the oiled treatments.

Ammonia: Ammonia in the stock solutions and test chambers followed the Seal Analytical Ammonia analyses in water and seawater procedure [Method No. G 171-96 Rev. 14 (Multitest

MT19)]. This method uses the Berthelot reaction, in which a blue-green colored complex is formed and measured at 660 nm. A complexing agent was used to prevent the precipitation of calcium and magnesium hydroxides. Sodium nitroprusside was used to enhance the sensitivity.

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A. Testing Protocol 1: Acute Toxicity Testing – Gametes, Embryos, Veliger Larvae, and Spat WAF Exposures

For all of the toxicity testing experiments described below, the placement of treatment chambers was randomized.

Final testing conditions were specified in the TCTs provided to Stratus Consulting during the test approval and test ID assignment process.

A.1 Gamete Exposures

Sperm and eggs were exposed to various concentrations of CEWAF, LEWAF, HEWAF, or dispersant for 30 minutes before being combined for assessment of fertilization rates and subsequent embryonic and larval development. Fertilization rates were determined by the presence of the polar body and/or division of the egg into an embryo.

Eggs were taken from the stock solution after continuous and gentle mixing (by means of a rod with an attached perforated disc) using an adjustable-volume pipetter, and stocked in the beakers at a density of 20 eggs/mL. Each replicate jar (n = 4; 400-mL beaker) contained 200 mL of CEWAF, LEWAF, HEWAF, or dispersant solution (~ 4,000 eggs/replicate/treatment). In a separate container, 10 mL of sperm solution was added to 40 mL of CEWAF, LEWAF, HEWAF, dispersant solution, or seawater. Gametes were exposed to these solutions for 30 minutes before addition of the sperm (10 mL) solution to eggs for fertilization. The fertilization of eggs by sperm began immediately, first resulting in the formation of a polar body (30 minutes), and rapidly progressing to cell division and embryo formation (45 minutes).

Fertilization success was estimated by taking a subsample of 5–10 mL from the test chamber. At least 50–100 eggs/embryos were visually examined under a microscope to assess fertilization rate (% fertilization). Embryos remaining in the test chamber were allowed to divide and progress to the D stage for 24 hours. At the end of the 24-hour period, a subsample of 5–10 mL was taken from all test chambers and transferred into a 15-mL centrifuge tube and fixed with buffered 0.1% formalin. These samples were evaluated under a microscope for developmental abnormalities, and examined for developmental success, abnormalities, and viability of the embryos. Any abnormal development, as embryos developed into veliger larvae, was noted.

The live and dead veliger larvae in the 24-hour subsample were counted and recorded, although it should be noted that undeveloped/dead veliger larvae may have disintegrated and thus may not

have been counted. At least 50 larvae were counted for each replicate/treatment. Remaining larvae in the test chambers were allowed to progress to the veliger stage. After 96 hours, larval survival was assessed under a microscope. Prior to September 2012, veliger larvae remaining after 96 hours were collected on a sieve, rinsed, resuspended with 20 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 200 µL after homogenization from each 50-mL centrifuge tube and counted. Starting in September 2013, veliger larvae remaining after 96 hours were collected on a sieve, rinsed, resuspended with 30 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted. Water samples were collected daily to determine salinity, temperature, DO, and pH, and ammonia was tested in representative chambers at the start and the end of the experiment.

Photoenhanced toxicity exposures were carried out similarly, except that they were done using outdoor UV exposure systems as detailed in other University of North Texas (UNT) GLPP protocols.

A.2 Embryo Exposures

Two to four cell-staged embryos were taken from the stock solution after continuous and gentle mixing (by means of a rod with an attached perforated disc) using an adjustable-volume pipetter, and stocked in the beakers at a density of 20 mL. They were exposed to various concentrations (5 test treatments + control) of seawater, CEWAF, LEWAF, dispersant, or HEWAF in 400-mL beakers containing 200 mL of seawater. Stocking density of embryos was 20 embryos/mL (4,000 embryos/replicate/treatment). There were 4 replicates/treatment. At the end of 24 hours and 96 hours, a subsample of 5–10 mL was taken from all test chambers and transferred into a 15-mL centrifuge tube and fixed with buffered 0.1% formalin. These samples were evaluated for developmental abnormalities and were examined for developmental success, abnormalities, growth, and viability of the embryos and veliger larvae. Any abnormality in the development or morphology was noted. For example, failure to develop into a D-shaped veliger larvae, a lack of shell, or a deformed shell were noted as abnormalities. After 96 hours, larval survival was assessed: prior to September 2012, remaining veliger larvae were collected on a sieve, rinsed, resuspended with 20 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 200 µL after homogenization from each 50-mL centrifuge tube and counted. Starting in September 2013, the remaining veliger larvae were collected on a sieve, rinsed, resuspended with 30-mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted.

Water samples were collected daily to determine salinity, temperature, DO, and pH, and ammonia was tested in representative chambers at the start and the end of the experiment.

A.3 Veliger Larvae Exposures

Veliger larvae that were 24-hours old and D-shaped were collected on a 35-µm sieve and used in toxicity testing. Veliger larvae were taken from the stock solution after continuous and gentle mixing (by means of a rod with an attached perforated disc) using an adjustable-volume pipetter, and loaded in each exposure chamber at a density of 15/mL. There were 5 treatments with 4 replicates/treatment, which included HEWAF, LEWAF, CEWAF, and dispersant only exposures. All tests were conducted at approximately 25°C and 20 ppt with gentle aeration. Algae were provided as food when larvae were 1 and 3 days old at a ratio of 1×10^5 cells/mL of *Isochrysis* sp. At test initiation and after 48 hours of the exposure, a subsample of either 5 mL (prior to September 2012) or 10 mL (after September 2012) was taken from all test chambers and transferred into a 15-mL centrifuge tube and fixed with buffered 0.1% formalin. These samples were evaluated under a microscope for developmental abnormalities, and were examined for developmental success, abnormalities, growth, and viability of the veliger larvae. Any abnormalities in development or morphology, such as failure to progress toward an umbo stage or having a deformed shell, were noted. After 96 hours, larval survival was assessed: prior to September 2012, remaining veliger larvae were collected on a sieve, rinsed, resuspended with 20 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 200 µL after homogenization from each 50-mL centrifuge tube and counted. Starting in September 2013, the remaining veliger larvae were collected on a sieve, rinsed, resuspended with 30 mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted. Water samples were collected daily to determine salinity, temperature, DO, and pH, and ammonia in representative chambers at the start and the end of the experiment.

A.4 Umbo Larvae Exposures

Ten-day old umbo larvae were collected on a 90-µm sieve for use in toxicity testing. Umbo larvae were taken from the stock solution after continuous and gentle mixing (by means of a rod with an attached perforated disc) using an adjustable-volume pipetter, and loaded in each exposure chamber at a density of 6.7/mL or ≈ 2,000 larvae in 300 mL. There were 5–6 treatments with 4 replicates/treatment, which included CEWAF, dispersant only, and HEWAF exposures. All tests were conducted at approximately 25°C and 20 ppt with gentle aeration. A bispecific food diet of microalgae (*T. lutea* and *Chaetoceros muelleri*) was provided on day 0

and day 2 of the experiment at a ratio of 1×10^5 cells/mL. At test initiation (T0), and after exposure, a subsample of 10 mL was taken from the stock (T0) and from all test chambers (T48 and T96), transferred into a 15-mL centrifuge tube, and fixed with buffered 0.1% formalin. Each replicate was examined under a microscope, and approximately 50 individuals were randomly measured using an inverted microscope equipped with an Olympus DP73 camera and CellSens image analysis software. Mean shell lengths and larval growth were determined between T0 and T96. After 96 hours of exposure, larvae from each beaker were concentrated by filtering them through a 90-µm mesh, and preserved with 0.9 mL of 10% buffered formalin to obtain a final volume of 30 mL. Final survival was assessed by taking 5 subsamples of 300 µL (n = 5) from the concentrate (30 mL) of each of the 4 replicates after homogenization. Each subsample was examined under a microscope to evaluate live and dead larvae (translucent shell or opened valves). In addition, gut fullness or percentage of veliger larvae with food in the stomach was assessed for each sample. Water samples were collected daily to determine salinity, temperature, DO, and pH; and ammonia was sampled in representative chambers at the start and end of the experiment.

A.5 Spat Exposures

Early spat (5–15-mm length, ~ 30 days post-hatch) were exposed to either HEWAF, CEWAF, dispersant treatments, or control treatments consisting of FSW for up to 14 days (see test-specific TCTs). For oyster spat, each test chamber consisted of 15–25 individuals (see test-specific TCTs). Exposures were conducted under static-renewal conditions. Exposure media were renewed every other day and water quality was monitored throughout the exposure period. Survival was assessed at each water change.

A.6 Sperm Activity Assays – Flow Cytometry

Flow-cytometry analyses were performed using a Cytomix FC500 (Beckman Coulter) equipped with a 480-nm argon laser. Collected data were analyzed with WinMDI 2.9 software. Analysis of membrane integrity, MMP, and reactive oxygen species (ROS) production of spermatozoa were measured according to Rolton et al. (2015).

Sperm motility and viability

The percentage of motile spermatozoa was assessed using a two-step dilution in a salt sperm activating solution – "Moti-gigas" (Brizard et al., 2004) – and observed through a dark field microscope. Flow-cytometry analyses were performed to assess gamete health as previously developed by Dr. Soudant's laboratory (co-PI; Haberkorn et al., 2010). An aliquot of 200 µL of spermatozoa suspension (diluted at 2 x 10^5 cell mL^{-1}) from each selected male oyster was

transferred into a tube containing FSW. Spermatozoa DNA was stained with two fluorescent DNA specific dyes, SYBR-14 and propidium iodide, in the dark at 18°C for 10 minutes before flow-cytometric (FCM) analysis. Propidium iodide permeates only spermatozoa that lose membrane integrity and are considered to be dead (necrotic), whereas SYBR-14 permeates both dead and live cells. Fluorescence of SYBR-14 and propidium iodide was measured at 500–530 nm (green) and at 550–600 nm (orange), respectively, by flow cytometry. This method distinguishes viable, "dying" (propidium iodide partially incorporated), and dead cells. By counting the cells stained by SYBR-14 green and cells stained by propidium iodide, it was possible to estimate the percentage of dead cells in each sample.

Mitochondrial membrane potential

The MMP of oyster spermatozoa was measured using the potential dependent JC-10 assay kit. This probe enters selectively into mitochondria and exists as two forms – monomeric or aggregate – depending upon membrane potential (Reers et al., 1991). The JC-10 monomer form predominates in cells with low MMP and emits in the green wavelength (525–530 nm). The JC-10 aggregate form accumulates in mitochondria with high membrane potential and emits in the orange wavelength (590 nm). JC-10 forms can change reversibly.

A sample of the spermatozoa suspension was diluted in FSW containing JC-10. An aliquot of 200-mL spermatozoa mixture at 2×10^5 cell mL^{-1} was stained with JC-10 (final concentration 5 $\mu molL^{-1}$, for 5 minutes) and then diluted at 1:10 to stop the reaction prior to FCM analysis. Samples were incubated for 10 minutes at room temperature. Fluorescence intensities of JC-10 monomers and aggregates were quantified, respectively, by FL1 (green) and FL2 (orange) detectors of the flow cytometer. The JC-10 aggregate/monomer ratio is assumed to be proportional to MMP (Reers et al., 1991; Cossarizza et al., 1996). Membrane potential of active cells was estimated by the ratio of aggregate/monomer (i.e., orange/green fluorescence ratio).

Reactive oxygen species production

Determination of oxidative activity was performed at room temperature (20–22°C) using 2'7'-dichlorofluorescein diacetate (DCFH-DA), a membrane-permeable, non-fluorescent probe. Inside cells, the -DA radical is first hydrolyzed by esterase enzymes. Intracellular hydrogen peroxide (H_2O_2) and superoxide ion (O_2-•) then oxidize DCFH to the fluorescent 2',7'-dichlorfluorescein (DCF) molecule. Oxidation of DCFH can also be mediated by nitrite radicals (NO_2 or N_2O_3) and various oxidase and peroxidase enzymes. DCF green fluorescence, detected on the FL1 detector of the flow cytometer, is proportional to the total oxidative activity of cells, including ROS. DCFH-DA (10 µM final) was added to a 200-µL suspension of 2×10^5 spermatozoa mL^{-1} for 30 minutes. The oxidative activity was expressed as fluorescence arbitrary units (AUs).

Acrosomal integrity

Analysis of acrosomal integrity of spermatozoa was adapted from Thomas et al. (1997) and Donaghy et al. (2010). The acrosome is an acidified organelle that can be specifically stained by LysoTracker probes, which spontaneously enter the acrosome. Once the acrosome is no longer intact, the dye then diffuses out of the organelle, resulting in a decrease of associated fluorescence. An increase in the volume of the acrosome meanwhile results in a higher fluorescence intensity. After exposure to dispersant, CEWAF, HEWAF, and 200 mL of spermatozoa at 2 x 10^5 cell mL^{-1} were incubated with LysoTracker Red DND-99 (Molecular Probes, Invitrogen; final concentration of 1 $\mu molL^{-1}$ for 10 minutes). Acrosomal activity was expressed as the level of red fluorescence, detected on the FL3 detector of the flow cytometer.

B. Testing Protocol 2: Chronic Toxicity Testing – Adult Oysters

Adult oysters collected from the field (e.g., Estero Bay) were exposed to similar concentrations of oil and/or dispersant for an extended period (e.g., 30 days). Adult oysters were exposed to a slurry of *Deepwater Horizon* (DWH) oil and algae. Oil was mixed with the algae (shellfish diet) with and without dispersant and fed to adult oysters daily, with gentle aeration to stimulate feeding. Control treatments were fed algae only, without oil and/or dispersant. Oysters from all treatments were sampled periodically and analyzed for *P. marinus* prevalence, inflammatory responses, hemocyte immune responses, etc. Representative samples of oysters from each treatment were analyzed for tissue bioaccumulation, histology, and other endpoints, at predetermined points (e.g., day 14) and at the end of the experiment.

Final testing conditions were specified in TCTs provided to Stratus Consulting during the test approval and test ID assignment process.

B.1 Preparation of Contaminated Algae

A shellfish diet (Reed Mariculture) was used to feed oysters for the duration of in vivo exposures. Adult oysters were fed 0.5 mL of algae/oyster each day. DWH oil (concentrations varied by test and were specified in the TCTs) and/or dispersant (10 or 20 mg/oyster/day) was added to the vortex of spinning algae in a beaker and stirred using a stir bar for 30 minutes to allow the oil to adsorb to the algae. Control oysters received only algae (0.5 mL/oyster each day). The amount of algae added to each test chamber was adjusted based on the number of oysters in each test tank. Any mortality in oysters and the amount of algae added to each tank were logged.

B.2 *P. marinus* Prevalence and Intensity

Differences in disease expression (i.e., prevalence/percentage of infected oysters) and intensity of disease in various treatments were examined. Disease prevalence, intensity of *P. marinus*, growth, and survival among adult oysters were used as end points. The prevalence and intensity of *P. marinus* in adult oysters were analyzed using Ray's fluid thioglycollate medium technique (Ray, 1954; Volety et al., 2000, 2003, 2009). Samples of gill and digestive diverticulum were incubated in the medium for 5–7 days. Prevalence of infection was calculated as a percentage of infected oysters. The intensity of infection was recorded using a modified Mackin scale (Mackin, 1962), in which 0 = no infection, 1 = light, 2 = light-moderate, 3 = moderate, 4 = moderate-heavy, and 5 = heavy infection (Volety et al., 2009).

B.3 Inflammation

Inflammatory responses were histologically determined by observation of potential hemocyte infiltrations in tissues using histological techniques. Oysters were opened and underwent gross examination for abnormalities, and a section of soft tissues that included all organs was excised and fixed in Davidson's solution prior to sectioning. For histologic sectioning, a 3–5 mm thick band of tissue was cut transversely with a razor blade in such a manner as to contain portions of mantle, gill, digestive tubule, and gonad. Dissected tissue was fixed for at least 1 week in Davidson's fixative and stored in 70% ethanol for at least 24 hours before paraffin embedding. After embedding, sections were made with a microtome, and slides were stained with hematoxylin and eosin prior to observation (Fisher et al., 2000; Oliver et al., 2001).

B.4 Hemocyte Populations, Concentration, and Viability

In oysters, three main hemocyte populations can be observed: blast-like cells, hyalinocytes, and granulocytes. The concentration and percentage of each hemocyte subpopulation were determined in collected hemolymph using flow cytometry (Donaghy et al., 2009).

Cellular parameters (viability, oxidative activity, MMP, phagocytosis, and lysosomal content) were analyzed on circulating cells of both exposed and non-exposed oysters. Mitochondrial respiration was assessed through the evaluation of both the oxidative activity and the membrane potential (Donaghy et al., 2009; Haberkorn et al., 2010).

Populations, concentration, and viability of hemocytes were evaluated using a double-staining procedure that included SYBR Green I and propidium iodide. Hemolymph was diluted in FSW containing SYBR Green I and propidium iodide and incubated for 120 minutes in the dark at 10°C prior to FCM data acquisition.

SYBR Green I is a membrane-permeable fluorescent dye that binds to double-stranded DNA and then emits green fluorescence (520 nm). Detection of this fluorescence allows distinction between single cells and aggregates, as well as debris. Hemocyte morphology is based upon relative FCM parameters, Forward Scatter (FSC), and Side Scatter (SSC). FSC and SSC commonly measure particle size and internal complexity, respectively. Morphological parameters are expressed in FCM AUs, and total hemocyte count (THC) is reported as the number of cells per milliliter of hemolymph. Membranes of viable cells do not allow propidium iodide to penetrate, whereas altered membranes are permeable by propidium iodide. Dead cells are characterized by loss of membrane integrity and are therefore double-stained by SYBR Green I and propidium iodide. Hemocyte mortality is expressed as the percentage of double positive cells.

B.5 Lysosome Quantification

The presence of lysosomes in hemocytes was determined using LysoTracker Red, a membrane-permeable, fluorescent red probe (emission maximum at 590 nm) that accumulates within lysosomal compartments. Hemolymph was diluted in FSW containing LysoTracker Red. Mixed solutions were incubated for 60 minutes in the dark, at room temperature. Tubes were then transferred and held on ice until FCM analysis. Relative intracellular lysosomal quantity was expressed as the level of red fluorescence (Donaghy et al., 2009; Haberkorn et al., 2010).

B.6 Phagocytosis Capacities

An oyster hemolymph sample was mixed with an equal volume of FSW containing fluorescent latex beads (2.0 μm in diameter). After 120 minutes of incubation at room temperature in the dark, the reaction was stopped by placing tubes on ice until FCM analysis. The percentage of phagocytic cells, defined as the percentage of cells that have internalized three or more of the fluorescent beads (Donaghy et al., 2009; Haberkorn et al., 2010), was then determined by flow cytometry.

B.7 Intracellular Oxidative Activity

The intracellular oxidative activity, including the production of ROS, was determined using DCFH-DA. A solution of DCFH-DA was added to hemolymph diluted in FSW. The mixtures were then incubated at room temperature for 120 minutes prior to FCM analysis. The oxidative activity was expressed as fluorescence AU (Donaghy et al., 2009; Haberkorn et al., 2010).

B.8 Mitochondrial Membrane Potential

Estimation of MMP was performed using JC-10. Hemolymph was diluted in FSW containing JC-10 and incubated for 30 minutes in the dark at 20°C prior to FCM analysis. Relative MMP intensity was expressed as the ratio between the levels of green fluorescence (FL1 detector of the flow cytometer) and MMP orange fluorescence (FL2 detector) (Donaghy et al., 2009; Haberkorn et al., 2010).

B.9 Water Sampling for Chemical Analysis

Water samples were collected throughout exposure and analyzed for PAHs at ALS Environmental.

The water samples were taken directly from the tank in 250-mL amber bottles. Sample volume, container type, and labeling and handling methods followed the guidelines provided in the QAPP.

Spot check water samples were sometimes collected when the tank water in treatments was renewed. Samples were made by filling the required type and number of sample bottles with water from one tank before the renewal process. Spot check samples were collected from the water column under the water surface, and tank detritus was avoided. Spot check water samples were not filtered. Sample volume, container type, and labeling and handling methods followed the guidelines provided in the QAPP, and samples were sent to ALS Environmental for PAH analysis.

Oyster tissue samples were collected at 14 days and at the end of the exposure period. Oyster samples were held at 4°C and sent to ALS Environmental, where the oysters were shucked and PAH and total lipid analyses on the composite samples were conducted.

B.10 Archiving Water and Tissue Samples

Archiving water and tissue samples (including any unused material) followed the protocols outlined in the QAPP. During the acute toxicity testing, subsamples (~ 10 mL) of the solution containing gametes, embryos, or veliger larvae were fixed using formalin for the analyses of fertilization success, gametogenic and larval development, developmental abnormalities, and survival. Any unused material from the formalin-fixed samples was retained and stored at room temperature for further analyses. Any unused tissue during or at the end of the experiments that was not designated for any specific analyses was pooled from the same replicate tank/exposure chamber and frozen at -20°C.

B.11 Renewal Procedures

Tank water was renewed every Monday, Wednesday, and Friday and renewals occurred after water sampling. See test-specific TCTs and bench sheets for information regarding the exact days samples were collected. All of the water in each tank was removed and replaced with new, clean water. After filling the tank, algae/oil mixtures were added.

C. Testing Protocol 3: Chronic Toxicity Testing – Dietary Exposure of Adult Oysters to Assess Impacts on F-2 Generation

Effects of DWH oil and/or dispersant on the F-2 generation were examined using adult oysters. Reproductively active broodstock of oysters were exposed to DWH oil and/or dispersant using algae as carrier particles for 2–4 weeks in 40-L aquaria. A concentration of oil (see test-specific TCTs) was added to the algal diet where the PAHs adsorb to the algal particles, as described in the previous section. Oysters ingest these PAHs during filtration and ingestion of algal particles. This method was successfully used by Chu and Hale (1994) and Choy et al. (2007) to deliver PAHs to *C. virginica* and *C. gigas* oysters.

Oysters were fed a shellfish diet daily and water was changed every Monday, Wednesday, and Friday during the exposure period. After the exposure period, oysters were induced to spawn, and reproductive and developmental success (i.e., fertilization success, embryonic development, metamorphosis to veliger larvae/umbo larvae) were determined at 1, 24, 48, 72, and 96 hours. In addition, histological analyses were used to examine the gonadal state of a subset of oysters from different treatments. The gametogenic stage was identified under a microscope according to Fisher et al. (1996) and the National Academy of Sciences (1980). This approach has been modified and successfully used by the PI's group (Volety et al., 2003, 2009; Volety, 2008). For histological sectioning, a 3–5 mm thick band of tissue was cut transversely with a razor blade in such a manner as to contain portions of mantle, gill, digestive tubule, and gonad. Dissected tissue was fixed for approximately 1 week in Davidson's fixative and stored in 70% ethanol for at least 24 hours before paraffin embedding. After embedding, sections were made with a microtome, and slides were stained with hematoxylin and eosin. Gonadal portions of the sections were observed by light microscopy to determine gender and gonadal condition.

All analytical chemistry sampling was conducted as described in Section B.9, "Water Sampling for Chemical Analysis," with the exception that tissue samples were collected at both 2-week and 4-week assessment time points. Water renewals followed the methods described above for adult chronic exposures.

D. Testing Protocol 4: Acute Toxicity Testing – Gametes, Embryos, and Veliger Larvae – Sediment Elutriate Exposures

D.1 Preparation of Sediment Elutriates

Bioassays were conducted using sediment elutriates according to modified protocol from Chu and Hale (1994) and Geffard et al. (2003). Seawater (25 ppt) was added to contaminated (LAAR38-B0123-SX401) or reference (LAAR42-C0208-SX403) sediment in a ratio of 10:1 and mechanically stirred (300 RPM) for 6 hours. Sediment was allowed to settle for 12 hours. For the preparation of sediment elutriates, supernatant (100% stock) was siphoned off and mixed with FSW in a dilution series to nominal concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5625, and 0 (control) percent elutriate.

Gametes, embryos, and veliger larvae of *C. virginica* were exposed to varying dilutions of the sediment elutriate described above. Protocols for exposure were similar to those described in FGCU GLPP Appendix A: *Acute Toxicity Testing – Gametes, Embryos, Veliger Larvae, and Spat WAF Exposures*. After 96 hours, larval survival was assessed. The remaining veliger larvae were collected on a sieve, rinsed, resuspended with 30-mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted.

D.2 Water Sampling Schedule

Water samples were collected for chemical analyses of PAHs and fluorescence. Specifically, water chemistry samples were collected from the highest nominal exposure treatment concentration (50% supernatant stock) after diluting and mixing, just before test initiation. Water chemistry samples were not filtered. PAH water chemistry samples were collected, handled, and analyzed as discussed in the QAPP and sent to ALS Environmental. Observations of oil sheen and turbidity in water chemistry samples or exposure waters were noted in data reporting tables for each test.

Water samples were collected for fluorescence analysis of dilution splits. Specifically, a standard curve was created using a fluorescence sample from the highest exposure treatment concentration after diluting and mixing, just before test initiation. Additional fluorescence analysis samples were collected from each treatment and analyzed using the standard curve to verify nominal dilution concentrations. Fluorescence sampling and analyses were conducted

in-house as discussed in the QAPP. If fluorescence samples were not taken and analyzed, additional water chemistry samples were taken from each dilution series. These samples were analyzed for PAHs or sent to the analytical laboratory for extraction and archival.

E. Testing Protocol 5: Whole Sediment Exposures – Adult Oyster Toxicity Testing

Adult oysters collected from the field (e.g., Estero Bay) were exposed to field-collected or prepared sediments potentially impacted with oil for a total of 14 days. Reference sediments were used as a negative control. Adult oysters were exposed to a sediment:water slurry added to the aquaria, with gentle aeration using a mini-pump to help maintain suspension of particles within the aquaria. The slurry was added every 3 days, just after the water renewal, at a rate of 3-g sediment/oyster (e.g., 60 g/aquaria, given 20 oysters/replicate).

All adult oysters were maintained in the exposure aquaria throughout the exposure period. Both reference and exposure treatments were similarly maintained. Water quality was maintained via static renewal conditions, where approximately 95% of the water and sediment particulates was removed and renewed every 3 days. Water suspended particulates were removed by siphoning. Care was taken to remove as much of the sediment as possible at each renewal. New sediment:water slurry was added to the aquaria just after renewal of water.

During exposures, the light cycle was held at 12L:12D and oysters were fed daily with a commercially prepared shellfish micro-algae diet at a rate of 0.5 mL of algae diet/oyster. On the days that the sediment:water slurry was added, the algae was added just after the slurry. The amount of algae and slurry added to each test chamber was adjusted based on the number of oysters in each test tank.

Adult oysters were inspected for mortalities and general health each day. Oysters from all treatments were sampled periodically and analyzed for *P. marinus* prevalence, inflammatory responses, and hemocyte immune responses as described in FGCU GLPP Testing Protocol 2, *Chronic Toxicity Testing – Adult Oysters,* above. After the exposure period, oysters were induced to spawn, and reproductive success (fertilization success, embryonic development, metamorphosis to veliger/pediveliger larvae) was determined as described in FGCU GLPP Testing Protocol 3, *Chronic Toxicity Testing – Dietary Exposure of Adult Oysters to Assess Impacts on F-2 Generation*. In addition, histological analysis was used to examine the gonadal state of a subset of oysters from different treatments. Representative samples of oysters from each treatment were also analyzed for tissue bioaccumulation, histology, etc., at the end of the experiment. Specific endpoints assessed for each test were listed in the TCTs provided to Stratus Consulting during the test approval and test ID assignment process. Dead oysters were removed from exposure tanks and archived according to the QAPP. Numbers of individual oysters used for endpoint assessments depended on the total number of oysters that remained after sediment exposures.

E.1 Sediment Preparation

Test sediments, provided by the National Oceanic and Atmospheric Administration (NOAA) in buckets or bags, were used as collected or blended before being used for toxicity testing. Table E.1 provides a list of sediment types that were tested. When not used, sediments were stored frozen, under strict COC. Care was taken to not defrost the entire sediment lot when aliquoting volumes needed for tests at hand. Excess aliquoted sediment was saved and archived.

Table E.1. Sediment types that may be used in adult sediment exposures

Loomis II 2012 (reference) (LAAR42-C0208-SX403)
Blend: 12.5% Black Hole 2011, 87.5% Loomis II 2012
Black Hole 2012 (LAAQ43-C0409-S61317-A)
Black Hole 2011 (LAAR38-B0123-SX401)

When required, test sediment blending was done prior to aliquoting sediments. Blending ratios were made on a weight-to-weight basis before adding water.

E.2 Test Preparation

Sediments were prepared for testing 48 hours before test initiation. For tests performed in triplicate, 1.3 kg of sediment was removed from the freezer (the blend was 12.5% Black Hole 2011 and 87.5% Loomis II 2012) and placed into a large glass jar or stainless steel mixing bowl. An equal volume (1.3 L) of 20 ppt artificial saltwater was added to the frozen sediment. The jar was covered, wrapped in aluminum foil, and allowed to thaw and equilibrate for 24 hours at room temperature. After equilibration, the sediment:water slurry was thoroughly homogenized using stainless steel spoons. For each treatment, homogenized sediment was placed into 3 separate clean jars for individual tank dosing. In-between filling each replicate jar for tank dosing, an aliquot of slurry was added to a sample jar that was then sent to ALS Environmental for analyses. Slurry doses were stored in the refrigerator until test initiation. At the initiation of the test and every 3 days after test initiation, one of the jars of sediment:water slurry was emptied into the aquaria. Each replicate jar contained 3 g of sediment slurry/oyster. To ensure complete transfer of the slurry, the jar was rinsed several times by dunking the jar into tank water.

During the aliquot process, an additional 1,000 g of sediment were placed into two 8-oz jars (495 g each) for chemical analysis. The two sample jars were filled simultaneously by adding an aliquot to each sample jar after every two scoops of sediment were added to the dosing jars. The jars of slurry were stored at 4°C until used to dose tanks.

E.3 Analytical Chemistry Sampling

Both sediment and water samples were collected during this experiment. Analytical chemistry samples were handled, labeled, and shipped to ALS Environmental according to the QAPP, with the following specifications in Sections E.3.1 and E.3.2.

E.3.1 Initial sediment sampling

Sediment samples were collected from each treatment group (sediment type) when preparing tanks for sediment exposures. When preparing sediments for dosing the tanks, sediment samples from each treatment group and control were also collected for analytical chemistry (two 8-oz jars). These initial sediment samples were analyzed for PAHs, total organic carbon (TOC), total extractable hydrocarbons plus alkanes, and particle grain size at ALS Environmental.

E.3.2 Initial and spot check water sampling

An initial composite water sample was collected from each treatment group 30 minutes after addition of the first slurry. Sufficient water was collected for a filtered and unfiltered composite water sample. A second set of spot check composite water samples was collected approximately 1.5 days after the initial slurry addition. A third set of composite water samples was collected just prior to the water renewal and new slurry addition on the third day. This sampling scheme was repeated once more at the end of the test. Samples were sent to ALS Environmental and extracted and archived for the possibility of future analyses.

At each water-sampling time point, a subsample from each replicate tank within each treatment (sediment type) was collected and composited into a clean decontaminated glass mixing container. Subsamples were collected by dipping a clean, decontaminated glass beaker into each tank. The same transfer beaker was used for all tanks within a single treatment. A separate clean, decontaminated beaker was used when collecting subsamples from each additional treatment. After mixing subsamples, the composite was used to fill the required analytical chemistry containers for requested chemical analyses (see the QAPP). This process was repeated for each treatment group and control. Water samples were sent to ALS Environmental.

F. Testing Protocol 6: Multiple Stressors – Gametes, Embryos, and Veliger Larvae Toxicity Testing

Gametes, embryos, and veliger larvae of oysters were exposed to various concentrations of oil prepared following CEWAF and HEWAF methods, under two temperatures and two salinities. Gametes, embryos, and veliger larvae were exposed in an aqueous matrix with salinities of 5 ppt and 25 ppt at 25°C and 30°C (i.e., 5 ppt at 25°C and 30°C and 25 ppt at 25°C and 30°C) in 400-mL exposure chambers for 4 days, with no water renewal (static exposure). Three replicates were used for each treatment; each replicate contained 4,000 eggs and fertilized embryos or 3,000 veliger larvae. Survival, growth, and normal development were used as end points for veliger larvae. Veliger larvae were fed with cultured microalgae on day 0 and day 2 of the exposure (for more details, see FGCU GLPP Testing Protocol 1). Prior to September 2012, after 96 hours, larval survival was assessed. The remaining veliger larvae were collected on a sieve, rinsed, resuspended with 20-mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking 3 subsamples of 200 µL after homogenization from each 50-mL centrifuge tube and counted. After September 2012, after 96 hours, larval survival was assessed. The remaining veliger larvae were collected on a sieve, rinsed, resuspended with 30-mL of seawater in 50-mL centrifuge tubes, and preserved in formalin for later estimation of final survival. Final survival was assessed by taking three subsamples of 300 µL after homogenization from each 50-mL centrifuge tube and counted.

G. Testing Protocol 7: Multiple Stressors – Spat Toxicity Testing

Oyster spat from the experimental hatchery of Auburn University, Alabama, were exposed to DWH oil prepared following CEWAF and HEWAF methods, under two temperatures and three salinities. Early spat (10–15 mm, ~ 1 g individual wet weight) were exposed in 500 mL of solution in 600-mL beakers, at a stocking density of 15 spat/beaker. A range of 5 concentrations of HEWAF were tested (100%, 25%, 5%, 2.5%, and 0.5%) with a seawater control, with 3 replicates/concentration and 5 replicates/control. Three different salinities (prepared by the addition of distilled water into FSW), 5‰, 10‰, and 25‰, and two temperatures, 25°C (room temperature) and 30°C (incubator), were tested for each concentration of oil and control. The exposure lasted 7 (CEWAF) to 10 days (HEWAF), and was conducted under static-renewal conditions (exposure media changed every 2 days). Effects of various treatments on early spat were determined by examining the survival at each water change, using a 2-mm sieve. Beakers were cleaned and wiped using Kimwipes, and refilled with new exposure media.

Water samples (analytical chemistry) as well as ammonia and fluorescence were taken at each water change from each dilution, and water quality (temperature, salinity, pH, and DO) was monitored daily. Each test chamber was aerated to maintained oxygen levels above 3 mg/L and the light photoperiod was maintained at 12-hours light/12-hours dark (LD 12h:12h) using fluorescent lamps. Oyster spat in each test chamber were fed a ration of 2 mL of Shellfish Diet/TW1200 daily.

In order to measure the clearance rate, a single species algal paste, called Instant Ocean Thalasiossira (TW1200), was used. Unlike Shellfish Diet, which is a mix of 4 species of microalgae, TW1200 cells have a square shape and are large (20–40 μm), which makes them easy to count and to discriminate from oil droplets. On day 0, day 2, and day 6, oyster spat in each beaker were fed a ration of 2 mL of TW1200 and 1.5-mL subsamples were taken at T0, T4, T8, T12, and T24 after algae was added. Aliquots were fixed by adding a drop of Glutaraldehyde and cell counts were later determined under a microscope using a counting hemocytometer.

Some individuals were examined for digestive histopathology based on methods described in Lowe et al. (1981) and Winstead (1995). Additionally, 6 individuals/treatment were selected for digestive tubule measurements. For these measurements, the digestive system of an individual oyster was split into 4 quadrants and photographs taken of each quadrant. CellSens software was used to measure approximately 7–8 tubules/quadrant/oyster. Two measurements were taken per tubule; the first (A1) is the inner tubule surface area and the second (A2) is the total tubule surface area. The tubule ratio was determined by calculating A1/A2.

H. Testing Protocol 8: Whole Sediment Intertidal Exposure – Adult Oyster – Spiked Sediment

Adult oysters collected from the field (e.g., Estero Bay) were exposed to sediments collected at Estero Bay and spiked with oil for a total of 14 days. Sediment that had not been spiked was used as a negative control along with a seawater-only control. Adult oysters were exposed to a 1–2 cm thick layer of spiked sediment at the bottom of the aquaria; gentle aeration and mixing by hand before feedings helped maintain some suspension of particles within the aquaria.

All adult oysters were maintained in the exposure aquaria throughout the exposure period. Both controls and exposure treatments were similarly maintained. Water quality was maintained via static renewal conditions, where approximately 95% of the water was removed and renewed every 3 days. Water was removed by siphon, and care was taken to disturb the sediment as little as possible. During exposures, the light cycle was held at LD 12h:12h and oysters were fed a commercially prepared shellfish micro-algae diet at a rate of 0.5 mL of algae diet/oyster/day. Immediately prior to every feeding (except directly after water changes where the sediment is suspended due to filling), the water above the sediment was mixed for 10 seconds by hand with a stainless steel long-handled spoon starting from the top of the aquaria to just above the oysters, and then back to the top of the aquaria.

Final testing conditions, including numbers and size of replicates, were specified in TCTs provided to Stratus Consulting during the test approval and test ID assignment process.

Adult oysters were inspected for mortalities and general health during water changes. If *P. marinus* prevalence was being assessed (see test-specific TCTs), oysters from all treatments were sampled on day 14 and were analyzed for *P. marinus* prevalence, as described in FGCU GLPP Testing Protocol 2, *Chronic Toxicity Testing – Adult Oysters*. If immune response was being assessed (see test-specific TCTs), oysters from all treatments were sampled and immune response was assessed, as described in FGCU GLPP Testing Protocol 2, *Chronic Toxicity Testing – Adult Oysters*. If reproductive success was being assessed (see test-specific TCTs), after the exposure period, oysters were induced to spawn and reproductive success (fertilization success, embryonic development, metamorphosis to veliger/pediveliger larvae) were determined as described in FGCU GLPP Testing Protocol 3, *Chronic Toxicity Testing – Dietary Exposure of Adult Oysters to Assess Impacts on F-2 Generation*. Representative samples of oysters from each treatment could be collected for histology and/or tissue bioaccumulation at the end of the experiment. Specific endpoints assessed for each test were listed in the TCTs provided to Stratus Consulting during the test approval and test ID assignment process. Dead oysters were removed from the exposure tanks and archived according to the QAPP. Numbers of individual oysters used for endpoints assessments depended on the total number of oysters that remained after sediment exposures.

Preparation of sediments

Oil was mixed into uncontaminated Estero Bay sediments using a Cuisinart stand mixer. For each treatment, 7 kg of sediment was weighed and thawed overnight. Oil was added to each sediment treatment following treatments listed in the appropriate TCT. Oil was mixed into the sediment for 30 minutes at a moderate speed (5 on mixer), scraping the sides of the bowl with a metal spatula every 2–4 minutes as needed. The oil-sediment mixture was weighed and placed into 10-gal aquaria (L = 50.5 cm, W = 25.5 cm, H = 32 cm) for each treatment (2,200 g sediment/aquarium). When preparing tanks, sediment samples for the analytical laboratory from each treatment group were prepared by aliquoting approximately 100 g of sediment into the analytical chemistry sediment jar, between filling each replicate tank with sediment, so that each 8-oz sample jar was at least three-quarters full. Sediment samples were stored at 4°C until shipment to ALS Environmental for analysis. The sediment in the tanks was allowed to settle in the tanks overnight under static conditions prior to the addition of the oysters.

Sediments were prepared for testing prior to test initiation and were stored at 4°C until used to dose the tanks. After the sediment had been added to each tank, the tanks were placed into the exposure system, covered with foil, and allowed to settle for another 24 hours. After the settling period, oysters were randomly assigned to each tank and aeration began. Sediments were not removed or replaced throughout the exposure period. Effort was made to not disturb sediments when conducting water changes and water sampling.

Analytical chemistry sampling

Both sediment and water samples were collected during this experiment. Analytical chemistry samples were handled, labeled, and shipped to ALS Environmental according to the QAPP, with the following specifications:

1. ***Initial sediment sampling:*** Sediment samples were collected from each treatment group (including the control) in one full 8-oz jar/treatment. The sample jars were filled by adding an aliquot to each the jar after filling each tank for dosing. These initial sediment samples were analyzed for PAHs and TOC at ALS Environmental.

2. ***Water sampling:*** An initial composite water sample was collected from each treatment group just prior to the first water renewal.

   Water samples were taken throughout the exposure; at each water sampling time point, a composite water sample consisting of a subsample from each replicate tank was collected and placed into a clean decontaminated glass mixing container for each treatment. Subsamples were collected by dipping a clean, decontaminated glass beaker into each tank. The same transfer beaker could be used for all tanks within a single treatment. A

clean, decontaminated beaker was used when collecting subsamples from each additional treatment. After mixing subsamples, the composite was used to fill the required analytical chemistry containers for requested chemical analyses (see the QAPP). This process was repeated for each treatment group and control. Water samples were analyzed for PAHs at ALS Environmental.

3. ***Final sediment sampling:*** Composite sediment samples were collected from each treatment group at the end of the experiment. First, all or most of the water was siphoned from each tank. Next, all of the sediment from each tank within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were then mixed using a stainless steel scoop or similar utensil until uniform in color and consistency. Once the sediment was thoroughly mixed, a scoop was used to fill the analytical chemistry sediment jar (8-oz glass). Sediments were sent to ALS Environmental for PAH analysis.

Any changes to the analytical sampling plan described in this section were documented in the laboratory notebooks and/or test results reporting tables.

The standard COC forms provided in the QAPP were used to request chemical analyses and relinquish samples to ALS Environmental.

I. Testing Protocol 9: Exposure of Veliger Larvae Using Dietary Pathways (Oil + T-Iso)

Veliger larvae that were 5 days old were used in chronic toxicity testing. Veliger larvae that were larger than 90 µm (retained on a 55-µm sieve) with a D-shape were used in the assays. Larval oysters were exposed to a slurry of DWH oil and algae (T-Iso) for 14 days, in 1,500-mL glass jars. Oil was prepared using the HEWAF method, mixed with the algae for 6 hours (see below for more details), and fed to larval oysters every other day after water renewal with gentle aeration to stimulate feeding. Control treatments were fed algae only without oil. There were 5 treatments with 4 replicates/treatment (see TCTs). All tests were conducted at 25°C, with salinities of 25 to 30 ppt, with gentle aeration. Just before each renewal, a subsample of 10 mL was taken from all test chambers, put into 15-mL centrifuge tubes, and fixed with buffered 0.1% formalin. These samples were evaluated using a microscope to examine abnormality, growth, and viability of the veliger larvae. Any abnormality in the development or morphology was noted. For example, failure to progress toward a pediveliger stage or a deformed shell was noted as an abnormality. Shell length was measured every 2 days (minimum of 200 individuals/concentration) to assess growth. In addition, gut fullness or percentages of veliger larvae with food in the stomach was assessed for each sample.

At the end of the exposure (day 14), larval survival was assessed. Remaining veliger larvae were collected on a sieve, rinsed, and resuspended with seawater in 50-mL centrifuge tubes. Final survival was assessed by taking 3 subsamples of 150 or 300 µL (see bench sheets) after homogenization from each 50-mL centrifuge tube and counted. Lastly, remaining veliger larvae were centrifuged and stored in plastic centrifuge tubes and stored at -80°C for later biochemical analysis (PAH content in larval tissue).

See TCTs for nominal treatment concentrations, replicates, and other test details.

Preparation of contaminated algae

T-Isochrysis (CCMP 1324) was used to feed oyster veliger larvae for the duration of exposures. DWH oil prepared as HEWAF was added to algae in a beaker (300 mL), gently stirred (250 RPM) using a stir bar for 6 hours to allow the oil to adsorb to the algae in the dark, and covered with Parafilm. Control oysters received only algae. Algae + oil solutions were added to each exposure vessel at test initiation and at each water change. See TCTs for feeding regime and algae concentrations.

Flow cytometry

Each exposure solution (oil + algae) was analyzed using flow cytometry: SSC, FSC, and fluorescence were used to determine complexity and size of the particles.

Renewal procedures

Exposure vessel water was renewed on Monday, Wednesday, and Friday by pouring the entire contents (water, veliger larvae, algae, and oil) through a Nitex screen filter (55 µm). The screen separated the veliger larvae from the exposure media. Retained veliger larvae were gently washed into a cleaned exposure vessel containing fresh seawater filtered at 0.1 µm. After filling exposure vessels, algae/oil mixtures were added at a feeding regime of 150,000 cells/mL of culture. During every water change and feeding day, new solutions of contaminated or control algae (HEWAF + T-Iso) were prepared and added to the exposure vessels. Control treatments received non-contaminated algae (no HEWAF), prepared the same way as the contaminated algae solutions (6 hours spinning, covered, in the dark).

Water for chemical analysis

Water samples were collected throughout the 2-week exposure and sent to ALS Environmental for PAH analysis. Two distinct sets of water samples were collected. The first set was referred to as sham samples and the second as effluent water samples.

A single sham sample was taken from one pseudo replicate from each treatment group, including controls at test initiation (day 0), for a total of five sham water samples for each test. The sham exposure vessel, volume, and oil preparation were the same as respective treatment exposure vessels, except that the sham vessels did not contain larval oysters. Each sham was prepared when all other test replicates were prepared. Sham water samples were taken from under the water surface in a well-mixed exposure vessel and were not filtered or decanted. If a surface slick was present in the sham vessel when the sample was taken, it was noted on the test Analytical Sample Inventory Bench Sheet.

Effluent water samples were taken during two time points during the 2-week exposure. The first set of effluent samples was taken during the first water renewal at day 3 (72 hours after feeding). The second set of effluent samples was taken on day 5 (48 hours after feeding). A total of 8 effluent water samples for each test; no samples were collected from control vessels.

In each exposure treatment, all replicate exposure vessel effluent (approximately 6 L) was composited into a single mixing vessel (with veliger larvae filtered out). The effluent water sample was taken from under the water surface in the well-mixed composite and was not filtered or decanted. If a surface slick was present in the composited effluent when the sample was taken, it was noted on the test Analytical Sample Inventory Bench Sheet.

J. Testing Protocol 10: Pediveligers WAF and Spiked Sediment Settlement Assessments

Pediveligers were exposed to HEWAFs, CEWAFs, or spiked sediment and settlement success was assessed.

J.1 Pediveliger Exposure to WAF

Pediveligers (retained on 200-µm Nitex screen sieve; about 12–15 days post fertilization) were exposed to 450 mL of HEWAF or CEWAF for 3 days (see test-specific TCTs). WAFs were prepared following the protocols in the QAPP.

Each test consisted of five treatments and a seawater control, with four replicates of each treatment (see test-specific TCTs). Prior to test initiation, two settlement plates, consisting of HardieBacker Cement Board tiles (120 mm × 58 mm), were placed vertically into each testing beaker. To help condition the tiles, the cement tiles were soaked in seawater a minimum of 2 weeks prior to test initiation. At test initiation, pediveligers were stocked into the exposure beakers at a density of approximately 1,000/beaker. To accurately estimate stocking densities, a "dummy beaker" was loaded at the same stocking density, for each treatment. These dummy beakers were sieved immediately after the test initiation and the total number of pediveligers that were stocked into the beaker was recorded on the appropriate bench sheets.

During the exposure, aeration (gentle bubbling) was supplied for 30 minutes every 2 hours using a timer-controlled air pump. Pediveligers were fed microalgae at approximately 100,000 cells T-Iso/mL on days 0 and 2.

J.2 Endpoint Assessment of WAF Exposure

The developmental success of pediveligers was determined by their progression to spat (i.e., settlement success), and mortality was assessed at the end of the experiment. To assess settlement rates, settlement plates and container walls were observed under magnification using a dissecting microscope, and newly settled oysters were counted. Any pediveligers remaining in the water column were collected on a sieve (150 µm), rinsed, and resuspended in 29.1 mL of seawater in a 50-mL centrifuge tube. Then, 0.9 mL of 10% formalin was added to the 50-mL centrifuge tube for later estimation of survival. The total volume of solution in the 50-mL centrifuge tube was 30 mL. Final survival was assessed by taking 3 subsamples of 1,000 µL from each 50-mL centrifuge tube after homogenization to estimate the total number of oyster remaining.

J.3 Pediveliger Exposure to Spiked Sediment

Pediveligers (retained on a 200-µm Nitex screen sieve; about 12–15 days post-fertilization) were exposed to sediment spiked oil in 600-mL beakers (350 mL of overlying water) for 3 days. Spiked sediment was prepared following the guidelines in Section I.4.

Each test consisted of five spiked-sediment treatments, an unspiked sediment control, and a seawater control, with four replicates of each treatment (see test-specific TCTs). Prior to test initiation, each container was loaded with 150 g of sediment (≈ 1.5 cm). Following the loading of the exposure chambers with sediment, two settlement plates, consisting of HardieBacker Cement Board tiles (120 mm × 58 mm), were placed vertically into each testing beaker. The tiles were in contact with the sediment approximately 0.5-cm deep. The tiles were soaked in seawater a minimum of 2 weeks prior to test initiation. At test initiation, pediveligers were stocked at a density of approximately 1,000/beaker. To accurately estimate stocking densities, a dummy beaker was loaded at the same stocking density for each treatment. The dummy beakers were sieved immediately after the test initiation and the total numbers of pediveligers that were stocked into the beakers were recorded on the appropriate bench sheets.

During the exposure, aeration (i.e., gentle bubbling) was supplied for 30 minutes every 2 hours using a timer-controlled air pump. Oysters were fed microalgae at approximately 100,000 cells T-Iso/mL on days 0 and 2.

J.4 Sediment Preparation

J.4.1 General guidelines

The control sediments were prepared using the same technique used for spiked sediments, with the exception of adding oil. Each sediment-oil concentration was prepared separately. Equipment was cleaned and prepared, using the *Decontaminating Glassware* SOP in the QAPP. Unused sediments were stored in a Ziploc bag in the dark at 4°C (short-term) or in the freezer (-20°C; long term).

J.4.2 Preparation of sediment

- After sediment was thawed, all the debris (grass, shells, etc.) was removed from the sediment and placed into a mixer bowl
- Using a Cuisinart SM-70 7-quart stand mixer, the sediment was homogenized by mixing for 2 minutes at low speed (1).

J.4.3 Mixing oil into sediments

The appropriate amount of oil was weighed out as outlined below.

- A weigh boat and two or three Kimwipes were tared on the top loading balance. Using a stainless steel spatula, slightly more than the desired mass of oil was placed onto the weigh boat. The oil was transferred onto the sediment in the mixing bowl and placed in several areas around the bowl using a spatula. Any oil remaining on the spatula was wiped with the tared Kimwipes. The weigh boat was reweighed with the Kimwipes to calculate and record the actual mass of oil transferred.

- The oil was mixed into the sediment at medium speed (5) with the Cuisinart mixer. Every 2 to 4 minutes, the mixer was briefly stopped and the sides of the mixing bowl were scraped with a putty knife.

- Once the mixing was complete, the mixer paddle was scraped down with a putty knife to remove all excess oiled sediment. The oiled sediment was either transferred from the mixing bowl into the test containers using a stainless steel spoon or stored in the dark at 4°C until test initiation.

J.4.4 Sediment and overlying water sampling

Sediment samples for the analytical laboratory were collected from each treatment group by adding approximately 100 g of sediment into the analytical chemistry sediment jar between filling each replicate tank with sediment. Each 8-oz sample jar was filled at least three-quarters full. Sediment samples were stored at 4°C until they were shipped to ALS Environmental for analysis. After the addition of sediment, the exposure beakers were allowed to settle overnight under static conditions prior to the addition of the oysters.

After the test was completed, overlying water from each replicate was carefully siphoned out of the beaker, composited by treatment, and sent to ALS Environmental for analysis.

J.5 Endpoint Assessment of Sediment Exposure

The developmental success of pediveligers was determined by their progression to spat (i.e., settlement success), and mortality was assessed at the end of the experiment. To assess settlement rates, settlement plates and container walls were observed under magnification using a dissecting microscope, and newly settled oysters were counted. Larvae remaining in the water column and/or settled on the sediment were collected on two sieves, 150 µm (to retain live and dead pediveligers) and 250-µm (to retain newly settled spat). Both were rinsed and the remaining

material was re-suspended in 48.5 mL of seawater in a 50-mL centrifuge tube. Then, 1.5 mL of 10% formalin was added to the 50-mL centrifuge tube for later estimation of survival. Separate 50-mL centrifuge tubes were prepared according to sieve size. Final survival was assessed by taking 3 subsamples of 1,000 µL from each 50-mL centrifuge tube after homogenization.

K. Testing Protocol 11: Cellular Responses of Spermatozoa and Oocytes to CEWAF and HEWAF and Impacts on Fertilization and Early Development

Gametes (spermatozoa and oocytes) were exposed to chemically and mechanically dispersed oil, and cellular effects were evaluated using FCM assays; impacts on fertilization and early development were also examined.

K.1 Cellular Characteristics of Exposed Gametes Using Flow-Cytometry

All FCM analyses were performed on an EasyCyte 6HT cytometer (Guava Merck Millipore) equipped with a 100-µm capillary opening, a 488-nm argon laser, and 3 fluorescence detectors: green (525 nm ± 15), yellow (583 nm ± 13), and red (680 nm ± 15). Samples were run for 30 seconds at a flow rate of 0.59 µL s^{-1}.

K.1.1 FCM analysis of sperm solution

A. Sperm exposure

Sperm suspensions stripped from 3 male oysters (Allen and Bushek, 1992) were exposed separately to two concentrations of HEWAF (see test-specific TCTs), and 2 concentrations of CEWAF (see test-specific TCTs) for 30 minutes at 25°C, prior to FCM analysis. An aliquot of 160 µL of prepared WAF was mixed with 40 µL of sperm suspension to obtain a final concentration of ≈ 1 x 10^6 cells mL^{-1}. Suspension of exposed spermatozoa (200 µL) were then used for FCM assays including morphology, viability, mitochondrial membrane potential, and ROS production using specific fluorescent probes, according to Le Goïc et al. (2013a, 2013b). Sperm incubated in FSW were used as controls.

B. Spermatozoa morphology and viability

Spermatozoa morphology was based upon relative FCM measurements of Forward Scatter (FSC: relative cell size) and Side Scatter (SSC: relative cell complexity).

Viability of spermatozoa was evaluated using a dual-staining procedure with SYBR-14 and propidium iodide (Live/Dead Sperm Viability kit, *Molecular Probes*). After 20 minutes of

exposure to two different concentrations of HEWAF or CEWAF, sperm were stained with both SYBR-14 (final concentration of 1 $\mu molL^{-1}$) and propidium iodide (final concentration of 10 $\mu g\ mL^{-1}$) for 10 minutes in the dark at 25°C. See more details in Testing Protocol 1, Section A.6.

C. Spermatozoa ROS production

Prior to FCM analysis, the spermatozoa suspension (200 µL) was incubated with WAF and DCFH-DA (final concentration of 10 $\mu molL^{-1}$) simultaneously in the dark for 30 minutes total incubation time. See more details in Testing Protocol 1, Section A.6.

D. Spermatozoa mitochondrial membrane potential (MMP)

The MMP of spermatozoa was measured using the potential-dependent JC-1 (BD MitoScreen, *BD Bioscience*). This is the same principle as JC-10, which is described in Testing Protocol 1, Section A.6.

Aliquots of 200 µL of spermatozoa (adjusted at a final concentration of $\approx 1 \times 10^7$ cell mL^{-1}) were exposed for 30 minutes to oil treatments and incubated with JC-1 (final concentration of 5 $\mu molL^{-1}$ for 10 minutes before the end of the exposure in the dark at 25°C), and then diluted at 1:10 to stop the reaction prior to FCM analysis (Le Goïc et al., 2013b).

K.1.2 FCM analysis of oocytes solution

A. Oocyte exposure

Oocyte suspensions were stripped from 3 females (Allen and Bushek, 1992) and exposed to 2 concentrations of HEWAF (see test-specific TCTs) and 2 concentrations of CEWAF (see test-specific TCTs) for 30 minutes at 25°C, prior to FCM analysis. To reach 8,000 oocytes mL^{-1}, oocyte suspensions were left to settle for a few minutes and collected using a Pasteur pipette. A total of 40 µL of oocyte suspensions were exposed separately to 160 µL of WAF (ratio of 1:4 oocyte to WAF). Oocytes incubated in FSW were used as controls. Suspensions of exposed oocytes were then analyzed by flow cytometry for morphology, viability, and ROS production using specific fluorescent dyes according to Le Goïc et al. (2014).

B. Oocytes morphology and viability

Values from FSC and SSC detectors were used as descriptors of oocyte morphological characteristics. To assess viability, aliquots of 200 µL of exposed oocytes (final concentration of 8,000 mL^{-1}) were stained with propidium iodide (final concentration of 10 $\mu g\ mL^{-1}$) for 10 minutes in the dark at 25°C. Propidium iodide identifies cell mortality by penetrating oocytes

with a compromised membrane, and emitting in the red fluorescence range. Results were expressed as percentages of live cells.

C. Oocytes ROS production

ROS were measured using 2'7'-dichlorofluorescein diacetate (DCFH-DA), a membrane-permeable, non-fluorescent probe. Aliquots of 40 µL of oocytes (at 8,000 mL^{-1}) were incubated with 160 µL of WAF and DCFH-DA (final concentration of 10 $\mu molL^{-1}$) simultaneously for 30 minutes in the dark at room temperature before FCM analyses. Inside cells, the -DA radical was first hydrolyzed by esterase enzymes. Intracellular hydrogen peroxide (H_2O_2) and superoxide ion (O_2-•) then oxidized DCFH to the fluorescent 2',7'-dichlorfluorescein (DCF) molecule. DCF green fluorescence, detected on the FL1 detector of the flow-cytometer, is proportional to the total oxidative activity of cells, including ROS.

K.2 Fertilization and Embryo Assay Using 24-Hour Static Exposure

Pooled sperm from 3 males and pooled eggs from 3 females were exposed for 30 minutes separately to the same 2 concentrations of HEWAF (see test-specific TCTs) and CEWAF (see test-specific TCTs) (n = 4 replicates for each concentration). Sperm suspensions (10 mL at a concentration of ≈ 1 x 10^6 cells mL^{-1}, n = 4 replicates) were incubated in 40 mL HEWAF or CEWAF (see test-specific TCTs). Simultaneously, about 4,500 oocytes were incubated in 200 mL HEWAF or CEWAF (n = 4 replicates; see test-specific TCTs). After the 30-minute incubation, the 200-mL egg solutions from each exposure replicate were fertilized with 10 mL of sperm from corresponding sperm exposure replicates. In addition, oil-exposed oocytes were cross-fertilized with sperm incubated in FSW.

Control groups consisted of sperm incubated in FSW fertilized with oocytes incubated in FSW.

Exposure beakers were maintained in darkness at 26°C ± 1 and at a salinity of 23 ± 2 ppt for 24 hours. At 1-hour, 6-hours, and 24-hours post-fertilization, 10-mL subsamples were taken and preserved with the addition of 300 µL of 10% buffered formalin for later measurements (fertilization success, abnormality, mortality). To determine fertilization success (%), the presence of the first cell cleavage of embryos (a minimum of 50 embryos were examined for each replicate) was verified under light microscopy (see Testing Protocol 1, Section A.1) using the 1-hour subsamples. For the 6- and 24-hour subsamples, embryo abnormality was evaluated according to the following criteria: (6-hour) embryos that did not reach the blastula stage, with abnormally shaped cells, delayed and/or arrested development (polar body to 2–3 cells) (Thompson et al., 1996; Carriker, 2001); and (24-hour) D-larvae with indented shell margin, incomplete shell, protruded mantle, convex hinge, and arrested development at the embryo stage

(His et al., 1997) (see Testing Protocol 1 Sections A.1 and A.2). Larval mortality was also assessed at the end of the 24-hour exposure by the observation of opened valves and/or translucent shells (no clear internal organization), as well as unfertilized eggs.

L. Testing Protocol 12: Spat Filtration Rate Measurements

Oyster spat from the experimental hatchery at Auburn University, Alabama, were exposed to DWH oil prepared following the HEWAF methods described in the QAPP. Early spat (10–15 mm, ~ 1 g individual wet weight) were exposed to increasing concentrations of HEWAF solution (100%, 25%, 15%, and 5% seawater control), with 3 replicates/concentration. The exposure lasted 24 hours and was conducted under static conditions.

To measure clearance rates, each beaker of oysters was fed *ad libitum* at the start of the trial with 100 mL of *T-lutea*/beaker. Residual algal cells were assessed throughout the exposure (T_0 and T_{24}) by taking 1.5-mL aliquot from each replicate and then fixing it by adding 100 µL of Glutaraldehyde. After thorough agitation, three 10-µL subsamples were taken from the fixed aliquots and observed under a microscope using a counting hemocytometer. Residual algal cells were then averaged for each sampling point and 24-hour clearance rates were determined for each HEWAF condition. To compare among different treatments, clearance rates (CRs) were normalized per gram of whole dry tissue weight of oyster, and determined using the following equation, adapted from Coughlan (1969):

$$CR = (V/(n \times t)) \times \ln (C_{ti}/C_{tf}))/\varpi$$

where CR is the clearance rate in L h^{-1} g^{-1}; V is the volume of each container in L; n is the number of spat; t is the time of experiment in h; C_{ti} and C_{tf} are the initial and final counts of *T. lutea* cells at t_i or t_f; and ϖ is the mean individual dry weight of spat in g (ϖ = 0.0005 g).

M. Testing Protocol 13: Oyster Embryos Exposure to DWH Oil Slick

This test was conducted at the University of North Carolina, Wilmington (UNCW). Oyster embryos were exposed to DWH oil slicks and mortality and abnormalities were assessed.

M.1 Preparation of Sieve Baskets to Hold Embryos

Sieve baskets were prepared to hold oyster embryos during the allocated exposure durations (see test-specific TCTs). Sieves were made of 3" PVC rings that had two holes drilled into the sides approximately 5 cm from the top of the ring, to allow for a metal hook to hold it in place on the beaker. A 20-µm mesh was placed on the opening opposite of the hook. This was done by cutting out a portion of the mesh that was larger than the PVC ring, spreading aquarium grade epoxy along the rim of the PVC ring, and then placing the 20-µm mesh onto this rim. This was allowed to dry for at least 48 hours before being placed in water (Figure M.1).

Figure M.1. Sieve basket.

Source: UNCW.

M.2 Preparation of the Test Chambers

Tests were conducted in 600-mL beakers. Using a metal hook, a sieve basket was placed into each beaker. Beakers that were assigned to the "no sieve control" group did not receive a sieve basket. After the placement of the sieve baskets into the appropriate beaker, 350 mL of filtered sea water was added to each beaker (Figure M.2). The test chambers were placed in an incubator and gently aerated.

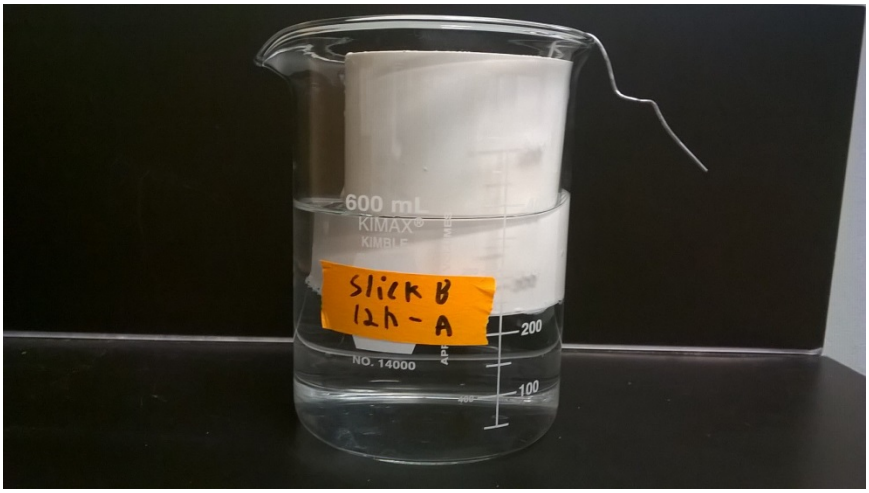

Figure M.2. Experimental beaker with a sieve basket.

Source: UNCW.

M.3 Preparation of the Oil Slick

Oil slick PVC rings were made of a 2" PVC ring that had two holes drilled into the sides approximately 5 cm from the top of the ring to allow a metal rod to hold it in place in the beaker while the slick formed. Approximately 2 g of oil was loaded into the inside ring of the PVC pipe. The 2" PVC pipe was then placed inside the 3" sieve basket and allowed to make contact with the water for 4–6 hours.

M.4 Loading the Test Chambers

Embryos were added to the test chamber by pipetting the desired number of embryos (see test-specific TCTs) inside the 3" sieve basket but outside of the 2" PVC ring with the slick. The PVC ring was then removed, leaving a thin oil slick at the surface. This time point represented the initiation of a test.

M.5 Exposure

For different treatments, sieve baskets were held in close proximity to the thin oil slick for different durations of time. For example, for the "instant touch" treatment, immediately after the

initiation of a test, the sieve basket was raised up to touch the oil slick and then gently dropped to the bottom of the test beaker. For the 4-hour treatment, the sieve basket remained in close proximity to the oil slick for 4 hours and then gently dropped to the bottom of the beaker. The same procedure was repeated for the rest of the treatment time points (see test-specific TCTs). For all treatments, embryos were fed 15,000 cell/mL *T. Iso* at 24 hours and 72 hours.

M.6 Test Termination

After 96 hours, the embryos were fixed and put into properly labeled 50-mL centrifuge tubes by lifting the sieve out of the test chamber and placing it over a treatment-specific, run-off receptacle; pouring all of the water through the sieve to catch any embryos that may have swam over the top of the test sieve PVC; and then the sieve was turned upside down over an open 50-mL centrifuge tube and new seawater was run across the underside of the sieve to resuspend the embryos. This was done until there was 30–35 mL of the new seawater in the tube, and then 900 µL of 10% formalin was added to the tube to fix the embryos. This procedure was repeated for all replicates in the test group over the same runoff receptacle; once all of the replicates from the treatment levels were fixed, an ammonia sample was taken from the runoff receptacle. This was repeated for each treatment level.

M.7 Mortality and Abnormality Assessments

Fixed embryo samples were left undisturbed for at least 12 hours to allow the embryos to settle to the bottom of the tube. To count embryos, a 1-mL aliquot of the settled embryos was transferred to a rafter slide and then covered with a clear glass slide. From that aliquot, oysters were assessed for mortality and abnormality. The results were recorded on the appropriate bench sheet. This process was repeated three times per replicate. This sampling effort allowed for the assessment of the majority of the oysters in the sample.

N. Testing Protocol 14: Oyster Embryo Exposure to DWH Oil Slick and UV

This test was conducted at UNCW in collaboration with the University of North Texas. Oyster embryos were exposed to DWH oil slicks and UV mortality was assessed.

N.1 Preparation of UV Set-Up

UV lamps were set up in an area in which they could be securely fastened and readjusted as needed. The height of the lights above the exposure dishes was adjusted to alter the intensity at the table surface to mimic a sunny summer day (100% UV exposure). A 50% UV exposure treatment was also set up by using a metal wire mesh above the test chamber platform that blocked 50% of the UV light. The UV control group was set up behind a curtain.

N.2 Preparation of the Oil Slick

Oil slick PVC rings were made from 2" PVC rings with two holes drilled approximately 5 cm from the top of the rings. This allowed a metal rod to hold the PVC ring in place in the beaker while the slick formed. Approximately 2 g of oil was loaded into the inside ring of the PVC ring. The ring was then placed inside a 400-mL beaker filled with 200 mL of seawater and allowed to soak for 4–6 hours so that an oil slick could form at the surface of the water.

N.3 Exposure

Embryos were added to the test chamber by pipetting the desired number of embryos (see test-specific TCTs) between the test beaker and the 2" PVC ring with the slick. The PVC ring was then removed, leaving a thin oil slick at the water's surface. This time point represented the initiation of a test. The UV lamps remained on throughout the duration of the test and UV levels were monitored (see test-specific TCTs).

N.4 Test Termination

When the testing period concluded (see test-specific TCTs), each test chamber was drained through a 20-µM sieve to capture the embryos. Each sieve was then rinsed with approximately 35 mL of seawater that was captured in a 50-mL centrifuge tube and fixed with 900 µL of 10% formalin.

N.5 Mortality and Abnormality Assessments

Fixed embryo samples were left undisturbed for at least 12 hours to allow the embryos to settle to the bottom of the tube. To count embryos, a 1-mL aliquot of the settled embryos was transferred to a rafter slide and then covered with a clear glass slide cover. Oysters in the aliquot on the slide were assessed for mortality using a microscope. The results were recorded on the appropriate bench sheet. This process was repeated three times per replicate. This sampling effort allowed for the assessment of the majority of the oysters in the sample.

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3. University of Southern Mississippi General Laboratory Procedures and Practices

3.1 Introduction

The University of Southern Mississippi Gulf Coast Research Laboratory (GCRL) conducted a series of toxicity tests to identify the toxicological impacts of the 2010 *Deepwater Horizon* oil spill on resident fish and invertebrate species in the Gulf of Mexico. This chapter describes General Laboratory Procedures and Practices (GLPP) used at GCRL.

3.2 Methods

3.2.1 Water

Artificial seawater (ASW) used at GCRL was prepared using a formulation from Fritz Aquatics (Mesquite, Texas). To make the ASW, approximately 900 gal of unbuffered well water was mixed with 1 bucket of Fritz Pro Aquatics Super Salt Concentrate and 80 pounds of Tru-Soft Water Softener Salt Pellets (United Salt Corporation, Houston Texas). The mixed ASW was recirculated through a 10–25-µm filter before transfer into another large reservoir within the toxicology building where the water was filtered and temperature-adjusted. This water was used for husbandry, to prepare exposures, and also as dilution water in flow-through exposures.

3.2.2 Test organism sources and husbandry

This section describes the sources and husbandry for test organisms used for toxicity tests. Tests were conducted with sheepshead minnow (*Cyprinodon variegatus*), speckled seatrout (*Cynoscion nebulosus*), blue crab (*Callinectes sapidus*), southern flounder (*Paralichthys lethostigma*), grass shrimp (*Palaemonetes pugio*), fiddler crab (*Uca longisignalis*), white shrimp (*Litopenaeus setiferus*), red snapper (*Lutjanus campechanus*), Atlantic croaker (*Micropogonias undulatus*), and red drum (*Sciaenops ocellatus*).

Sheepshead minnow

Wild-type sheepshead minnow were in stock in the Shoemaker Toxicology Laboratory (STL) at the University of Southern Mississippi, housed in 80-gal (200-L) raceways under recirculating conditions with a 10% water change each week. Fish were fed pelleted feed at a rate of 2% body weight daily and were available on demand.

Speckled seatrout

Speckled seatrout were in culture at the Cedar Point Aquaculture Facility at GCRL. They were transported to the STL at least 4 days prior to use and acclimated to 15-ppt salinity prior to exposure initiation. Seatrout eggs were obtained from tank spawns of captive adults maintained under controlled environmental conditions similar to previously described methods (Arnold et al., 1978). Eggs were incubated at a density of 1/mL in 120-L conical-bottomed tanks on a recirculating system at 25-ppt salinity and 27°C. At 33 hours post-hatch, larvae were stocked at a density of 15/L into 1,500-L conical-bottomed black tanks (diameter = 1.5 m) filled with approximately 25-ppt salinity ASW, *Artemia* nauplii, and 0.05 × 106 cells/mL T-iso. Gentle aeration was applied from the bottom center of the tanks. Light intensity at the water surface was approximately 400 lux from standard fluorescent bulbs with constant illumination. Dissolved oxygen (DO) was maintained above 4.5 mg/L, water temperature was maintained at approximately 28.0°C, and salinity was maintained at approximately 25 ppt. Total ammonia (NH_3-N), nitrite (NO_2-N), and alkalinity were measured daily using Hach (Hach Co., Loveland, Colorado) test strips. Alkalinity was maintained at > 150 mg/L by addition of sodium bicarbonate. The pH was measured daily using a YSI 556.

Blue crab

Ovigerous female blue crabs were collected from waters of the Mississippi Sound in an area south of the mouth of Biloxi Bay. Crabs were collected using industry standard crab traps with a soak time of less than 48 hours (preferred). Laboratory personnel accompanied a local fisherman to select healthy females with early bright orange sponges. The females were placed in individual coolers containing aerated site water for transport to GCRL. They were held in quarantine tanks for 24 hours, where they were acclimated to culture conditions (25°C and 28-ppt salinity) and checked for disease (white spot syndrome virus and *Hematodinium* sp.) before being placed in hatching tanks. After approximately 1 week, the zoeae were collected, counted, and stocked (approximately 100,000 per tank) in 1,400-L recirculating culture systems equipped with biological and mechanical filtration. Depending on their stage, the zoeae were fed a diet of rotifers (*Brachionus rotundiformis*) and Instant Algae (*Nannochloropsis*, *Isochrysis*, and Shellfish Diet), newly hatched and enriched *Artemia*, and frozen copepods (Cyclop-eeze). The zoeae molted through 7 zoeal stages to megalopae. When the culture contained ~ 85% megalopae, the systems were drained and the megalopae were collected.

Grass shrimp

Grass shrimp were collected by dip netting in marshes adjacent to the GCRL shore, and stored in 800–4,000-L tanks (15-ppt ASW, 26°C, 16:8 L:D cycle) in the STL quarantine facility. Grass shrimp were held a minimum of 7 days prior to testing, and fed commercial flake food twice daily. Ovigerous females were separated daily from the general population and put into a separate 1,600-L aquarium under the same conditions and feeding regime. Water quality

parameters (temperature, salinity, DO, ammonia) were measured twice weekly in the shrimp-holding aquaria.

Southern flounder

Southern flounder juveniles were obtained from the University of Texas Marine Science Institute (UTMSI) and transported to GCRL in 32-ppt salinity ASW at 20.5°C. They were put into two Living Streams systems with high-flow recirculating water. Juveniles were acclimated to 15-ppt salinity prior to experimentation, and held at 15-ppt salinity for a minimum of 1 week prior to experimentation. They were fed twice daily with commercial flake food and *Artemia* nauplii.

Southern flounder larvae were obtained from the Texas Parks and Wildlife Department and were held in 200-L aquaria at 30-ppt salinity until they were used in experiments. They were fed algae (*Nannochloropsis* spp.), rotifers, and *Artemia* nauplii depending on size.

Fiddler crab

Fiddler crab zoeae were obtained from Auburn University and were used immediately in toxicity testing. See the Auburn University GLPP for fiddler crab collection and culture procedures.

White shrimp

White shrimp were obtained from Florida Organic Aquaculture, Vero Beach, Florida, and transported to STL in 30-ppt salinity ASW at 20°C. They were put into two Living Streams systems with high-flow recirculating water. Shrimp were acclimated to 24°C and 30-ppt salinity water prior to experimentation and held at these conditions a minimum of 4 days prior to experimentation. They were fed twice daily with commercial shrimp pellet food and frozen mysids.

Red snapper

Red snapper were in culture at the Cedar Point aquaculture facility at GCRL, and 8- to 12-week-old fingerlings were transported to STL at GCRL at least 4 days before use and acclimated to 30-ppt salinity before exposure initiation. Captive cultures were started from adult males collected from the field. Broodstocks were reared at Cedar Point and were induced for gamete maturation using previously described methods (Arnold et al., 1978). Eggs were collected by strip spawning, then incubated at a density of 1/mL in 120-L conical-bottomed tanks on a recirculating system at 30 ppt and 26°C. At 14-hours post-fertilization, larvae were stocked at a density of 50/L into 1,000-L conical-bottomed black tanks (diameter = 1 m) filled with 30-ppt salinity seawater. Gentle aeration was applied from the bottom-center of the tanks. Light intensity at the water surface was approximately 1,000 lux from standard fluorescent bulbs with constant illumination under a 16 L:8 D photoperiod. DO was maintained above 4.5 mg/L, water

temperature was maintained between 26°C and 28.0°C, and salinity was maintained at approximately 30 ppt. Alkalinity was maintained at > 150 mg/L with the addition of sodium bicarbonate.

Atlantic croaker

Atlantic croaker were in culture at the Cedar Point Aquaculture Facility at GCRL. They were transported to the STL at least one month before use and housed in a 500-gallon circular tank. Fish were originally housed at 26 ppt salinity; the salinity was dropped 1–1.5 ppt every other day until a salinity of 18 ppt was reached. Fish were housed at approximately 28°C and DO was maintained at approximately 6.0 mg/L. Fish were fed Silver Grow Out Feed by Zeigler daily.

Red drum

Juvenile red drum were obtained from Texas Parks and Wildlife aquaculture facility in Lake Jackson, Texas. Upon arrival at GCRL, fish were transferred into living streams. Fish were held for approximately 2 weeks before testing. Salinity was dropped from 29 ppt to 15 ppt in the two-week span in the Living Stream quarantine system. Salinity was dropped 1 to 1.5 ppt per day to reach the experimental design salinity of 15 ppt. Fish were fed Otohime Fish Diet by Reed Mariculture daily.

Pathogenic bacteria

Vibrio anguillarum was used in bacterial exposures. *V. anguillarum* was procured from ATCC (Strain 19264), which was initially isolated from ulcerous lesions in cod. Isolates obtained from ATCC (Manassas, VA) were cultured in T1N3 bacterial media and bacteria were then frozen in glycerol for later use. Media was thawed to ambient temperature before use in toxicity tests.

3.2.3 Exposure media preparations

Toxicity testing at GCRL used multiple oils and preparation methods. There were two different water accommodated fraction (WAF) preparations, high energy (HEWAF) and chemically enhanced (CEWAF) WAFs, using four oil types: Slick A, Slick B, artificially weathered source oil, and source oil. Some tests included Corexit 9500-only as dispersant controls during CEWAF exposures where applicable [see test-specific test conditions tables (TCTs)]. Other tests included exposure to contaminated sediments. In all exposures, appropriate controls were performed. Control waters were managed similarly to WAF preparation methods, except that no oil was added. WAF exposure preparation methods were performed according to protocols described in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Sediment exposure preparation methods are included in the individual Testing Protocols listed in the appendix of the GCRL GLPP.

3.2.4 Testing methods

Embryonic, larval, and/or juvenile life stages of sheepshead minnow, speckled seatrout, grass shrimp, southern flounder, fiddler crab, blue crab, red snapper, Atlantic croaker, and red drum were exposed to HEWAF or CEWAF using aqueous exposures. Post-larval, juvenile and adult life stages of southern flounder, brown shrimp, and white shrimp were exposed to spiked sediment. Spiked sediment preparations were prepared as outlined in the GCRL Testing Procedures SOP: *Protocol for Preparation of Oil-Spiked Sediments*.

Acute aqueous exposure toxicity tests

Test solutions for acute toxicity exposures were made according to the QAPP. Acute exposures were performed according to GCRL GLPP Testing Protocol 1. Selected life stages of each species were exposed to multiple concentrations of WAF as acute static or static/renewal tests (see test-specific TCTs for exposure duration). Exposures consisted of different toxicant doses and appropriate controls, and each concentration was performed with 5–20 individuals per replicate, with the exception of blue crab megalopae, which were exposed individually in 20-mL borosilicate scintillation vials containing 20-mL test solution.

At the beginning of the exposure, HEWAF, CEWAF, and control stock solutions were sampled for hydrocarbon analysis and shipped overnight to ALS Environmental according to the QAPP. Dilution series archive and fluorescence samples were collected and handled according to the QAPP.

Test organisms that survived and were used during the tests, but not used for subsequent analyses, were sampled and archived. Water and tissue samples were archived at GCRL according to the procedures outlined in the QAPP. Samples requiring long-term storage were shipped to ALS Environmental for storage.

Chronic aqueous exposures

Chronic exposures were performed according to the protocols in *Definitive Chronic Exposures* (GCRL GLPP Testing Protocol 2)*, Exposure Systems: General Methods and Materials* SOP, *Calibration of flow-through exposure systems SOP*, *Operation and maintenance of the Hamilton PSD/2 SOP*, and the *ASTM International chamber and flow-through loading calculations SOP*. This was done by performing simultaneous exposures that subjected organisms to HEWAF or CEWAF.

At the beginning of the exposure, the HEWAF, CEWAF, and control stock solutions were sampled for hydrocarbon analysis and shipped overnight to ALS Environmental as described in the QAPP. Water and tissue samples were archived at GCRL according to the procedures outlined in the QAPP.

Dilution series archive and fluorescence samples were taken and handled according to the QAPP. When flow-through systems were used during chronic exposures, dilution water was periodically sampled for archival extractions and fluorescence analysis.

Test organisms that survived and were used during the tests, but not used for subsequent analyses, were sampled and archived.

Water and tissue samples were archived at GCRL according to the procedures outlined in the QAPP.

Aqueous exposure fertilization tests

Testing staff examined the effects of HEWAF and CEWAF on fertilization and reproductive success in sheepshead minnow and grass shrimp.

Assessments of effects of aqueous exposure on progeny

To assess reproductive effects, adult male and female individuals were exposed to sublethal concentrations of WAF for 4–7 weeks in a flow-through system. Individuals of each sex were sampled from each replicate aquarium and histologically evaluated for treatment-related effects on gonadal development.

Sediment exposures

Oil-contaminated sediments were prepared by adding oil to uncontaminated sediments using a Cuisinart stand mixer as outlined in the GCRL Testing Procedures SOP: *Protocol for Preparation of Oil-Spiked Sediments.*

3.2.5 Water quality monitoring

Water quality was monitored throughout each exposure according to the *Monitoring water quality parameters during static and flow-through exposures SOP*. See the QAPP and test-specific TCTs for required monitoring.

3.2.6 Analytical chemistry sampling

Analytical sampling and analysis of water and sediments were carried out following the procedures outlined in the QAPP.

3.3 Reporting and Testing Documentation

Data management, documentation, quality assurance/quality control (QA/QC), and reporting were handled as described in the QAPP.

3.4 General Testing Standard Operating Procedures

3.4.1 Exposure systems: General methods and materials

Purpose

The purpose of this standard operating procedure (SOP) is to provide guidelines on the material, methods, and construction of exposure systems used in our various studies. Generally, flow-through exposures were performed if the test duration was longer than 7 days; for exposure durations of less than 7 days, static or static/renewal was appropriate.

Procedures

Static and static renewal exposures

A. Static exposures were generally conducted:

   1. When the amount of test substance was limited and the requirements for a flow-through exposure exceeded available test material

   2. When the test substance was highly hazardous, and it was necessary to keep the waste produced to a minimum

   3. When a test organism could not tolerate the stresses of water changes.

B. Static exposures were typically limited to 96 hours or less. In cases where exposure time exceeded 96 hours, scheduled renewals were conducted. For sensitive test organisms that could not tolerate the stress of water changes, tests were run as static exposures.

C. Static exposures were generally conducted in glass vessels with a capacity appropriate for the test volume required to ensure that organism loading and water quality parameters were not compromised during the test. Static exposures were conducted in a temperature-controlled water bath, an incubator, a closed system such as a glove box, or under a laboratory hood. The specific lighting conditions for each test can be found in the test-specific TCTs. Light regimen depended on WAF type and duration of exposure.

Lighting, when present, was generally fluorescent. However, depending upon specifications in the test-specific TCTs, other types of lighting were used.

Flow-through exposures

A. Dosing apparatus:

1. The dosing apparatus was used for metering dilution water and toxicant stock in the proper proportions to each of the exposure aquaria

2. The dosing apparatus in the flow-through system incorporated a water delivery device, which provided either continuous or intermittent flow of test solution or dilution water.

B. The intermittent delivery flow-through system used in toxicology at GCRL was a water partitioning type. The stock delivery system generally used for this type of exposure system was the Hamilton PSD/2, which provided a calibrated volume of toxicant stock on impulse demand. The dosing apparatus was generally located above the exposure aquaria, and gravity aided in the dispensing of test media. Generally, glass tubing was used to transfer test media from the dosing apparatus into mixing chambers or mixing tubes prior to introduction into the exposure aquaria.

Dilution water

A. During flow-through exposures, diluent water was most often pumped continuously through polyvinyl chloride (PVC) pipes from a fiberglass holding reservoir to a wood or fiberglass headbox located above the dosing apparatus system. Excess dilution water was returned to the reservoir through an overflow standpipe or side drain in the headbox.

Toxicant stock

A. During flow-through exposures, the toxicant reservoirs were graduated cylinders, flasks, carboys, and/or other containers that met the individual needs of the stock and exposure. The injector devices transferred toxicant stock from the dispensing carboy to mixing containers through strands of mini-bore tubing.

Mixing chambers, splitter boxes, and exposure aquaria

A. Glass mixing chambers or mixing tubes were used to blend the dilution water and toxicant stock prior to introduction into exposure aquaria or splitter boxes. A splitter box was sometimes used to divide the exposure solution among two or more exposure vessels via glass tubing emerging from bored stoppers that were plugging holes drilled in the bottom or the sides of the boxes. Mixing chambers and splitter boxes were usually

constructed by toxicology personnel by cementing glass sections together with clear silicone adhesive. When volatile compounds were tested, the mini-bore tubing emerging from the injectors was threaded the length of the diluent delivery tubes to the mixing chambers. Because the mini-bore tubing terminates below the standing water level in the mixing chamber, the toxicant stock was delivered under the surface of the diluent water, thereby minimizing volatilization.

B. Exposure aquaria were generally constructed of glass, Plexiglas, or polycarbonate sections. The aquaria dimensions varied depending upon loading density, the number of treatment concentrations, and the number of replications at each treatment. Aquaria pieces were cemented together with either clear silicone cement or plastic solvent (for Plexiglas or polycarbonate) purchased locally. Bored stoppers plugged holes that were drilled on the sides or bottoms of exposure tanks to accommodate overflow tubes, which allowed the aquaria to drain into an effluent trough. The stoppers used for mixing containers, splitter boxes, and exposure aquaria were of a non-toxic material, generally silicone or neoprene.

Exposure chamber

A. An exposure chamber generally housed the test aquaria to minimize environmental disturbances to the test system, and to provide constant temperature and light conditions.

B. The exposure system contained the exposure aquarium in a temperature-controlled water bath inside an exposure chamber. The tanks in the water bath were on supports so that water could circulate under them to control temperature. Outside of the exposure chamber, water pumps that communicate with the water bath operated to circulate and distribute the temperature-controlled bath water. The photoperiod was maintained by fluorescent lights.

3.4.2 Calibration of flow-through exposure systems SOP

Purpose

The purpose of this SOP is to provide guidelines on the calibration of flow-through exposure systems.

General

Any exposure system that was used in the performance of a flow-through toxicity test was calibrated prior to the initiation of the test.

Procedures

A. A water partitioning system provided diluent water to all test concentrations and control(s) by the use of a single series of "water cells." The selected concentrations were achieved by adding various volumes of stock(s) by a series of injectors (usually PSD/2). Instructions for use of the PSD/2 are provided in *Operation and maintenance of the Hamilton PSD/2 SOP*. The volume of stock provided was achieved by altering the syringe size (50 µL to 25 mL), the plunger stroke length, and the number of injections per cycle (details regarding the injection volumes can be found in test-specific TCTs). The electrical pulses for injections were monitored by impulse counters, which tallied the number of injection commands received by the pump as a check of proper system function. Splitter boxes were used to divide test solutions among the replicated test chambers.

B. Calibration of the partitioning system involved a common volume for each treatment and control(s). Volume was checked and adjusted based on the total desired volume minus any addition resulting from the injected stock. Volume of each treatment concentration was checked and recorded 3 times following final adjustments, and the mean of the 3 measurements met protocol specifications or were ± 5% of the target volume, if not explicitly stated.

C. When replicate test chambers were used in a flow-through test system, splitter boxes were employed. The function of the splitter box was to provide its contents equally to each of the replicate test chambers for a specific treatment. If splitter boxes were necessary for a test, the splitter boxes had to be checked for delivery. The volume delivery difference between the replicates met protocol specifications or was within 10%, if not explicitly stated, based on the average of three measurements.

3.4.3 Operation and maintenance of the Hamilton PSD/2 SOP

Purpose

The purpose of this SOP is to direct a user in the proper operation and maintenance of the Hamilton PSD/2.

General

The compact PSD/2 performs accurate liquid dispensing operations at speeds ranging from 1 to 60 seconds per syringe stroke. Operating on a single 24-volt DC power supply with an optional DC/DC converter, each module is capable of functioning as a standalone unit via an onboard EEPROM (Electronically Erasable Programmable Read-Only Memory) with remote activation.

Programs downloaded from an external computer to the EEPROM are retained until erased or replaced, and instruction codes are executed each time remote activation occurs. All operations followed the PSD/2 Installation Guide and/or the Hamilton PSD/2 Operator's Service Manual.

Procedures

A. Power requirements and connection

A power supply that provides 24-V DC (+ 15%, -5%) @ 1A, 25 W was required for operation. Each module requires a maximum of 1 amp. Connection of the power supply to the PSD/2 module was made at the J4 port on the interface board on the back of the module. Connection to the power supply was accomplished with a 4-pin molexR connector.

B. External computer communications requirements and connection

The default settings for both the PSD/2 module and any external computer were used. In some cases, if the default setting was not used, that information was recorded in laboratory notebooks. A set of dual-inline package (DIP) switches are located on the interface board of the PSD/2 module. Except for switch 7, the switches come preset in the off position. The connection of the external computer to the PSD/2 module was made at the J8 (COM IN) port on the interface board on the back of the module. The connection was accomplished with a 4-pin molexR connector at one end of the cable for connection to the PSD/2 at the J8 port; the other end of the cable must be a DB 9 female plug connected to the computer.

C. Remote activation

Remote activation by closed contact was achieved through the TTL IN (J7) port on the interface board on the back of the PSD/2 module. This is an 8-pin molexR connection with the Pin 2 position designated as a trigger. A wire was connected to this position and a separate wire was connected to the Pin 7 position by means of an 8-pin molexR connection. The other ends of these wires were attached to a float switch, timer, or any other normally open contacts. When the contacts were closed, a set of stored instructions was remotely activated.

Downloading stored instructions to the EEPROM

1. Connect the DB 9 end of the communications cable to the external computer at the appropriate port (mouse port or COM 1). Plug the molexR end of this cable into the J8 port on the interface board on the back of the PSD/2 module.

2. Turn on the external computer and access the PSD2DEMO program from either the hard drive or a floppy disk.

3. Supply power to the PSD/2 module. Verify power to the module by the initialization (turning) of the valve motor. It is necessary to periodically interrupt this power later in this procedure. If there is a connection made at the TTL IN (J7) port at this time, ensure that there is no contact closure between the leads. If there has been contact closure, open it, make certain it remains open, and briefly interrupt the power before continuing.

4. Select the first option on the menu of the PSD2DEMO program: Manual ASCII Commands.

5. Type aXR <enter> to verify communications setup. The screen should echo the command and acknowledge (ACK) it. The module should initialize the valve motor and syringe drive. If these things do not happen and/or a screen error appears, check all connections, hit <esc>, interrupt the power to the module, and start again at Step 2.

6. If this is the first time computer communication has been accomplished with the PSD/2 module at hand, or if the user is uncertain of this, type aYSM1R <enter>. This command sets the syringe drive at full resolution (0–2,100 steps) and enables accurate volume adjustments.

7. Based on the amount of WAF that needs to be injected into the splitter box (see test-specific TCTs), choose the correct-size Hamilton syringe (dependent on injection amount) with a threaded hole in the thumb-plate of its plunger. Attach the syringe to the correct valve for the syringe size (part no. 39221 for syringes of 2.5 mL or higher, unless it is determined that the injection stroke speed is slow enough so as not to create excessive backpressure, or part no. 39298 for smaller syringes). The syringe twists clockwise to engage the Luer Lock fitting. The valve and syringe can then be put in place by confirming that the valve-locking handle is in the counter-clockwise position as far as possible; the valve with the syringe is pushed in position until it sits against the valve adapter; the valve-locking handle is rotated clockwise until it locks in a vertical position; and the thumbscrew on the syringe drive arm is secured into the end of the syringe plunger (this may require positioning of the plunger).

8. Type aXS12R <enter> to initialize again for syringe position. The inclusion of S12 in this command is to slow the syringe drive speed in case the dry syringe presents too much resistance for the syringe drive to operate at the default speed of S4. With large syringes of 10–25 mL capacity, the S or Speed Command may need to be increased beyond S12. This to be determined at the time of installation.

9. Type IP(xxxx)S12N5R <enter> with (xxxx) being some step number between 0 and 2,100. This step number is an estimate of what will draw the syringe to the proper volume. As in Step 5, the command should be echoed, acknowledged <ACK>, and executed. Determine the volume called for by the step number chosen and readjust as necessary. Repeat Steps 8 and 9 until the correct injection volume is achieved.

10. Type the following instruction codes in order with <enter> following each line. Fill the (xxxx) with the step number determined in Step 9. While these commands are being entered, the module will echo and acknowledge <ACK> on the computer screen, but it will not execute the functions.

    - a#SP9: Clears the EEPROM memory.
    - a#SP8: Starts the download mode, selects method 8.
    - aXS12R: Initializes the module on the first trigger after any power interrupt. The S or Speed may need to be increased and should be reflected in all S values past this command.
    - aIP(xxxx)S12N5: Sets the valve to the input port, draws syringe the number of steps chosen, and returns 5 steps at speed 12 (12 seconds for a full syringe stroke).
    - aOD(xxxx)S12R: Sets the valve to the output port, and moves the syringe drive backup (dispense) the same number of steps drawn at speed 12. The execute command placed here ensures that the draw and dispense strokes are executed as a unit on each trigger experienced after the first.
    - a#SP0: Ends the download mode.

11. Type <esc> to return to the menu and end communications.

12. Interrupt and re-establish power to the PSD/2 module.

13. Close the contact on the leads from the TTL IN port (J7). The module should initialize and stop. Close the contact again, and the module should execute a complete injection cycle as programmed. Once in place, with nothing connected to the COM IN (J8) port, only power (J4), and TTL IN (J7), the module should execute the downloaded set of functions as described each time remote activation by contact closure occurs. Be aware that the first contact closure after power interruption will trigger initialization only. Computer communication can be achieved only if Steps 1–4 above are followed carefully, and if no untimely contact closure by the remote activation system has occurred.

14. Any changes to downloaded instructions will have to be made by following all steps listed above, except Step 6. No editing options exist. The memory must be cleared and another download executed.

3.4.4 ASTM International chamber and flow-through loading calculations SOP

Purpose

The purpose of this SOP is to provide guidelines for calculating loading in chambers for static, renewal, and flow-through conditions. The number of organisms that were loaded per tank can be found in the test-specific TCTs.

General

1. Loading is the ratio of biomass (wet weight, blotted dry, in grams of whole body tissue) to the volume of solution in a chamber, or passing through it, in a 24-hour period. The loading in culture, testing, and growout was not so high as to affect the health of the animals by an accumulation of excretory waste material, a diminution in DO, or physical contact of organisms to each other. Therefore, loading was maintained below a maximum established level.

2. The loading of organisms in test chambers is generally based on values proposed in ASTM International (ASTM) E729-88a (*Standard Guide for Conducting Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians*). The guideline requires that in static or renewal tests, the loading in each test chamber shall not exceed 0.8 g/L at or below 17°C, and shall not exceed 0.5 g/L at higher temperatures. In flow-through tests at or below 17°C, the loading shall not exceed 10 g/L in the chamber at any time, and shall not exceed 1 g/L of solution passing through the chamber in 24 hours. At temperatures greater than 17°C, the loading in flow-through tests shall not exceed 5 g/L at any time or 0.5 g/L passing through the chamber in 24 hours. This information is summarized in Table 3.1.

Table 3.1. Maximum loading density guidelines

	Maximum loading density	
Holding conditions	**g/L at any time**	**g/L in 24 hours**
Static or renewal, ≤ 17°C	0.8	–
Static or renewal, > 17°C	0.5	–
Flow-through, ≤ 17°C	10	1
Flow-through, > 17°C	5	0.5

Procedures

A. Static or renewal conditions

For organisms maintained under static or renewal conditions, it was necessary to ensure that the maximum chamber loading at any time was not exceeded.

B. Determination of the acceptable number of organisms for a given vessel

1. To determine the acceptable loading (number of organisms) for a given chamber under static or renewal conditions, the volume of solution and the average organism wet weight were determined. The volume of solution was determined for an aquarium by length × width × solution depth. In a vessel that did not have right-angle dimensions, the solution volume was determined by volumetric addition of a liquid medium. The average organism wet weight was determined from a grab or otherwise random sample of organisms comparable to those to be maintained in the chamber. The organisms were preferably individually weighed, but sometimes a pooled weight was taken.

2. To determine the number of organisms that could be placed in the chamber, the solution volume was first multiplied by the appropriate maximum loading density determined from the table. The result was divided by the average wet weight of the organism. The resulting value was the approximate number of organisms that could be added to the chamber to not exceed loading.

C. Determination of the volume of solution necessary to hold a given number of organisms

To calculate the volume required to house a group of organisms in a static or renewal chamber, the total organism wet weight was divided by the maximum loading density. The final result was the minimum liters of solution needed to meet the ASTM guidelines.

D. Flow-through conditions

For organisms that were maintained under flow-through conditions, it was necessary to ensure both that the maximum chamber loading at any time was not exceeded and that the flow of water through the tank in 24 hours was sufficient. Chamber loading calculations were performed in the manner described previously for static or renewal conditions.

E. Determination of the acceptable number of organisms for a given flow rate (cycles/hour)

1. To determine the acceptable loading (number of organisms) for a given flow rate under flow-through conditions, the volume of solution entering a chamber each

day and the average organism wet weight must be known. In intermittent flow-through systems, the daily volume of solution was determined by multiplying the given cycles/hour by the volume of solution entering a chamber each cycle, by the 24 hours in a day. In a continuous flow-through system, the volume of solution per day was calculated by multiplying the flow rate in L/minute by 60 minutes in an hour, by 24 hours in a day.

2. To determine the number of organisms that could be placed in the chamber, the daily solution volume was first multiplied by the appropriate maximum loading density determined from Table 3.1. The result was divided by the average wet weight of the organism. The resulting value was the approximate number of organisms that could be added to the chamber to not exceed loading.

F. Determination of the flow rate (cycles/hour) necessary for a given number of organisms

To calculate the flow rate necessary for a given number of organisms in a flow-through chamber, the total organism wet weight was divided by the maximum loading density. The final result was the minimum volume of solution that must pass through the chamber each day to meet the ASTM guidelines. Intermittent flow rate in cycles/hour was then calculated by dividing this volume by the product of the volume of solution entering a chamber each cycle and the 24 hours in a day. Continuous flow rate in L/minute was calculated by dividing this volume by the 1,440 minutes in a day (60 minutes/hour times 24 hours/day).

3.4.5 Monitoring water quality parameters during static and flow-through exposures SOP

Purpose

The purpose of this SOP is to provide guidelines for monitoring water quality parameters during toxicity tests.

General

Chemical and physical data on test solutions are routinely generated during toxicity tests at times prescribed by the test protocol (see test-specific TCTs). Generally, the temperature, salinity, DO concentration, and pH were measured during the tests.

Equipment and materials

1. DO – YSI ProODO and DO probe

2. pH – pH probe (Hach PHW77-SS)

3. Salinity – Atago handheld refractometer (ATC-S/Mill-E)

4. Temperature – calibrated mercury-filled thermometer, a digital thermometer, or a temperature recorder

5. Total unionized ammonia – measured with Koch Ammonia Strips or Tetra Easy Strips.

Procedures

A. Monitoring methods

1. DO concentration – measured daily in one randomly selected replicate per concentration.

2. pH – measured daily in all exposure containers.

3. Salinity – measured daily in all exposure containers.

4. Temperature – for exposures performed in small volumes in environmental chambers, daily temperature readings were taken from a temperature-equilibrated beaker placed inside the environmental chamber. For exposures performed in larger tanks, daily temperature recordings were taken from each tank.

5. Total unionized ammonia – measured daily in one randomly selected replicate per concentration.

B. Recordkeeping

All chemical and physical measurements taken during tests were recorded on the appropriate bench sheets. Data collected by the probes were digitally stored and printed for a hard-copy reference in the data logbook. Measurements not within protocol/procedural specifications were reported to the study manager immediately.

Measuring devices or methods of determination were recorded for each occasion that the measurements were performed.

Decontamination procedures

DO meters and handheld refractometers were decontaminated after each use by gentle washing with warm soapy water, rinsing with deionized water, and careful rubbing with Kimwipes. When making multiple measurements across multiple treatments, care was taken to ensure that measurements were always made in order from lowest concentration to highest, starting with the controls.

Calibration procedures

DO meters and handheld refractometers were calibrated monthly following manufacturer protocols.

3.4.6 Protocol for preparing oil-spiked sediments SOP

Purpose

The purpose of this SOP is to provide instructions for the preparation of oil-spiked sediments for exposures.

General

- Controls were prepared using the same technique that was used for spiking sediment, with the exception of adding oil.
- Each sediment-oil concentration was prepared separately. For instructions on cleaning and preparing the equipment, refer to the Decontaminating Glassware SOP in the QAPP.
- Unused, prepared sediments were placed into a Ziploc bag and stored in the dark at 4°C (short-term) or in the freezer at -20°C (long-term).

Preparation of sediments

1. Sediments were allowed to thaw overnight at room temperature
2. All debris (sticks, etc.) was removed from thawed sediment and sediment was placed in a mixing bowl
3. Sediment was homogenized by mixing for 2 minutes at low speed (1).

Mixing oil into sediments

1. The appropriate amount of oil was weighed out as outlined below:

   Slick oil was weighed in a pre-cleaned aluminum weigh boat. A weigh boat was tared with 2 to 3 Kimwipes on the top loading balance. Using a stainless steel spatula, slightly more than the desired mass of oil was added onto the weigh boat. Using the spatula, oil was transferred onto the sediment in the mixing bowl and placed in several areas around the bowl (step 2). Any remaining oil was wiped off of the spatula with the tared

Kimwipes. The weigh boat was re-weighed with the Kimwipes to calculate and record the actual mass of oil transferred.

2. The mixer paddle was lowered into the bowl.

3. Oil was mixed into the sediment with a KitchenAid KM45 4.5 quart stand mixer at medium speed (4.5) for 30 minutes or with a Cuisinart SM-70 7-quart stand mixer, also at medium speed (5). The mixer was stopped briefly every 2 to 4 minutes to scrape the sides of the mixing bowl with a putty knife.

4. Once mixing was complete, the mixer paddle was scraped with a putty knife to remove all excess oiled sediment. The sediment was transferred from the mixing bowl to the test containers using a stainless steel spoon, or stored in the dark at 4°C until test initiation.

5. A sample of oil-spiked sediments was collected from each treatment. Samples were stored at 4°C before being shipped

3.4.7 RNA extraction and qPCR for gene expression analyses SOP

Purpose

This protocol describes in detail the experimental procedure for real-time qPCR using SYBR Green I.

General

Real-time qPCR determines relative amounts of genes of interest using a fluorescent reporter, SYBR Green.

Procedures

1. Best laboratory practices were followed to prevent contamination of samples. This included wearing gloves at all times and cleaning the work area and pipettes before prepping the samples or preparing the master mix (step 3).

2. All reagents (step 3) and samples were thawed on ice. Once they had thawed, all components were thoroughly mixed.

3. Enough of the following reaction master mixture was prepared to run each sample in triplicate.

   - Volume per single 20-µL reaction:
     - Two aliquots of 10-µL SYBR Green Mix
     - 1.8 µL of each forward and reverse primer (0.9 µm each)
     - µl H_2O (nuclease-free)
     - µl cDNA template

4. 1.0 µL of template, plus 19 µL of master mix were added to each of the 96 wells in a plate, and the plate was sealed with optically clear film. Samples were run in triplicate along with a no-template control to test for contamination.

	Temp (°C)	Time (s)
Initial denaturation	95	30
40 cycles of:		
Step 1	95	5
Step 2	58	15
Step 3	72	10

5. All qPCR reactions were amplified using the ABI 7500 Fast machine or similar.

6. An endogenous control, such as 18S, was run simultaneously with each gene of interest for all samples.

3.4.8 Method to extract DNA for microbial analyses SOP – conducted at Texas Tech University

DNA was extracted from tissues using a PowerSoil DNA Isolation Kit with minor adaptations (MoBio Laboratories). A thoroughly homogenized aliquot of each tissue sample was added to the PowerSoil bead tube. The extraction proceeded per the directions in the kit, resulting in 100 µL of DNA in the elution buffer (10 mM Tris). Concentrations of DNA in each sample were measured and recorded using a NanoDrop (Thermo) to account for total DNA used in qPCR.

3.4.9 Microbial diversity analysis SOP – conducted at Texas Tech University

The relationship between microbial communities in intestine and gill tissues of oil- versus non-oil-exposed fish was determined by 16S rRNA gene amplification and sequencing as described

by Dowd et al. (2008), targeting the V1–V3 regions. Sequences that failed to return at least half the expected amplicon length (or 250 bp, whichever was shortest) were removed from the data pool. All sequences were then denoised using an algorithm based on USEARCH and checked for chimeras using UCHIME (Edgar, 2010). After denoising and chimera checking, sequence data were separated into operational taxonomic units (OTUs) and annotated using the RDP classifier (Edgar et al., 2011) with GreenGenes v. 12.10 (MacDonald et al., 2012) used as a reference. Finally, relative abundances of taxa at each hierarchical taxonomic level were calculated using the summarize taxa.py QIIME script.

3.4.10 Gill histology image analysis methods SOP

Scope

The scope of this SOP was to provide protocols for gill histology sample preparation and analysis.

General

The purpose of histological analysis was to determine the effect exposure had on the cellular structure of a given tissue.

Histology sample preparation

The entire heads and body cavities of juvenile southern flounder were preserved in 10% neutral buffered formalin (NBF) for at least one week. Tissues were decalcified in Cal-Ex decalcifier (Fisher Scientific) overnight, rinsed for 18 hours in running tap water, and dehydrated in a series of graded ethanols for histological processing. Tissues were embedded in Paraplast and sectioned at 4 μm at two levels: the middle of the eye and completely through the eye. One section from each level was mounted on slides and stained with hemotoxylin and eosin. All areas of gill tissue from each fish on slides were photographed at 100x magnification using a Nikon Eclipse microscope and ACTcamera software. This process resulted in 4 to 12 photographs of gill tissue per fish. Each photograph was numbered with the test number, fish number, tissue type, and photograph number for that tissue type (e.g., ST133 1A6G1).

Photographs were analyzed to quantify the percentage of gill lamellae that presented with various histopathological conditions. Histopathological conditions recorded included telangiectasis in secondary lamellae, epithelial proliferation, swollen/fused lamellae, and the rank of telangiectasis. Ranks were defined based on the percentage of filaments presenting telangiectasis that were observed for each lamella: 0 = no telangiectasis; 1 = < 25% filaments presenting telangectasis; 2 = 25–50% filaments presenting telangectasis; 3 = 51–75% filaments presenting telangiectasis; and 4 = > 75% filaments presenting telangiectasis. Three photographs

were analyzed for each fish – the photographs to be analyzed were selected randomly using a random number table. All lamellae occurring in each photograph were inspected for histopathological conditions. To determine the percentage occurrence of each histopathological condition within the gill, the total number of lamellae present was divided by the total number of lamellae containing the histopathological condition. The mean rank of telangiectasis was determined by adding the rank scores for all lamellae and dividing that number by the total number of lamellae present.

3.4.11 Liver histology image analysis methods SOP

Scope

The scope of this SOP is to provide protocols for liver histology sample preparation and analysis.

General

The purpose of histological analysis was to determine the effect exposure had on the cellular structure of a given tissue.

Histology sample preparation

The entire head and body cavity of juvenile southern flounder were preserved in 10% NBF for at least one week. Tissues were decalcified in Cal-Ex decalcifier (Fisher Scientific) overnight, rinsed for 18 hours in running tap water, and dehydrated in a series of graded ethanols for histological processing. Tissues were embedded in Paraplast and sectioned at 4 µm at two levels: the middle of the tissue and completely through the tissue. One section from each level was mounted on slides and stained with hemotoxylin and eosin. All areas of liver tissue from each fish on a slide were photographed at 100x magnification using a Nikon Eclipse microscope and ACT camera software. This process resulted in 4 to 16 photographs of liver tissue per fish; there was no overlap of liver tissue in any photograph. Each photograph was numbered with the test number, fish number, tissue type, and photograph number for that tissue type (e.g., ST133 1A6L1).

Photographs were analyzed using Image J software to allow quantification of the percentage of liver tissue that presented with various histopathological conditions. Histopathological conditions recorded included macro/micro vesicular vacuolation, congestion, edema, eosinophilic inclusions in exocrine pancreas, and the amount of exocrine pancreas present. Three photographs were analyzed for each fish – the photographs to be analyzed were selected randomly using a random number table. A grid of 80 points (accessed through Plugins-Analyze-Grid) was superimposed on the selected photographs, and histopathological conditions occurring at each point were recorded. Any grid points intersecting non-liver tissue area were recorded as “total excluded

grids." The total number of excluded grid points was subtracted from the total number of grid points (240), and the resulting number of grid points containing liver tissue was used for calculations. To determine the percentage occurrence of each histopathological condition within the liver, the total number of grid points recorded for each condition from all three slides was divided by the total number of grid points containing liver tissue. To determine the percentage of eosinophilic inclusions in exocrine pancreas, the number of grid points containing this condition was divided by the number of grid points containing exocrine pancreatic tissue.

3.5 General Immunotoxicity Testing Standard Operating Procedures

3.5.1 Media preparation and QA/QC SOP

Purpose

The scope of this SOP was to provide protocols for the QC of all microbiological media, including equipment and protocols used for media preparation.

General

All media used in this project came pre-mixed, except for Phosphate Buffered Saline (PBS), which had multiple components. The manufacturer's instructions on preparation were printed on the bottle, were available from the manufacturer, and were printed on the specific QC forms for each media preparation.

Procedures

The following procedures were followed for equipment monitoring:

1. On a monthly basis, the autoclave was checked with a calibrated thermometer on a gravity 20 cycle and the temperatures were recorded.

2. On a monthly basis, the sterility of the autoclave was checked using duo-spore biological indicator strips. Strips were aseptically inoculated into Tryptic Soy Broth (TSB) tubes and incubated at 55°C (+ /-3°C) for seven days, per manufacturer instructions.

3. The sample incubator temperature was checked twice daily using a digital thermometer. The current temperature readings, along with the minimum and maximum temperatures stored in the thermometer memory, were recorded. The pH meter was calibrated before each use, using purchased pH 4, pH 7, and pH 10 standards. The before-correction and post-correction readings were recorded for each standard.

4. Before each use of the meter and after calibration, the pH meter was checked using the pH 7 standard. If a reading was off by more than 0.2 pH units, the meter was recalibrated.

The following procedures were followed for the preparation and QA of all media:

1. Ready-to-use mixtures were prepared following the manufacturer's instructions.

2. PBS was prepared by adding 0.58 g NaH_2PO_4-$2H_2O$, 2.5 g Na_2HPO_4-$2H_2O$, and 8.5 g NaCl to 1-L deionized water. The resulting solution was then autoclaved at 121°C for 15 minutes.

3. Once autoclaved, the PBS was allowed to cool to room temperature. An aliquot was removed to measure final pH. If the measured pH was not 7.4 +/- 0.2 pH units, the pH meter was checked using the pH 7 standard, and recalibrated if necessary. A second aliquot of PBS was collected and measured; if the pH was again not with the appropriate range of 7.4 +/- 0.2 pH units, the media was discarded and a new batch of PBS was prepared.

4. All plates were prepared in 100-mm disposable petri dishes at a 20-mL volume.

5. All agar plates were tested for sterility using an un-inoculated plate placed in the 33°C incubator for a minimum of 48 hours.

6. TSB was tested by placing un-inoculated broth into a sterile test tube and incubating it at 33°C for a minimum of 48 hours. PBS was tested by placing 1 mL into a sterile test tube that contained previously tested TSB; the tube was then incubated at 33°C for a minimum of 48 hours.

7. A positive control was prepared for each batch of agar by streaking challenge organisms onto a plate. Controls were checked for growth at after one and two days; if no observable growth was present after two days, the agar was discarded and re-prepared.

8. Positive controls on TSB consisted of inoculated tubes containing each of the challenge organisms that were incubated at 33°C for one to two days. Broth that did not produce turbid growth in two days' time was discarded and re-prepared. A 1-µL loop of challenge bacteria was inoculated into 100 µL of PBS and allowed to sit at room temperature for one hour. The PBS was then plated onto an MA plate, which was incubated 33°C for one to two days. Any PBS that did not produce growth was rechecked with a second aliquot. If the second aliquot failed to produce growth, the PBS was discarded.

9. All media was stored in the dark at room temperature.

10. Marine Agar plates were checked for surface moisture and dried if there was condensation present on the agar surface. Plates were also checked for contaminants and discarded if any were present.

3.5.2 Bacterial inoculation

Bacterial inoculation occurred after fish were exposed to oil-contaminated water or sediment. Bacteria (e.g., *Vibrio anguillarum*) were grown in overnight culture to a density of approximately 1 x 10^9 cfu/mL, as assessed by comparison to McFarland Standards. The bacteria were then introduced into the bacterial exposure tanks at the desired concentration by direct addition with a pipette. Bacteria and water were allowed to mix for approximately 30 minutes before fish were exposed. All samples destined for bacterial enumeration were documented using the appropriate bench sheet.

Final bacteria concentration and fish exposure duration were described in test-specific TCTs before beginning definitive tests. The concentration of bacteria for inoculation was determined for each species and, when appropriate, each cohort of fish using test performance information from preliminary dose-response exposures similar to those proposed in this GLPP. For example, we assessed survival rates and bactericidal capacity [using the Bacterial Killing Assay (BKA)] after exposing two flounders, per concentration, to four concentrations of *V. anguillarum*. The exposures lasted for 24-hrs and enabled us to determine the highest concentration (1 x 10^6 cfu/mL) in which all fish survive but with the greatest immune suppression.

3.5.3 Bacteriological analysis of sediment and water SOP

Scope

The scope of this SOP was to provide protocols for bacterial analysis of the water and sediment for pre- and post-pathogen challenge.

General

To obtain a quantitative measurement of bacterial loads before and after exposure, we plated water and sediment directly onto non-selective media. Non-selective media (Marine Agar) provided an understanding of the total bacterial population in the challenge tanks, while allowing phenotypic characterization to aid in further molecular analysis. In addition, water and sediment samples, if applicable, were removed and stored for later microbiomic analyses.

Procedures

Sediment analysis

a. For each sediment type (oiled sediment and non-oiled sediment), five Marine Agar (MA) plates were labeled, brought to room temperature, and checked for contaminants before use.

2. For each sediment type, six 50-mL conical tubes with PBS were prepared; one was labeled 1:2 and the remainder of the tubes were labeled for 10-fold dilutions, from 1:10 to 1:100,000.

3. Twenty millimeters of PBS were added to the 1:10 dilution, and 18 mL of PBS were added to each successive dilution (1:100–1:100,000).

4. Before adding sediment to the tanks, a 25-mL aliquot of sediment was aseptically collected from each pooled sediment batch (oiled sediment and non-oiled sediment) and placed into the appropriate 50-mL conical tube.

5. The conical tube was then filled to the top with PBS, capped, and shaken vigorously.

6. Five millimeters were then removed and placed into the tube labeled 1:10 dilution, which contained 20 mL of PBS.

7. After shaking vigorously, 2 mL were removed and placed into the tube labeled 1:100.

8. Steps 6 and 7 were repeated for the remaining dilutions (1:100–1:100,000).

9. Plates were labeled with the date and dilution.

10. For each dilution, 0.2 g of the well-mixed sediment slurry were plated onto each of the agar plates and carefully spread with a sterile spreader.

11. Slurry liquid was allowed to absorb before inverting the plates and placing them in the incubator.

12. The plates were incubated for 16–18 hours at ~ 25°C.

13. After 18 hours, colonies on the lowest dilution were counted.

14. Additionally, approximately 2 mL of sediment were collected aseptically into a 2-mL microcentrifuge tube and stored at -80°C for later microbiomic analysis.

Water analysis

1. 1 For each tank, two MA plates were labeled, brought to room temperature, and checked for contaminants before use.

2. For each tank, two 50-mL conical tubes were labeled and 20 mL of PBS were added to one.

3. Approximately 50 mL of water from each tank were collected in one conical by dipping the conical down toward the center of the tank without disturbing the sediment.

4. The cone was lifted and the water in the cone was vigorously mixed and 20 mL of suspended sediment and water were quickly transferred to the second conical, which contained 20 mL of PBS.

5. The PBS/water dilution was vigorously mixed and 500 µL were plated onto an MA plate.

6. The MA plates were incubated for 16–18 hours at ~ 25°C.

7. After 18 hours, colonies on the lowest dilution were counted.

8. Additionally, 100 mL of water were pooled from the replicate tanks of each treatment type (oiled sediment and non-oiled sediment), aseptically collected, filtered through a 0.20-um mesh filter, and stored at -80°C for later microbiomic analysis.

3.5.4 Fish dissection and sampling SOP

Scope

The scope of this SOP was to provide protocols for dissection and sample collection from fish that were challenged with pathogens.

General

Proper attire was worn at all times while handling the fish. This included, at a minimum, gloves, eye protection, proper clothing, and shoe protection. At no point during biological sample collection was the interior of the fish touched by anything other than autoclaved or alcohol-flamed scissors and forceps. During dissection, proper attire included, at a minimum, gloves and disposable or autoclavable clothing protection. All biological samples were weighed in sterile petri dishes. When possible, samples of each organ were taken and preserved for histological analysis.

Procedures

Fish measurement

Before euthanizing a fish, its weight and standard length (SL) were taken and recorded on the appropriate bench sheet.

Euthanization, blood collection, and visual inspection

1. Individually, fish were removed from their aquaria and visually inspected for lesions before being placed in the anesthetic bath. If any gross pathologies, including lesions, were present, their descriptions were recorded and photographed; lesions were swabbed for bacterial analysis before immersion in the anesthetic bath.

2. To euthanize a fish, it was placed in 50 mg/L solution of MS-222.

3. As soon as a fish stopped pushing water through its gills, blood collection was performed for the purpose of BKA, according to SOP "Bacteriological Analysis of Sediment and Water."

Dissection and sample collection

1. The exterior of the fish was cleaned with iodine and blood was collected from the caudal vein by cutting the tail and collecting the blood in a capillary tube.

2. Using alcohol-flamed scissors, the fish was cut open along the ventral side, from the proximal end to the anterior end and up the left side, removing the skin to expose the organs.

3. Using a newly flamed set of scissors and forceps, the liver, spleen, kidney, gills (upper and lower), and gut were removed and placed individually in 500 µL of RNALater for subsequent examination of gene expression via transcriptomic analysis, bacterial abundance via microbiome analysis, and qPCR quantification.

4. If necessary, tissue samples to be analyzed for bacterial abundance were diluted in PBS and plated on MA plates as in the "Bacterial Analysis of Sediment and Water SOP."

5. The remaining fish carcass was wrapped in aluminum foil, labeled, and stored at -20°C for archive according to the QAPP.

Histological preservation

1. When possible, organ samples for histology were taken after the bacteriological and toxicological samples.

2. To collect histological samples, a small cut of each organ was taken and then weighed.

3. The sample was placed into a histology cassette, which was then placed in 10% buffered formalin. Samples were labeled, stored, and shipped as described in the QAPP.

3.5.5 Bacterial analysis of tissue SOP

Scope

The scope of this SOP was to provide protocols for the biological analysis of samples that were collected from pathogen-challenged fish to provide quantitation of pathogenic infection of fish via lesions and bacterial load in selected organs.

General

Organs were plated directly onto selective and non-selective media to obtain a quantitative measurement of bacterial loads in the organs. Non-selective media (Marine Agar) provided an understanding of the total bacterial population of the organs, while selective media (TCBS) grew only specific bacteria with distinct phenotypic characteristics. This allowed for quick identification (ID) of the pathogens used in the study. The enrichment broth was used to increase the bacterial levels in the organs, which otherwise could have been too low to detect by direct plating.

Procedures

Dissection sample plating

1. For each fish, 10 TCBS and 10 Marine Agar plates were labeled with the appropriate sample ID as described in the QAPP. All TCBS plates were dried for 30 minutes at 33°C. Marine agar plates were checked for surface moisture and dried if necessary.

2. An extra 10 plates of each agar were prepared for any samples of opportunity (e.g., lesions).

3. Fourteen sterile 1.5-mL microfuge tubes were prepared for each fish: four with 1 mL of TB, three with 100 µL of PBS, six with 990 µL of PBS, and one with 35 µL of PBS.

4. All plates and tubes were labeled as described in the QAPP.

5. Blood (10 µL) and organs were removed and prepared at necroscopy, as described in the Fish Dissection and Sampling SOP.

6. Blood and organ samples for bacteriological analysis were split in half; one portion was placed into thioglycollate broth and the other into 95 µL (blood) or 100 µL (organs) of sterile PBS.

7. Both sets of organ samples were macerated by mashing with micropipette tips.

8. The macerated organs and blood in PBS were diluted, respectively, to 1:10 and 1:100 with PBS.

9. 100 µL of each sample were plated directly onto TCBS and Marine Agar plates using appropriate sterile techniques.

10. The thioglycollate broth tubes and plates were transported to the Marine Microbiology Laboratory and incubated at 33°C for 16–18 hours.

Colony analysis

1. Plates and tubes were removed from the incubator after 16–18 hours; the exact time was recorded on sample data sheets.

2. Plates were examined for growth; the lowest dilution containing countable colonies was examined and colonies were counted based on colony morphology for presumptive Pd and Vh on TCBS. Colony counts were back-calculated to CFU/g tissue.

    a. *Vibrio harveyi* produced yellow colonies on TCBS and *Photobacterium damselae* produced dark-green colonies.

3. A representative subsample of each morphology class was analyzed by genetic sequencing with universal 16S primers to verify presumptive identity.

4. If samples did not exhibit growth from direct plating, then 100 µL of broth from enrichment tubes exhibiting visible turbid growth were plated onto TCBS plates, incubated for 16–18 hours, and analyzed for colonies.

3.5.6 BKA SOP

Scope

The scope of this SOP was to provide protocols for the immunological analysis of samples collected from pathogen-challenged fish to provide quantitation of innate and adaptive immunity.

General

The purpose of this assay was to determine the effect the exposure had on both acquired and innate immunity.

Procedures

Bacteria culture preparation

1. T1N3 broth (T1N3) was prepared according to the Media Preparation & QA/QC SOP.

2. Approximately 20 mL of T1N3 was inoculated with *Vibrio anguillarum* and incubated overnight with shaking.

Blood collection

1. Fish were anesthetized with MS222.

2. Using a sterile gauze pad, the outside of the fish was wiped down using ethanol.

3. Using sterilized scissors, the tail was cut off, slicing the caudal vein in the process; blood was collected using a heparinized capillary tube.

4. Blood was displaced from the capillary tube into a 0.2 mL microcentrifuge tube that had been washed with sodium citrate or EDTA (for anti-coagulant properties). After 1.5 µL of blood was removed for each replicate (see below), the remaining blood was reapplied to a capillary tube, then centrifuged to remove plasma. Plasma was stored at -20°C.

BKA assay

1. Using a McFarland Standard, overnight bacterial culture was diluted to 1 x 10^5 CFU/mL with BHIB.

2. 1.5 µL of whole blood was placed into the prepared 35 µL PBS tube.

3. As a positive control, 12.5 µL bacteria to 36 µL PBS were prepared; as a negative control, 48 µL of PBS were prepared.

4. To each of the sample tubes, 12.5 µL bacteria were added.

5. The samples and controls were incubated at 30°C for 30 min.

6. The samples were removed from incubator and vortexed; 250 µL of sterile T1N3 broth was added to each sample and control. The mixture was vortexed and incubated at 30°C for 12 hours.

7. The absorbance at 450 nm was measured for all samples and controls.

8. The anti-microbial activity was determined by calculating:
1 – (Absorbance of sample/Absorbance of control).

3.5.7 Packed cell volume (PCV) SOP

Scope

The purpose of this SOP was to provide protocols for the indirect evaluation of red blood cell (RBC) volume using PCV, which estimated the proportion of hematocrit to total blood volume.

General

Blood was collected from sacrificed fish using heparinized capillary tubes and stored in EDTA-washed tubes. Centrifugation and PCV measurements were taken soon after blood collection (< 30 minutes).

Procedures

1. Blood collection

1.1 Fish were sacrificed as described in the Fish Dissection and Sampling SOP.

1.2 Once a fish was effectively euthanized, it was sliced just ahead of its tail with a razor blade. The blood was immediately collected with a capillary tube, tipping the fish so that it was above the capillary tube. The blood was collected in two heparinated capillary tubes so that the PCV tube was approximately 3/4 full. The blood was mixed well by an up-and-down motion.

1.3 Both ends of the PCV capillary tube were sealed using Crit-o-Seal.

2. Centrifugation of PCV capillary tubes

2.1 Making sure that the PCV capillary tubes were firmly secured to the rubberized edge of the single-speed capillary microcentrifuge, they were spun for 1 minute.

2.2 Figure 3.1 shows a capillary tube post-centrifugation. Centrifugation separated the total blood sample into RBCs (red layer) and plasma (clear layer). To estimate PCV, we measured the length of the hematocrit layer (red layer) and the length of total blood. PCV was hematocrit length/total blood length. The results for both capillary tubes needed to agree within four percentage points. If they did not, the hematocrit procedure was repeated. If they did agree, the two measurements were averaged.

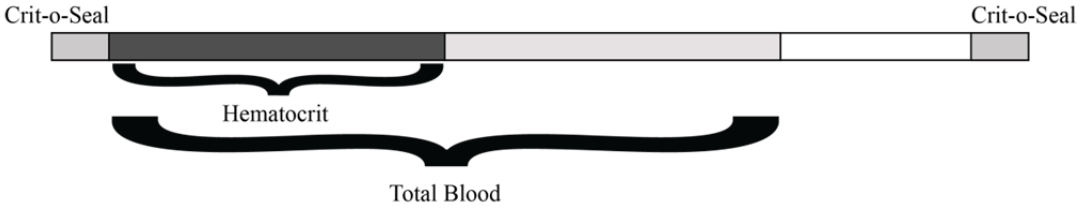

Figure 3.1. Capillary tube post-centrifugation.

2.3 Alternatively, a micro-capillary reader was used to quickly assess the proportion of hematocrit length to total blood length.

2.4 All capillary tubes were properly disposed of in biohazard sharps containers.

3.5.8 RBC count SOP

Scope

The purpose of this SOP was to provide protocols for the direct enumeration of RCBs using a hemocytometer.

General

Blood was collected from sacrificed fish using heparinized capillary tubes at the same time that samples were taken for PCV; blood was stored in EDTA-washed tubes.

Procedures

1. Blood was collected on two heparinated capillary tubes to prevent coagulation. The capillary tube was filled 2/3 full and mixed well by inverting it.

2. A 1:200 dilution was prepared with saline and mixed.

3. The dilution was plated on each side of the chamber to allow cells to settle for 3 minutes.

4. Four outer squares were counted, as well as the center square of the center square mm (sqmm), on each side of the hemocytometer using 40x objective and low light. Cells that were touching the upper and left limits were counted; cells touching the lower and right limits were not counted. If there were fewer than 100 cells in a sqmm, two or more 1-mm square areas were counted and the results were averaged. The center square was used for platelets and red cells.

   Total area counted = 0.4 mm^2
   Center square was divided into 25 squares; each square in the center square was 1/25 mm^2
   5/25 mm^2 counted on each side = 10/25 mm^2 OR 1/5 mm^2 + 1/5 mm^2 = 2/5 mm^2 = 0.4 mm^2

5. RBCs/mL were counted.

   $$\text{RBC} = \frac{\#\text{cells (both sides)} \times 200}{0.1\text{ mm} \times 0.4\text{ sqmm}} \quad \text{or} \quad \frac{\text{total \#cells} \times 200 \times 10\text{ mm}}{0.4\text{ sqmm}}$$

   Example:

   Side 1 305
   Side 2 315

   $$\frac{620 \times 200 \times 10}{0.4} = 3.10 \text{ million/cmm or } 3.10 \times 10^6/\mu L$$

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A. Testing Protocol 1: Definitive Acute Exposures

A.1 Equipment and Materials

CEWAFs, HEWAFs, and Corexit-only exposure solutions were prepared as described in the QAPP; spiked sediment was prepared according to the protocol listed in Section A.3.

A.1.1 Organisms for exposure

1. Sheepshead minnow (*Cyprinodon variegatus*), obtained from GCRL Toxicology laboratory culture

2. Speckled seatrout (*Cynoscion nebulosus*), obtained from GCRL Cedar Point Aquaculture facility

3. Blue crab (*Callinectes sapidus*), obtained from GCRL blue crab program and the University of Maryland

4. Grass shrimp (*Palaemonetes pugio*), obtained from marsh areas around GCRL

5. Southern flounder (*Paralichthys lethostigma*), obtained from Texas Parks and Wildlife

6. Fiddler crab (*Uca longisignalis*), obtained from Auburn University

7. White shrimp (*Litopenaeus setiferus*), obtained from Florida Organic Aquaculture.

A.2 Procedures

The following guidelines were followed when definitive toxicity tests were being planned:

1. Each definitive toxicity test consisted of different concentrations of HEWAF, CEWAF, Corexit, or spiked sediment plus a control, with 3 or 4 replicates per each treatment. The specific concentrations and number of replicates for each test can be found in the test-specific TCTs.

2. Generally 10–20 organisms per replicate were used for exposures, but fewer animals were sufficient if space or animal numbers were limited. The specific number of organisms used per replicate can be found in the test-specific TCTs.

3. The necessary volume of exposure water and corresponding container size from the loading were calculated based on the weight of the test organism. Refer to GCRL GLPP when making these calculations. Water quality (temperature, salinity, pH, DO, ammonia) parameters were recorded daily in at least 1 test container/treatment.

4. The location of test and all other pertinent data were recorded on the appropriate bench sheets.

5. Exposure duration details can be found in the test-specific TCTs. Vessel size/volume was in agreement with approximate body mass of test organisms. In general, fish embryo, larval fish, and crustacean zoeae acute exposures occurred in 15 × 75 Pyrex glass cups (100 mL/cup), and juvenile fish and crustacean exposures were performed in large Carolina bowls (500 mL/bowl). Megalopae exposures occurred in 20-mL borosilicate scintillation vials (1 individual per vial in 10-mL water). Juvenile seatrout were exposed in 10-gal aquaria (20-L water/aquarium), white shrimp juveniles were exposed in 2-gal aquaria (4-L water/aquarium), and adult grass shrimp were exposed in 1-gal jars (3.5-L water/jar) or in 30-gal aquaria (25 L/aquarium). Information regarding the feeding regime, water renewal schedule, and aeration schedule of a particular test can be found in the test-specific TCTs. Aeration was provided with compressed oxygen at a rate of no more than 100 bubbles per minute per aquarium.

6. During static renewal tests (see test-specific TCTs) the following methods were used to transfer organisms to fresh WAF or fresh control solutions during the water renewals:

    a. For the sheepshead minnow embryo and larvae tests, the test container was stirred and the water was rapidly poured through a square of 500-µm nitex mesh. The organisms on the mesh were counted, and the mesh was dipped into the new test container with fresh WAF or control solution to release all of the organisms.

    b. For the white shrimp tests, the shrimp were netted and the old WAF or control solution was discarded from the exposure chambers. Fresh WAF or control solution was added to the exposure chamber and the shrimp were returned to their exposure chamber.

    c. For the speckled seatrout and blue crab tests, 90% of the WAF or control solution was carefully pipetted out of each exposure chamber and then fresh WAF or control solution was added to the exposure chamber.

7. During certain tests that were performed on sheepshead minnow embryos and larvae, after a predetermined exposure duration, organisms were transferred to clean ASW for the remainder of the test (see test-specific TCTs). To transfer organisms, the WAF in the

test container was stirred and the water was rapidly poured through a square of 500-µm nitex mesh. The organisms on the mesh were counted, and the mesh was dipped into the new test container with clean ASW to release all of the organisms. The mesh was rinsed in hot water between replicates, and a new piece of mesh was used for each treatment.

8. For the sheepshead minnow embryo (< 48 hpf) tests, the exposure chambers were maintained on a rotary oscillator set at 70 rpm. Additionally, where heart rate was a listed endpoint, heartbeats of all viable embryos were counted and recorded during the daily observations. The heartbeats of each embryo were counted for 10 seconds using a dissecting microscope; the results were recorded on an appropriate bench sheet.

9. Organisms obtained from Cedar Point or the Blue Crab Hatchery were moved to STL at least 24 hours prior to test initiation for acclimation. All containers holding test organisms were inspected daily and mortality was recorded. Dead organisms were removed and frozen; organisms from each replicate container were labeled and frozen separately. At study termination, any living organisms were sacrificed and frozen, except in cases where tissues were collected for further analysis (see test-specific TCT for details on test endpoints).

A.3 SOP: Protocol for Preparation of Spiked Sediment

A.3.1 General guidelines

- Prepare the controls using the same technique used for spiked sediment, with the exception of adding oil.
- Prepare each sediment-oil concentration separately. For instructions on cleaning and preparing the equipment, refer to the *Decontamination SOP* in the QAPP.
- Enter all of the appropriate information into the bench sheets provided by Stratus Consulting.
- Place any unused prepared sediment into a Ziploc bag and store in the dark at 4°C (short term) or in a freezer at -20°C (long term). Store large quantities (> 5 kg) of prepared sediment in large metal pans, covered with aluminum foil, and stored in the dark at 4°C.

A.3.2 Glassware preparation

Prepare all of the equipment in accordance with the *Decontamination SOP* in the QAPP.

A.3.3 Preparation of sediments

- Allow the sediment to thaw.
- Remove all debris (grass, shells, etc.) from the thawed sediment, weigh, and place in a mixer bowl.
- Using a Cuisinart SM-70 7-quart stand mixer or a KitchenAid 5-quart stand mixer, homogenize the sediment by mixing for 2 minutes at low speed (1).

A.3.4 Mixing oil into sediment

- Weigh the appropriate amount of oil as outlined below:

  Slick oil should be weighed in a pre-cleaned aluminum weigh boat. Tare a weigh boat and 2–3 Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat. Transfer the oil into the mixing bowl, placing it in several areas around the bowl. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass of oil transferred.

- Place the sediment from the mixing bowl over the oil, and lower the mixer paddle into the bowl.
- Mix the oil into the sediment at medium speed with a Cuisinart SM-70 7-quart stand mixer or a KitchenAid 5-quart stand mixer. Stop the mixer briefly every 2–4 minutes to scrape the sides of the mixing bowl with the putty knife.
- Once the mixing is complete, scrape down the mixer paddle with the putty knife to remove all of the excess oiled sediment. Transfer the oiled sediment from the mixing bowl into bags or metal pans for storage using a stainless steel spoon. Store sediment in the dark at 4°C until it is ready for test initiation.
- Weigh oiled sediment for each replicate when transferring into test containers.

B. Testing Protocol 2: Definitive Chronic Exposures

B.1 Equipment and Materials

Refer to QAPP *Appendix A: Protocols and Standard Operating Procedures, A.1 Protocols for Preparing Water Accommodated Fractions* for making HEWAF and CEWAF stock solutions.

B.1.1 Organisms for exposure

Sheepshead minnow, obtained from GCRL Toxicology laboratory culture.

B.2 Procedures

Each definitive toxicity test consisted of exposures with different HEWAF and CEWAF concentrations plus a control (see test-specific TCTs for details). All chronic tests were flow-through to maximize water-quality parameters**.** Generally, 10 organisms per replicate were used for exposure, but fewer animals were sufficient if space or animal numbers were limiting (see test-specific TCTs for details). The necessary volume of exposure water and corresponding container size from the loading was calculated based on the weight of the test organism. Refer to *ASTM International chamber and flow-through loading calculations SOP* in the GCRL GLPP when making these calculations.

Water quality (temperature, salinity, pH, DO, ammonia) was recorded daily in each aquarium on the bench sheet.

Details regarding the light-cycle parameters and feeding schedules, during the exposures, were included in the test-specific TCTs. During the exposure, if the DO fell below 4 mg/L, aquaria had gentle aeration provided from compressed oxygen delivered through Pasteur pipettes. The background data for the organisms were acquired from the culture logs or chain-of-custody transfer sheets prior to or during the exposure and were maintained with the study data.

B.3 Exposure

Treatments were generated by injection of individual stocks of HEWAF, CEWAF, or dispersant into the dilution water, following protocols outlined in Manning et al. (1999). Appropriate volumes of the stock solutions were injected into splitter/mixing boxes by precision syringe pumps (see test-specific TCTs for details). Immediately after the injection of stock, 2 L of diluent water from a water partitioner were delivered to each splitter box/mixing chamber to

produce the desired concentrations. Each treatment splitter box/mixing chamber then delivered the appropriately diluted study concentration to each replicate aquarium through glass delivery lines. To ensure that the reduced flow into the aquaria did not result in a drop in DO, the partitioner was oxygenated with a gentle stream of oxygen immediately before delivery to the splitter boxes, and oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium. The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

Stock solutions were replaced every 48 hours with newly prepared stocks. Stock solutions were placed in foil-covered glass containers in a dark cabinet at 20°C. A sample of each stock solution was retained for analysis as specified in the QAPP.

The injector system was allowed to run a minimum of 3 days prior to initiation of the test to ensure that aquaria had the correct concentration of compound, to regulate flow rates, etc.

Test organisms were added to test aquaria in a random order that was assigned prior to test initiation. The day of addition was considered day 0 of the test.

All containers holding test organisms were inspected daily and mortality was recorded. Dead organisms were removed, frozen, and archived according to the QAPP; organisms from each replicate container were labeled and frozen separately. At study termination, any living organisms were sacrificed, frozen, and archived according to the QAPP.

C. Testing Protocol 3: Flounder Chronic Sediment Exposures

C.1 Organism for Exposure

Southern flounder were obtained from UTMSI.

C.2 Procedures

C.2.1 Experimental design

1. During the toxicity testing of field-collected sediments, southern flounder were exposed to sediments collected from different field sites. The number of sediments that were tested and the number of replicates per each sediment treatment were recorded in the test-specific TCTs. All sediment tests were performed in a flow-through system.

2. Ten to 15 organisms per replicate were used for exposure, but fewer animals were sufficient if space or animal numbers were limited (see test-specific TCTs for details).

3. The necessary volume of water for each exposure chamber and corresponding container size was calculated based on the weight of the test organism. See the GCRL GLPP *ASTM International chamber and flow-through loading calculations SOP* when making these calculations.

4. Water quality (temperature, salinity, pH, DO, and ammonia) parameters were recorded daily in each test container/treatment. If the DO fell below 4 mg/L, aquaria had gentle aeration from oxygen delivered through Pasteur pipettes.

5. The length of the exposures, the light-cycle parameters, and the feeding schedule can be found in the test-specific TCTs.

6. The data pertaining to the acquisition and maintenance of the southern flounder were maintained in the culture logs or chain-of-custody transfer sheets prior to or during the exposure and were maintained with the study data.

C.2.2 Preparation of sediment/exposure

Sediments were prepared for testing 48 hours prior to test initiation. For tests performed in quadruplicate, 1.3 kg of sediment was removed from the freezer and placed into a large glass jar. An equal volume (1.3 L) of 15-ppt salinity ASW was added to the frozen sediment. The jar was covered, wrapped in aluminum foil, and allowed to equilibrate for 24 hours. After equilibration, the sediment:water slurry was homogenized by hand, and 500 g of the slurry was added to each of the 4 replicate tanks in 100-g aliquots. After the slurry was added to each tank, the tanks were placed into the exposure system, covered, and allowed to settle for an additional 24 hours. After the settling period, individual fish were randomly assigned to each tank. Uncontaminated ASW was dripped into the system on a regular basis for the duration of the test. Sediment samples were collected before and after the experiment and overlying water samples were collected throughout the experiment. Analytical chemistry samples were handled, labeled, and shipped to ALS Environmental according to the QAPP.

All containers holding test organisms were inspected daily and mortality was recorded. Dead organisms were removed, frozen, and archived according to the QAPP; organisms from each replicate container were labeled and frozen separately. At study termination, any living organisms were sacrificed, frozen, and archived according to the QAPP.

C.2.3 Water sampling

Analyses of polycyclic aromatic hydrocarbons (PAHs) in the water of each treatment were performed at ALS Environmental. Water samples were collected from each replicate aquarium and combined into 1 sample/treatment at each collection time point. Water samples were collected by pulling water directly from the tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface while not disturbing the sediment by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles.

C.2.4 Final sediment sampling

Composite sediment samples were collected from each treatment group at the end of the experiment. First, all or most of the water was siphoned from each tank. Next, all of the sediment from each tank within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were mixed using a stainless steel scoop or similar utensil until they reached a uniform color and consistency. Once the sediment was thoroughly mixed, the jars for analytical chemistry sediment samples were filled. Sediment samples were sent to ALS Environmental for PAH analysis.

C.2.5 Test termination

At the end of the exposure period, fish were removed from the aquaria and sacrificed with MS-222. Fish were weighed and measured. Liver tissue and gill tissue from a subset of the total number of fish were placed in Eppendorf tubes with 1 mL of RNAlater for potential subsequent RNA extractions. Tissues were stored at -80°C until processing. The remaining whole fish were collected and fixed in 10% NBF for potential histological analyses.

D. Testing Protocol 4: Effects of Chronic Exposure to HEWAF and CEWAF from Oil Slick A on Growth, Reproduction, and Gene Expression of Grass Shrimp

D.1 Test Organisms

Grass shrimp that were wild-caught in Ocean Springs, Mississippi, marshes.

D.2 Acclimation/Holding

Fully mature grass shrimp were used in this study and were in GCRL laboratory culture for a minimum of 1 week prior to experimentation. Shrimp were held in 100% dilution/culture water prior to test initiation and fully acclimated to its characteristics. Shrimp were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study. Adult shrimp were fed *Artemia* nauplii and a commercial fish food twice daily during the holding and test periods.

D.3 Procedure

D.3.1 Preparation of stock solutions

1. HEWAF – the HEWAF solution was prepared following standard protocols described in the QAPP. The HEWAF stock was stored in glass Erlenmeyer flasks in the dark and used as the source for injection into test aquaria. New HEWAF stock was prepared every 48 hours.

   Analysis of PAHs in each HEWAF stock was done by ALS Environmental. Additionally, water samples from each dilutor/splitter box (one/treatment concentration – see below) and 1 tank per treatment were sampled during the study period and sent to ALS Environmental.

   Water samples were collected by pulling water directly from splitter boxes and tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface by dunking a sample bottle or transfer beaker into the tanks, or using a siphon to directly fill the sample bottles.

2. CEWAF – the CEWAF solution was prepared following standard protocols described in the QAPP. The CEWAF stock was stored in glass Erlenmeyer flasks in the dark and used as the source for injection into test aquaria. The new CEWAF stock was prepared every 48 hours.

   Water samples from each dilutor/splitter box (one/treatment concentration – see below) and 1 tank per treatment were sampled during the study period. Water samples were collected by pulling water directly from splitter boxes and tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface by dunking a sample bottle or transfer beaker into the tanks, or using a siphon to directly fill the sample bottles. Analysis of PAHs and dioctyl sodium sulfosuccinate (DOSS) were done by ALS Environmental.

D.3.2 Experimental design

Adult female and male grass shrimp were held in individual Nitex cages in 48.3 cm (L) × 37.5 cm (W) × 12.9 cm (D) aquaria with an overflow drain of 8 cm, providing a maximum water volume of 24.4 L during the exposure and subsequent egg collections. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Details regarding dosing, number of individuals per replicate, and number of replicates can be found in test-specific TCTs.

Shrimp were isolated individually in the test aquaria into retention chambers constructed from 10-cm glass petri dish bottoms with a collar of 500-µm nylon mesh with a 10-cm diameter disposable petri lid. The exchange of water within the chamber was ensured by fluctuating the water level within the aquaria 4 to 6 cm periodically with a self-starting siphon. Retention chambers were maintained in a minimum depth of approximately 7 ± 1 cm dilution water.

A flow-through test system was used for this evaluation. Treatments were generated by injection of individual stocks of HEWAF and CEWAF into the dilution water. New HEWAF and CEWAF stocks were prepared every 48 hours for injection. Appropriate volumes of the stocks were injected into splitter/mixing boxes by precision syringe pumps (see test-specific TCTs for details). Immediately after injection of stock, 2 L of diluent water from a water partitioner was delivered to each splitter box/mixing chamber to produce the desired concentrations. Each treatment splitter box/mixing chamber delivered 500 mL of the appropriately diluted study concentration to each replicate aquarium through glass delivery lines. To ensure that test aquaria maintained adequate DO levels, the partitioner was oxygenated with a gentle stream of oxygen immediately before delivery to the splitter boxes, and oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium. The flow-through system was initiated 3 days

prior to the addition of shrimp to the aquaria to ensure that the water in all aquaria was at the correct treatment concentration at the start of the experiment.

The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

1. *Adult exposure*. The exposure was initiated with 15 female and 9 male grass shrimp in individual chambers introduced into each study aquarium. After 4 and 14 days exposure, 3 female shrimp from each aquarium were removed and preserved. The temperature was at 27 ± 1°C and the salinity was maintained at a nominal 15 ppt.

2. *Reproduction*. Following 14 days of HEWAF and CEWAF exposure to male and female adult grass shrimp, spawning pairs were established to monitor fecundity of control and treatment shrimp. Spawning pairs consisted of 1 female and 1 male shrimp placed into the same individual chamber (n = 9 pairs/replicate aquarium). Shrimp pairs were checked daily beginning on day 15 of the experiment for evidence of molts and ovigerous females. Ovigerous females were allowed to remain in the chamber for 7–8 days prior to sacrifice. Seven-eight days after first observation of a clutch of eggs, the female was removed and sacrificed in ice water, and all eggs were removed and counted. The female was weighted and measured, and the hepatopancreas tissue was removed as described above. Twenty embryos were isolated into each of 2 retention chambers (10-cm petri dish with a collar of 300-µm nylon mesh). One chamber was returned to the original aquarium, and 1 chamber was placed into a control aquarium. Embryos were observed once daily until hatch; the percent of successful hatching was determined. Exposure to HEWAF and CEWAF continued throughout this portion of the experiment. The carcasses of each spawning pair were wrapped in foil, given a unique ID number, and stored at -20°C. Hatched larvae were placed into Eppendorf tubes with 500 µL of RNAlater for potential subsequent RNA extractions.

D.3.3 Temperature and lighting

Temperature was regulated at 28°C, and for each single test the temperature remained constant within ± 1°C. A 12-hour light and 12-hour dark photoperiod was maintained.

D.3.4 Test initiation

Test shrimp were distributed to the 12 test aquaria in groups of 24 individual chambers (15 females, 9 males in each treatment replicate). The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

D.3.5 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 28°C. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment; thus each aquarium was monitored every 5 days.

D.3.6 Biological data

Daily observations of the presence of molts or occurrence of mortality were recorded and reported for all treatments and controls. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. If dead organisms were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

E. Testing Protocol 5: Effects of HEWAF from Oil Slick A on Growth, Reproduction, and Gene Expression of Grass Shrimp

E.1 Test Organisms

Grass shrimp, wild-caught in Ocean Springs, Mississippi, marshes.

E.2 Acclimation/Holding

Fully mature grass shrimp that were used in this experiment were maintained in GCRL laboratory culture for a minimum of 1 week prior to experimentation. Shrimp were held in 100% dilution/culture water prior to test initiation and fully acclimated to its characteristics. Shrimp were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study. Adult shrimp were fed *Artemia* nauplii and a commercial fish food twice daily during the holding period.

E.3 Procedure

E.3.1 Preparation of stock solutions

The HEWAF solution was prepared following the standard protocols described in the QAPP. The HEWAF stock was stored in a 10-L glass jar in the dark and used as the source for injection into test aquaria. New HEWAF stock was prepared every 48 hours.

Analysis of PAHs from each batch of stock HEWAF was done at ALS Environmental. Additional composite water samples were taken from exposure tanks within each treatment and the control splitter box during the study.

Water samples were collected by pulling water directly from splitter boxes and tanks; samples were not collected from the outflows. Water samples were collected from under the water surface by dunking a sample bottle or transfer beaker into the tanks or by using a siphon to directly fill the sample bottles.

E.3.2 Experimental design

Adult female (n = 5) and male (n = 2) grass shrimp were held in 10-L glass aquaria with an overflow drain of 8 cm providing a maximum water volume of 8 L during the exposure and egg collections. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Each replicate contained 7 shrimp. Shrimp (adults and embryos) were exposed to treatments for the duration of the experiment.

To facilitate mating, shrimp were allowed to freely interact in the 10-L aquaria. A thin layer of calcium carbonate (crushed coral) sediment was placed on the bottom of each aquarium. When gravid females were observed, they were removed and isolated from the mating aquaria within 48 hours of appearance of an egg mass. Isolated individuals were placed into retention chambers (constructed from 10-cm glass petri dish bottoms with a collar of 300-µm nylon mesh). The retention chambers were placed into a larger glass "brooding" aquarium [48.3 cm (L) × 37.5 cm (W) × 22.9 cm (D), with an overflow drain of 19 cm providing a maximum water volume of 34.4 L] of the same HEWAF treatment level. The water level within the aquaria fluctuated 4 to 6 cm periodically with a self-starting siphon to ensure the exchange of water in the chamber. Retention chambers were maintained in a minimum depth of approximately 7 ± 1 cm dilution water.

A flow-through test system was used in this evaluation. Treatments were generated by injection of individual stocks of HEWAF into the dilution water. New HEWAF stocks were prepared every 48 hours for injection. Appropriate volumes of the stocks (see test-specific TCTs for treatment concentrations) were injected into splitter/mixing boxes by precision syringe pumps. Immediately after injection of stock, 2 L of diluent water from a water partitioner was delivered to each splitter box/mixing chamber to produce the desired concentrations. Each treatment splitter box/mixing chamber delivered 500 mL of the appropriately diluted study concentration to each replicate 10-L mating aquarium through glass delivery lines. The system cycling rate was maintained at approximately 1 volume addition every 4 hours, which provided approximately 95% replacement every day. To ensure that test aquaria maintained adequate DO levels, oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium. The flow-through system was initiated 3 days prior to the addition of shrimp to the aquaria to ensure that the water in all aquaria was at the correct treatment concentration at the start of the experiment.

The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

1. *Adult exposure.* The exposure was initiated with 5 female and 2 male grass shrimp introduced into each of the study mating aquaria. Females with an egg mass 24–48 hours old were isolated into individual retention chambers (10-cm petri dish with a collar of 300-µm nylon mesh) and placed in brooding aquaria (1 aquarium/treatment).

Temperature was maintained at 27 ± 1°C and salinity was maintained at a nominal 15 ppt.

2. *Reproduction:* Seven-eight days after isolation in the brooding aquaria, the female was removed and sacrificed in ice water, and all eggs were removed and counted. The female was weighed and measured, and hepatopancreas tissue was placed in Eppendorf tubes with 500 µL of RNAlater for subsequent RNA extractions. Tissues were stored at -80°C until processing. Twenty embryos were isolated into the retention chamber and returned to the female brooding aquaria. The remaining embryos were homogenized in Stat-60 and stored at -80°C. Isolated embryos were observed once daily until hatch; the percentage of successful hatching was determined. Exposure to HEWAF continued throughout this portion of the experiment. The carcasses of each female were wrapped in foil, given a unique ID, and stored at -20°C.

E.3.3 Temperature and lighting

Temperature was maintained at approximately 27°C. For each single test, the temperature remained constant within ± 1°C. A 12-hour light and 12-hour dark photoperiod was maintained.

E.3.4 Test initiation

Test shrimp were distributed to the mating test aquaria in groups of 7 individuals per aquarium (5 females, 2 males in each treatment replicate). The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

E.3.5 Diet

During the exposure, shrimp were fed dry pellet food (B2) twice daily.

E.3.6 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 27°C ± 1°C. Ammonia, DO, temperature, and pH were monitored daily in all mating and brooding aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment; each aquarium was monitored every 5 days.

E.3.7 Biological data

Observations of the presence of molts or occurrence of mortality were recorded and reported for all treatments and controls. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at least once daily during the exposure. If dead organisms were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

The length and weight of female shrimp at the initiation of the experiment (day 0) were determined from 20 female shrimp randomly chosen from the same culture aquarium as the experimental shrimp. These shrimp were sacrificed in ice water, weighed, measured, wrapped in aluminum foil, and frozen at -20°C.

F. Testing Protocol 6: Effects of WAF on Growth, Reproduction, and Gene Expression of Sheepshead Minnow

F.1 Test Organisms

Sheepshead minnows from the GCRL laboratory culture.

F.2 Acclimation/Holding

Fully mature fish used in this study were in 100% dilution/culture water prior to test initiation and fully acclimated to its characteristics. Fish were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs for details). Adult fish were fed *Artemia* nauplii and a commercial fish food twice daily during the holding and test period.

F.3 Preparation of Stock Solutions

The WAF solutions were prepared following standard protocols described in the QAPP. New stock solutions were prepared every 48 hours. Analysis of PAHs was done by ALS Environmental.

F.4 Experimental Design

Adult female and male sheepshead minnow were held in 48.3 cm (L) × 37.5 cm (W) × 22.9 cm (D) aquaria with an overflow drain of 19 cm providing a maximum water volume of 34.4 L during the exposure and subsequent egg collections. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Each treatment contained a minimum of 9 male and 11 female fish.

A flow-through test system was used in this evaluation. Treatments were generated by injection of individual stocks of WAF into the dilution water. New WAF stocks were prepared every 48 hours for injection. Appropriate volumes of the stocks were injected into splitter/mixing boxes by precision syringe pumps (see test-specific TCTs for details). Immediately after the injection of stock, 2 L of diluent water from a water partitioner was delivered to each splitter box/mixing chambers to produce the desired concentrations. Each treatment splitter box/mixing

chamber then delivered the appropriately diluted study concentration to each replicate aquarium through glass delivery lines. To ensure that test aquaria maintained adequate DO levels, the partitioner was oxygenated with a gentle stream of oxygen immediately before delivery to the splitter boxes. The flow-through system was initiated 4 days prior to the addition of fish to the aquaria to ensure the water in all aquaria was at the correct treatment concentration at the start of the experiment.

The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

F.4.1 Adult exposure

The exposure was initiated with at least 11 female and 9 male sheepshead minnow introduced into each study aquaria. After 4 days exposure, 1 male and 1 female fish from each aquarium was removed and sacrificed and placed in cryo vials and flash-frozen with liquid nitrogen or placed in RNAlater for possible RNA extractions. After 14 days of exposure, 4 female and 4 male fish from each aquarium were removed and sacrificed for tissues for possible histology and gene expression as described above. Temperature was maintained at 27 ± 1°C and salinity maintained at a nominal 15 ppt.

F.4.2 Reproduction

Following 14 days of exposure to male and female adult sheepshead minnow, spawning groups were selected to monitor fecundity of control and treatment fish. Spawning groups consisted of 3 females and 2 males placed in a 20 × 40 × 20 cm chamber with a 0.5-cm polyvinyl mesh bottom to allow passage of eggs out of the chamber. Fish selected for spawning from each replicate treatment were the most robust females and males in all treatments to ensure maximum production. The remaining males and females were placed in the aquaria outside the spawning chambers. Seven days following the placement of fish into the spawning chambers, a 300-µm mesh tray was placed under each chamber to collect spawned eggs. This delay in the collection tray placement allowed for establishment of territories before assessing egg production. During this time, any fish within the chamber that died were replaced with females or males not selected earlier that have been housed in the aquarium outside the chamber. Eggs were harvested daily following placement of the egg collection tray for 11 consecutive days, rinsed with dilution water, counted, and microscopically assessed for viability. Exposure to WAF continued throughout this portion of the experiment. At the end of the egg collection period, female and male spawners were removed from their respective chambers, measured for SL, wet weighed (0.001 g, total weight and gonad weight), and sacrificed. Gonadal tissue (whole testis, half ovary) was placed into individually labeled cassettes and preserved in 10% NBF for histological

analysis. Liver tissue and the other half of the ovarian tissue were placed in cryo vials and flash-frozen with liquid nitrogen or placed in RNAlater for possible RNA extractions. Tissues were stored at -80°C until processing.

F.4.3 F1 generation

Progeny were reared from eggs harvested from each of the spawning groups. Retention chambers containing 20 embryos isolated from each of the spawning groups were used to monitor embryo survival, larval survival, and growth of hatch larvae. One group of 20 embryos was isolated from each replicate. Isolation of progeny began on the first day of embryo collections. Each group of 20 embryos came from a single day's collection to ensure embryos of the same age and maintained at the same density were isolated into retention chambers. Embryos for progeny evaluations were isolated into retention chambers (100-mm petri dish bottoms with a 15-cm tall 40-µm mesh nylon collar) and placed in aquaria from which they came. Embryos were removed from the aquaria daily and counted through hatch. Dead embryos were discarded and live embryos returned to the retention chamber. This procedure was repeated until all living embryos hatched. After hatching, juvenile fish were fed *Artemia* nauplii twice daily. Survival was monitored daily for 10 days post-90% hatch (15 days post-isolation). At the end of the monitoring period, half of the fry were sacrificed and measured (SL, weight) for growth and flash-frozen or placed in RNAlater for possible RNA extraction. The other half were sacrificed and preserved in 10% NBF for histological analysis.

F.4.4 Temperature and lighting

Temperature was regulated at approximately 27°C and for each single test the temperature was constant within ± 1°C. A 12-hour light and 12-hour dark photoperiod was maintained.

F.4.5 Test initiation

Test fish were distributed to the test aquaria in groups of 11 females and 9 males in each treatment replicate. Addition of fish was done in an impartial manner until the required numbers were distributed to each test chamber. The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

F.4.6 Diet

During the exposure fish were fed *Artemia* nauplii and commercial flake food twice daily.

F.4.7 Water quality

Ammonia, DO, temperature, pH, and salinity were monitored daily in 1 replicate aquarium for each treatment; each aquarium was monitored every 5 days.

F.4.8 Biological data

Observations of mortality were recorded and reported for all treatments and controls. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a minimum of once daily during the exposure. If dead fish were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

G. Testing Protocol 7: Flounder Chronic Spiked Sediment Exposures

G.1 Test Organisms

Southern flounder from UTMSI.

G.2 Acclimation/Holding

Juvenile fish used in this study were in 100% dilution/culture water for 4 days prior to test initiation and fully acclimated to its characteristics. Fish were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs). Fish were fed *Artemia* nauplii and a commercial fish food twice daily during the holding and test periods.

G.3 Procedure

G.3.1 Preparation of sediments

Oil was mixed into uncontaminated sediments (ALAJ46-C1127-SB701B) using a KitchenAid stand mixer. For each treatment, 2 kg of sediments was weighed and thawed overnight. Details regarding loading rates (grams of oil/kg of sediment) used for each treatment can be found in test-specific TCTs. Oil was mixed into the sediment for 30 minutes at moderate speed (4.5 on mixer), scraping the sides of the bowl with a metal spatula every 2–4 minutes as needed. The oil-sediment mixture was weighed and placed into mesh cages (10-cm petri dish with a 20-cm column of 2-mm nylon mesh) for each treatment (75-g sediment/cage). When preparing tanks, sediment samples for chemical analysis from each treatment group were prepared by aliquoting 50 g of sediment into the analytical chemistry sediment jar between filling each replicate tank. Samples were stored at 4°C until shipment. Cages were placed in treatment aquaria and sediment was slowly added to each aquarium to a depth of 15 cm. The sediment was allowed to settle in the cages overnight under static conditions prior to beginning flow-through water and the addition of the fish.

G.3.2 Analysis of water and sediment samples

Analyses of PAHs in the water of each treatment were done by ALS Environmental. Filtered water samples were collected from each replicate aquarium and combined into 1 sample/treatment at each collection time point; the same was done for unfiltered samples.

Methods for filtration can be found in the QAPP Appendix A. Water samples were collected by pulling water directly from the tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface while not disturbing the sediment by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles. Additionally, duplicate water samples were collected from each replicate aquarium for fluorescence analysis; 3.5 mL of water was added to 3.5 mL of 100% ethanol (ETOH) and sample vials were stored at 4°C until analysis within 4 days.

G.3.3 Final sediment sampling

Composite sediment samples were collected from each treatment group at the end of the experiment. First, all or most of the water was siphoned from each tank. Next, all of the sediment from each tank within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were mixed using a stainless steel scoop or similar utensil until they were a uniform color and consistency. Once sediment was thoroughly mixed, the jars for analytical chemistry samples were filled. Sediment samples were sent to ALS Environmental for PAH analysis.

G.4 Experimental Design

Juvenile southern flounder were held in 10-gal aquaria with an overflow drain of 10 cm providing a maximum water volume of 20 L during the exposure. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Details regarding the number of treatments and the number of replicates per treatment can be found in the test-specific TCTs. Each treatment contained juvenile flounder in individually numbered mesh cages containing 75 g of prepared sediment.

Each fish was weighed (0.001 g) and measured (SL) and assigned to a mesh cage; flounder were randomly selected for all treatments. Test day 0 began with the initial exposure of the test organisms to the test substance. At the end of the exposure period, fish were removed from the aquarium and sacrificed with MS-222. Fish were weighed and measured, and liver tissue and gill tissue from 4 fish/replicate were placed in Eppendorf tubes with 1 mL of RNAlater for potential subsequent RNA extractions. Tissues were stored at -80°C until processing. Tissues were also removed from the remaining 4 fish/replicate and placed into individually labeled cassettes and fixed in 10% NBF for histological analysis.

The flounder in each mesh cage was video recorded twice each week to provide images from which growth measurements were collected. A 7-cm etched glass rod or a 2.5-cm stir rod was placed into each cage during recording to provide a reference for making measurements from the

video. For each recording session, prior to recording each cage, a label with the name of each treatment was filmed to indicate which treatment was being filmed. The label at the top of each cage contained a small piece of tape with the cage ID (example B-2-7 = treatment B, replicate 2, cage 7). For each treatment, recording always began with replicate 1 followed by replicates 2, 3, and 4, respectively. Any fish that died during the experiment was removed from its mesh cage, although the cage remained in the tank for the duration of the experiment. A piece of marking tape was placed at the end of each mesh cage where a fish had been removed so that when the video was taken of that cage, the viewer could easily identify that there was no longer a fish in that cage. The sequence of recording always followed the recording scheme described above regardless of the presence of fish (i.e., there may be occasions when a video was taken of an empty mesh cage). The video for each week was stored on separate secure digital (SD) cards that were backed up at GCRL prior to shipment of the SD card to Stratus Consulting for growth measurements.

G.5 Test Design

A flow-through test system was used in this evaluation. Clean ASW (15-ppt salinity) flowed from a head box partitioner into 6 splitter boxes and then into the aquaria. To ensure test aquaria maintained adequate DO levels, oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium.

G.5.1 Fish exposure

The exposure was initiated with 8 southern flounder introduced into each study aquarium. Each flounder was in an individual mesh cage containing 75 g of sediment. After 60 days of exposure, all fish from each aquarium were removed and sacrificed. Tissues were removed for possible gene expression, microbiome, or histology analyses as described above.

G.5.2 Temperature and lighting

Temperature during the test was maintained at 22 ± 1°C, and salinity maintained at a nominal 15 ppt. A 12-hour light and 12-hour dark photoperiod was maintained.

G.5.3 Test initiation

Test fish were distributed to the test aquaria in groups of 8 juveniles in each treatment replicate (1 fish/cage). Fish were added to each cage in a random order that was assigned prior to test initiation. The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

G.5.4 Diet

During exposure, fish were fed *Artemia* nauplii and/or commercial pellet food twice daily.

G.5.5 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 22 ± 1°C. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment; each aquarium was monitored every 5 days.

G.5.6 Biological data

All fish were weighed and measured at test initiation and at test termination. Observations of mortality were recorded daily for all aquaria. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a minimum of once daily during the exposure. If dead fish were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C. Measurements of gene expression and histopathology followed the same methods described in Testing Protocol 12. Although the fish were removed, the cage of the dead fish remained in the tank for the duration of the test. All fish were video recorded twice weekly to provide images that were analyzed for growth measurements.

H. Testing Protocol 8: Effects of Spiked Oiled Sediment on Growth, Reproduction, and Gene Expression of Grass Shrimp

H.1 Test Organisms

Grass shrimp from Ocean Springs, Mississippi, marshes.

H.2 Acclimation/Holding

Grass shrimp used in this study were in 100% dilution/culture water for 14 days prior to test initiation and fully acclimated to its characteristics. Shrimp were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs). Shrimp were fed *Artemia* nauplii and a commercial fish food twice daily during the holding and test periods.

H.3 Procedure

H.3.1 Preparation of sediments

Oil was mixed into uncontaminated sediments (ALAJ46-D0515-SB701B) using a Cuisinart stand mixer. For each treatment, 4 kg of sediments was weighed and thawed overnight. Oil was added to each sediment treatment following the test-specific TCTs. Oil was mixed into the sediment for 30 minutes at a moderate speed (4.5 on mixer), scraping the sides of the bowl with a metal spatula approximately every 4 minutes as needed. The oil-sediment mixture was placed into four 20-L aquaria/treatment (1 kg/aquarium). Test sediment was placed into the analytical chemistry 8-oz glass sediment jars for analysis by ALS Environmental; approximately 50 g of sediment was placed in the jar after each replicate tank was filled. Sediments in the jars were stored at 4°C until shipment to ALS Environmental. Water was slowly added to each aquarium to a depth of 15 cm. The sediment was allowed to settle overnight under static conditions prior to beginning flow-through water, aeration, and the addition of the shrimp.

H.3.2 Analysis of water and sediment samples

Analysis of PAHs in the water of each treatment was done by ALS Environmental. Water samples were collected by pulling water directly from the tanks. Samples were not collected

from the outflows. Care was taken to collect water samples from under the water surface while not disturbing the sediment. This was done by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles. Additionally, duplicate water samples were collected from each replicate aquarium for fluorescence analysis, 3.5 mL of water was added to 3.5 mL of 100% ETOH, and sample vials were stored at 4°C until analysis within 4 days.

H.3.3 Final sediment sampling

Composite sediment samples were collected from each treatment group at the end of the experiment. First, most of the water was siphoned from each tank. Next, all of the sediment from each tank within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were then mixed using a stainless steel scoop or similar utensil until uniform color and consistency was reached. Once sediment was thoroughly mixed, a scoop was used to fill the analytical chemistry sediment jar (glass 8-oz). Sediments were sent to ALS Environmental for PAH analysis.

H.4 Experimental Design

Grass shrimp were held in 5-gal (20-L) aquaria with an overflow drain of 10 cm providing a maximum water volume of 10 L during the exposure. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Each replicate aquarium with sediment on the bottom contained 12 female and 6 male grass shrimp. Ovigerous females were placed in clean brooding aquaria (maximum volume of 28 L) containing 15 cages; females were placed in brooding tanks following a randomized block design. Additionally, there was a single egg incubation aquarium [48.3 cm (L) × 37.5 cm (W) × 22.9 cm (D) with an overflow drain of 19 cm providing a maximum water volume of 34.4 L] containing 24 incubation cups with no sediments (100-mm petri dish bottoms with a 15-cm tall 40-µm mesh nylon collar) for incubating grass shrimp embryos prior to hatch.

H.5 Test Design

A flow-through test system was used in this evaluation. Clean ASW (15-ppt salinity) flowed from a head box partitioner into 7 splitter boxes and then into the aquaria. The flow rate for brooding aquaria was 1,000 mL/aquarium/cycle, and flow rate for the embryo incubation aquaria was 2 L/cycle. To ensure test aquaria maintained adequate DO levels, oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium.

H.5.1 Shrimp exposure

The exposure was initiated with 12 female grass shrimp introduced into each of 16 study aquaria (4 replicates of 4 sediment treatments), and after 7 days, 6 male grass shrimp were added to each tank. Ovigerous females were removed from the aquaria and placed into individually isolated cages in brooding aquaria; females were added to brooding aquaria following a randomized block design. Ovigerous females were sacrificed after incubating their embryos for 9 days and embryos were transferred to incubation cups with no sediment in an incubation aquarium until 24 hours post-hatch. After 28 days exposure, all unmated females from each aquarium were removed and sacrificed; the hepatopancreas was collected and placed in RNAlater for possible subsequent RNA extraction. Hepatopancreas tissue was also collected from ovigerous females at time of sacrifice. Temperature was maintained at 27 ± 1°C, and salinity maintained at a nominal 15 ppt.

H.5.2 Test initiation

Test shrimp were distributed to the 16 test aquaria in 2 rounds of 6 shrimp each, such that all treatment aquaria contained 6 shrimp before the next 6 shrimp were added. The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

H.5.3 Diet

During exposure, shrimp were fed *Artemia* nauplii and/or commercial pellet food twice daily.

H.5.4 Temperature and lighting

Temperature was regulated at 27°C and for each single test the temperature was constant within ± 1°C. A 12-hour light and 12-hour dark photoperiod was maintained.

H.5.5 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 27 ± 1°C. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment, such that each aquarium was monitored every 5 days.

H.5.6 Biological data

A representative subsample of female shrimp from the culture aquaria was weighed and measured at test initiation. All female shrimp were weighed and measured upon sacrifice. Observations of mortality and presence/absence of molts and eggs were recorded daily for all aquaria. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a minimum of once daily during the exposure. If dead shrimp were discovered, they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C. Ovigerous females were observed daily, and were sacrificed 9 days after the first appearance of embryos; embryos were counted and a subsample of 20 was isolated until 24 hours post-hatch.

I. Testing Protocol 9: Effects of HEWAF, CEWAF, and Corexit on Gill and Gut Microflora of Sheepshead Minnow

I.1 Test Organisms

Sheepshead minnow from GCRL laboratory culture.

I.2 Acclimation/Holding

Adult fish used in this study were in 100% dilution/culture water for 4 days prior to test initiation and fully acclimated to its characteristics. Fish were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs for details). Fish were fed *Artemia* nauplii and a commercial fish flake food twice daily during the holding and test periods.

I.3 Procedure

I.3.1 Preparation of stock solutions

The stock solution was prepared following standard protocols from the QAPP. New stock solution was prepared every 48 hours.

I.3.2 Sampling

Samples were sent to ALS Environmental for possible analyses of PAHs and DOSS.

Water samples were collected by pulling water directly from splitter boxes and tanks. Samples were not collected from the outflows. Care was taken to collect water samples from under the water surface. This was done by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles. Additionally, duplicate water samples were collected from each replicate aquarium for fluorescence analysis once weekly at 2 and 46 hours post new stock; 3.5 mL of water was added to 3.5 mL of 100% ETOH and sample vials were stored at 4°C until analysis within 4 days.

I.4 Experimental Design

Adult female and male sheepshead minnow were held in 5 gal aquaria with an overflow drain of 10 cm providing a maximum water volume of 10 L during the exposure. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions.

Thirty-two fish (16 males, 16 females) from the culture population were individually weighed (0.001 g) and measured (SL) at test initiation to obtain time 0 data for growth. Males and females were impartially assigned to the test aquaria, and then exposed for 14 days to the selected treatments and controls. Test day 0 began with the initial exposure of the test organisms to the test substance. After 7 days of exposure, 2 males and 2 females from each aquarium were removed and sacrificed with MS-222. Fish were weighed (0.001 g) and measured (SL), and gonadal tissues removed and weighed (0.001 g). Gill and gut (entire stomach and intestine) tissues were placed in Eppendorf tubes with 1 mL of RNAlater for possible subsequent microflora analysis. Additionally, liver and gonadal tissues were removed from each fish and placed into Eppendorf tubes with 1 mL of RNAlater for possible RNA extractions. Tissues were stored at -80°C until processing. The remaining fish carcasses were given a unique ID number, wrapped in aluminum foil, and frozen at -20°C. After 14 days of exposure, 2 female and 2 male fish from each replicate of each treatment were sacrificed, weighed (0.001 g), measured (SL), and sampled as described above for gill, gut, liver, and gonadal tissues. Tissues were stored at -80°C until processing. The remaining fish carcasses were given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

I.5 Test Design

A flow-through test system was used in this evaluation. Treatments were generated by injection of individual stocks of HEWAF, CEWAF, or Corexit into the dilution water (see test-specific TCTs). New stocks were prepared every 48 hours for injection. Appropriate volumes of the stocks were injected into splitter/mixing boxes by precision syringe pumps. Immediately after injection of stock, 2 L of diluent water from a water partitioner was delivered to each splitter box/mixing chamber to produce the desired concentrations. Each treatment splitter box/mixing chamber then delivered 500 mL of the appropriately diluted study concentration to each replicate aquarium through a glass delivery line. To ensure test aquaria maintain adequate DO levels, oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium. The flow-through system was initiated 4 days prior to addition of fish to the aquaria to ensure that the water in all aquaria was at the correct treatment concentration at the start of the experiment.

The test had a control treatment with the same dilution water, conditions, procedures, and organisms, except that dilution water without test substance was used as the control.

I.5.1 Fish exposure

The exposure was initiated with 8 sheepshead minnow (4 males, 4 females) introduced into each aquarium. After 7 days exposure, 2 male and 2 female fish from each aquarium were removed and sacrificed for possible microflora and gene expression analyses as described above. After 14 days of exposure, the remaining 2 female and 2 male fish from each aquarium were removed and sacrificed for potential microflora and gene expression analyses as described above. Temperature was maintained at 27 ± 1°C, and salinity maintained at a 15 ppt.

I.5.2 Temperature and lighting

Temperature was regulated at 27 ± 1°C for each test and a 12-hour light and 12-hour dark photoperiod was maintained.

I.5.3 Test initiation

Test fish were distributed to the test aquaria in rounds of 4 fish each, such that 4 males were placed into 1 aquarium in all treatments prior to placement of the 4 females into each replicate aquarium. The maximum loading rate in the treatment aquaria was greater than 1.0 g tissue/L.

I.5.4 Diet

During the exposure, fish were fed *Artemia* nauplii and commercial flake food twice daily.

I.5.5 Water quality

Temperature was measured in 1 test chamber continuously throughout the test and was maintained at 27 ± 1°C. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was monitored daily in 1 replicate aquarium for each treatment, such that each aquarium was monitored every 5 days.

I.5.6 Biological data

A subsample of culture fish were weighed and measured at test initiation, and all fish were weighed and measured during sampling at days 7 and 14. Observations of mortality were recorded daily for all aquaria. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a

minimum of once daily during the exposure. If dead fish were discovered they were removed, given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

J. Testing Protocol 10: Effects of Field Collected Sediment on Survival, Growth, and Gene Expression of Grass Shrimp

J.1 Test Organisms

Grass shrimp from Ocean Springs, Mississippi, marshes.

J.2 Acclimation/Holding

Shrimp used in this study were fully acclimated to 100% dilution/culture water for at least 4 days prior to test initiation. Shrimp were within 2°C of the test temperature for the 48 hours immediately prior to initiation of the study (see test-specific TCTs). Shrimp were fed *Artemia* nauplii and a commercial flake food twice daily during the holding and test periods. The diet was supplemented with frozen adult *Artemia*.

J.3 Procedure

J.3.1 Preparation of sediments

Frozen, field-collected sediments (contaminated and reference) were used for this exposure. Frozen sediment (2,000 g) was weighed and placed in a 5-L glass container with 500 mL of ASW (15 ppt) and allowed to thaw for 18 hours at room temperature in the dark. The sediment/water solution was stirred to thoroughly mix into a homogeneous solution, and equally distributed into each replicate aquarium in 100–150 g portions (total of ~ 500 g sediment/aquaria). Any water that was remaining in the glass container was divided equally among the replicates. The remaining sediment (50–100 g) was put into a glass sample jar for analysis by ALS Environmental. The aquaria with sediments were placed in a water bath and 8 L of 15 ppt seawater was gently added to each aquarium so that the sediment was not stirred up. The sediment settled for 24 hours prior to the addition of shrimp to the system.

J.3.2 Analysis of water

Analysis of PAHs in the water of each treatment was done by ALS Environmental. Water samples were collected from each replicate aquarium and combined into 1 sample/treatment at

each collection time point. Water samples were collected by pulling water directly from the tanks. Samples were not collected from the outflows. Water samples were collected from under the water surface while not disturbing the sediment by dunking a sample bottle or transfer beaker into the tanks or using a siphon to directly fill the sample bottles. Additionally, duplicate water samples were collected from each replicate aquarium for fluorescence analysis. Next, 3.5 mL of water was added to 3.5 mL of 100% ETOH, and sample vials stored at 4°C until analysis was performed within 1 week.

J.4 Experimental Design

Adult grass shrimp were held in 10-gal aquaria with an overflow drain of 10 cm providing a maximum water volume of 10 L during the exposure. Each aquarium was covered with a foil lid. The study was conducted under flow-through conditions. Six different sediment types were used for the exposure with 4 replicates each and 15 shrimp in each replicate (see test-specific TCTs). A minimum total of 360 grass shrimp were needed to initiate the experiment.

J.5 Test Design

Clean ASW (15-ppt salinity) flowed from a head box partitioner into 6 splitter boxes and then into the aquaria at a rate of 500 mL/aquarium/cycle. To ensure test aquaria maintained adequate DO levels, the partitioner was oxygenated with a gentle stream of compressed oxygen immediately before delivery to the splitter boxes, and oxygen was bubbled at a rate of 1 bubble/second into each replicate aquarium.

J.5.1 Test initiation

Test shrimp were distributed to the 24 test aquaria. Each individual shrimp was weighed in a beaker of water prior to being added into a treatment aquarium; a mean weight of the 15 shrimp/aquarium was calculated. The addition of shrimp was done in a random manner until the required numbers were distributed into each test aquarium. The maximum loading rate in the treatment aquaria was no greater than 1.0 g tissue/L.

J.5.2 Shrimp exposure

The exposure was initiated with 15 grass shrimp introduced into each of 24 study aquaria (4 replicates of 6 sediment treatments). At the end of the exposure period, shrimp were removed from the aquaria and sacrificed by immersion in ice water. Shrimp were weighed (0.001 g),

measured (mm CL), and hepatopancreas tissue from 6 shrimp/replicate were placed in Eppendorf tubes with 0.5 mL of RNAlater for possible subsequent RNA extractions. Tissues were stored at -80°C until processing. All remaining shrimp were wrapped in acetone-rinsed foil, labeled, and frozen at -20°C.

J.5.3 Temperature and lighting

Temperature was maintained between 26°C and 28°C. A 12-hour light and 12-hour dark photoperiod was maintained.

J.5.4 Diet

During the exposure, shrimp were fed commercial flake food once daily and a mixture of *Artemia* nauplii and frozen adult *Artemia* shrimp once daily.

J.5.5 Water quality

Temperature was measured in 1 test aquarium continuously throughout the test. Ammonia, DO, temperature, and pH were monitored daily in all aquaria. Salinity was measured in 1 replicate aquarium per day such that each aquarium per treatment was measured every 5 days.

J.5.6 Biological data

Observations of mortality were recorded daily for all aquaria. An organism was considered to be dead if there was no visible movement and if touching with a probe produced no reaction. Observations were made at a minimum of once daily during the exposure. If dead shrimp were discovered, they were removed, with each shrimp given a unique ID number, wrapped in aluminum foil, and frozen at -20°C.

K. Testing Protocol 11: Effects of Exposure to HEWAF on the Immune Function Fish

K.1 Organisms for Exposure

1. Red Snapper (*Lutjanus campechanus*) were obtained from the Cedar Point aquaculture facility at GCRL

2. Atlantic croaker (*Micropogonias undulates*) were obtained from the Cedar Point aquaculture facility at GCRL

3. Red drum (*Sciaenops ocellatus*) were obtained from the Texas Parks and Wildlife aquaculture facility in Lake Jackson, Texas.

K.2 Procedures

K.2.1 Preparation of stock solutions

HEWAF stock solutions were prepared according to the QAPP.

K.2.2 Experimental design

These experiments were designed to analyze how exposures to oil in aqueous solutions affect fish immune response to acute pathogenic insults. Oil exposures were performed for the duration of the experiments, except for the period when fish were removed for pathogen exposure and immediately placed into a bacterial exposure tank containing ASW and bacteria. After bacterial exposure, the fish were removed and placed in their original WAF exposure tanks. All fish were monitored for the times specified in test-specific TCTs. At regular intervals post-bacterial exposure, some fish were removed from each tank and sacrificed for tissue sampling (see test-specific TCTs). At each sampling event, fish were weighed and measured, visually assessed for the presence of skin lesions, and dissected aseptically according to the *General Immunotoxicity Testing* SOPs. During necropsies, blood and tissue samples were collected for assessing various endpoints (see test-specific TCT).

Juvenile fish were separated into different treatment groups: no oil/no pathogen, oil only (no pathogen, see test-specific TCTs for oil concentrations), pathogen only (no oil), and oil + pathogen. The number of replicates per treatment and the number of fish per replicate are described in the test-specific TCTs.

For each test, pertinent information concerning fish species, life stage, numbers of organisms, treatment levels, exposure durations, oil loading rates, bacteria species, inoculation loading rates, and analytical sampling requirements is included in the test-specific TCTs.

K.2.3 Endpoints

Various endpoints were assessed throughout the course of each test. Some endpoints, such as survival, growth, and gross pathology observations were performed on live fish on a regular basis. Other endpoints, such as internal gross pathology observations and histopathology sampling required sacrificing individual fish and thus were conducted only once per fish. Fish were sacrificed at various time points after the pathogen challenge and sampled. These time points were specified in test-specific TCTs. A description of potential endpoints for each test is below (see test-specific TCTs).

Survival: Survival of each fish was assessed daily by observing movement in response to gentle prodding or similar external stimuli. Mortality observations were recorded on the appropriate bench sheets.

Growth: Growth was assessed by measuring the total weight and length of each fish before being exposed to oil and at the end of the experiment just before it was euthanized. Initial weights (to the nearest tenth of a gram) and lengths (to the nearest millimeter) were taken from un-anesthetized fish immediately after removal from the tank. All dead fish were removed, weighed, and measured as above, and preserved as archived tissue samples, as described in the QAPP. Growth data were recorded on the appropriate bench sheet.

Gross pathology: A general analysis of fish health was conducted regularly on live fish during the course of the exposure (external) and during necropsies (internal). Gross external assessments of live fish were conducted twice daily. Observers looked at fish eye and skin color, as well as the surface of the skin and fins for presence of lesions or other external abnormalities. Behavior observations included lethargy, erratic swimming, and feeding vigor. External gross pathology assessment results were record on the appropriate bench sheet. Fish were not handled during these external assessments. Internal gross pathology assessments were conducted on euthanized fish during necropsies. Internal organs were assessed for gross pathological changes. These changes included changes in organ size, texture, shape, or color as described in the *Fish Dissection and Sampling* SOP. Internal gross pathology assessment results were record on the appropriate bench sheets.

Immune response: Blood was collected according to the *Bacterial Killing Assay* SOP. Blood sampling was documented on the appropriate bench sheets. Blood was analyzed for bactericidal

potential using the BKA in accordance with the *Bacterial Killing Assay* SOP and for cytokine levels in accordance with the *Cytokine Analysis of Plasma SOP*.

Bacterial infection: Some tissues from kidney, spleen, liver, gills, intestine, and any samples of opportunity (e.g., ascites and lesions) were aseptically sampled for microbiomic response. Aseptic samples were taken immediately after a fish was necropsied and blood was taken using freshly decontaminated dissection equipment. Sample collection procedures are described in the *Bacterial analysis of tissue* SOP. Aseptic sampling was documented on the appropriate bench sheets.

Gene expression: Some tissues from liver, kidney, and spleen were collected for gene expression using transcriptomics or qPCR. If collected, tissue samples were preserved in RNALater as described in the *Fish Dissection and Sampling* SOP. For sample containers and storage requirements, refer to the QAPP. Gene expression sampling was documented on the appropriate bench sheets. Gene expression analyses followed the *RNA extraction and qPCR for gene expression analyses* SOP.

Histopathology: Some tissues from the spleen, liver, kidney, and any samples of opportunity (e.g., lesions) were collected for histopathological analyses according to the *Fish Dissection and Sampling* SOP. Histopathology samples were collected after all blood, bacterial infection, and gene expression samples were taken. The QAPP provides details on histology sample containers, fixative, and storage requirements. Sample collection information was recorded using the appropriate bench sheets. Histological analyses were done using the *Gill Histology Image Analysis Methods* SOP and the *Liver Histology Image Analysis Methods* SOP.

Raw seawater bacterial characterization: The bacterial content of the water in the circulating system was tested before adding fish and at the end of the observatory period. This was accomplished via bacterial plating (see the *Bacteriological Analysis of Sediment and Water* SOP), qPCR quantification (see the *RNA extraction and qPCR for gene expression analyses* SOP), and microbiomics (see the *Microbial Diversity Analysis* SOP).

L. Testing Protocol 12: Effects of Exposure to Spiked Sediment on the Immune Function Fish

L.1 Organisms for Exposure

Southern flounder were obtained from UTMSI.

L.2 Procedures

L.2.1 Sediment preparation/exposure

Oil was mixed into uncontaminated sediments using a KitchenAid stand mixer (*Protocol for Preparation of Oil-Spiked Sediments*). For each replicate, the appropriate amount of sediment was weighed and thawed overnight (see test-specific TCTs). Details regarding loading rates (grams of oil/kg of sediment) used for each treatment can be found in test-specific TCTs. Oil was mixed into the sediment for 30 minutes at moderate speed (4.5 on the mixer); the sides of the bowl were scraped with a metal spatula every 2–4 minutes as needed. For test 900, the oil mixture was placed directly into the tanks. For test 904, the oil-sediment mixture was weighed and placed into mesh cages (10-cm petri dish with a 20-cm column of 2-mm nylon mesh) for each treatment (approximately 75-g sediment/cage). When preparing the tanks, sediment samples for chemical analysis from each treatment group were prepared by taking 50-g aliquots of sediment into the analytical chemistry sediment jar, in-between filling each replicate tank. Samples were stored at 4°C until shipment. Cages were placed in treatment aquaria and sediment was slowly added to each aquarium. The sediment was allowed to settle in the cages overnight under static conditions before beginning flow-through water and adding the fish.

L.2.2 Experimental design

These experiments were designed to analyze how exposures to spiked sediment affect fish immune response to acute pathogenic insults. Oil exposures were performed for the duration of the experiments, except for periods when fish were removed from the oil exposure tanks and placed into a bacterial exposure tank containing ASW and bacteria or a control tank containing no bacteria. After exposure, the fish were removed and placed back into their original WAF or control tanks. All fish were monitored for the times specified in test-specific TCTs. At regular intervals post-bacterial exposure, fish were removed from each tank and sacrificed for tissue sampling (see test-specific TCTs). At each sampling event, fish were weighed and measured, visually assessed for the presence of skin lesions, and dissected aseptically according to the

General Immunotoxicity Testing SOPs. During necropsies, blood and tissue samples were collected for assessing various endpoints.

For each test, there were the following treatment groups: no oil/no pathogen, oil only (no pathogen, see test-specific TCTs for oil concentrations), pathogen only (no oil), and oil + pathogen. The number of replicates per treatment and the number of fish per replicate are described in the test-specific TCTs.

For each test, the pertinent information concerning fish species, life stage, numbers of organisms, treatment levels, exposure durations, oil loading rates, bacteria species, inoculation loading rates, and analytical sampling requirements is included in the test-specific TCTs.

L.2.3 Endpoints

Various endpoints were assessed throughout the course of each test. Some endpoints, such as survival, growth, and gross pathology observations were performed on live fish on a regular basis. Other endpoints, such as internal gross pathology observations and histopathology, sampling required sacrificing individual fish and thus were performed only once per fish. Fish were sacrificed at various time points after the pathogen challenge and sampled. These time points were specified in test-specific TCTs. A description of potential endpoints is below (see test-specific TCTs).

Survival: Survival of each fish was assessed daily by observing movement in response to gentle prodding or similar external stimuli. Mortality observations were recorded on the appropriate bench sheets.

Growth: Growth was assessed by measuring the total weight and length of each fish before being exposed to oil and at the end of the experiment just before fish were euthanized. Initial weights (to the nearest tenth of a gram) and lengths (to the nearest millimeter) were taken from un-anesthetized fish immediately after removal from tank. All dead fish were removed, weighed, and measured as above, and preserved as an archived tissue sample, as described in the QAPP. Growth data was recorded on the appropriate bench sheet.

Gross pathology: A general analysis of fish health was conducted regularly on live fish during the course of the exposure (external) and during necropsies (internal). Gross external assessments of live fish were conducted twice a day. Observers looked at fish eye and skin color, as well as the surface of the skin and fins for presence of lesions or other external abnormalities. Behavior observations included lethargy, erratic swimming, and feeding vigor. External gross pathology assessment results were record on the appropriate bench sheet. Fish were not handled during these external assessments. Internal gross pathology assessments were conducted on euthanized fish during necropsies. Internal organs were assessed for gross pathological changes.

These changes included changes in organ size, texture, shape, or color as described in the *Fish Dissection and Sampling* SOP. Internal gross pathology assessment results were record on the appropriate bench sheets.

Immune response: Blood was collected according to the *Bacterial Killing Assay* SOP. Blood sampling was documented on the appropriate bench sheets. Blood was analyzed for bactericidal potential using the BKA in accordance with the *Bacterial Killing Assay* SOP or for cytokine levels in accordance with the *Cytokine Analysis of Plasma* SOP.

Bacterial infection: Some tissues from kidney, spleen, liver, gills, intestine, and any samples of opportunity (e.g., ascites and lesions) were aseptically sampled for microbiomic response. Aseptic samples were taken immediately after a fish was necropsied and blood was taken using freshly decontaminated dissection equipment. Sample collection procedures are described in the *Bacterial analysis of tissue* SOP. Aseptic sampling was documented on the appropriate bench sheets.

Gene expression: Some tissues from liver, kidney, and spleen were collected for gene expression using transcriptomics and/or qPCR. If collected, tissue samples were preserved in RNALater as described in the *Fish Dissection and Sampling* SOP. For sample containers and storage requirements, refer to the QAPP. Gene expression sampling was documented on the appropriate bench sheets. Gene expression analyses followed the *RNA extraction and qPCR for gene expression analyses* SOP., the *RNA sequencing analysis* SOP, and the *Bioinformatics and Transcriptomics* SOP.

Histopathology: Some tissues from the spleen, liver, kidney, and any samples of opportunity (e.g., lesions) were collected for histopathological analyses according to the *Fish Dissection and Sampling* SOP. Histopathology samples were collected after all blood, bacterial infection, and gene expression samples were taken. The QAPP provides details on histology sample containers, fixative, and storage requirements. Sample collection information was recorded using the appropriate bench sheets. Histological analyses were done using the *Gill Histology Image Analysis Methods* SOP and the *Liver Histology Image Analysis Methods* SOP.

Raw seawater bacterial characterization: The bacterial content of the water in the circulating system and exposure sediment was tested before adding fish and at the end of the observation period. This was accomplished via bacterial plating (*Bacteriological Analysis of Sediment and Water* SOP), qPCR quantification (*RNA extraction and qPCR for gene expression analyses* SOP), and microbiomics (*Microbial Diversity Analysis* SOP).

L.2.4 RNA sequencing analysis SOP (National Center for Genomic Research)

Total RNA samples were processed by taking an aliquot for QC analysis to determine the amount of RNA and the integrity of the RNA using Qubit and Bioanalyzer, respectively. All samples passed QC and were made into sequencing libraries using the Illumina TruSeq RNA Sample Preparation Kit. Total RNA went through poly-A selection reaction, in which the mRNA is pulled down using poly-T oligo-attached to magnetic beads. The pulled-down mRNA was fragmented and randomly primed in a one-step reaction. The randomly primed mRNA was then taken through first-strand synthesis using a reverse transcriptase enzyme. The product then underwent a second strand synthesis using a second strand master mix that contained DNA polymerase I and RNase H. The synthesized second strand was end repaired using End Repair Mix (converted overhangs generated from fragmentation into blunt ends), followed by the addition of an A-base on the 3' end of the double-stranded cDNA molecule. The addition of A-base prepared it for the ligation of Illumina adapters, which had a T-base on its 3 end. After the ligation of the sample with uniquely barcoded adapters, the resulting product was taken through 15 cycles of PCR amplification. QC was performed on the Nanodrop to determine the amount and on the Bioanalyzer for fragment size determination of the library and for any adapter dimers.

L.2.5 Bioinformatics and Transcriptomics SOP (National Center for Genomic Research)

Bioinformatics was performed on the Illumina HiSeq 2000 50-nt paired-end sequencing results of the 52 RNA flounder samples (average reads per sample was 24.5 M). First, the sequence reads were processed to create an optimized *de novo* transcript assembly. Using an iterative approach, an annotated assembly was created containing a minimum amount of contigs, or transcripts. The reads were then aligned to this newly created reference and counted to perform differential expression analysis. The differential expressed transcripts were than filtered using standard criteria, and pathway analysis was performed. The details of each step are explained below.

1. *De Novo* Transcriptome Assembly

The National Center for Genome resources *de novo* assembly pipeline has three main components: the assembly of transcripts or contigs, the annotation of genes and transcripts, and peptide annotation.

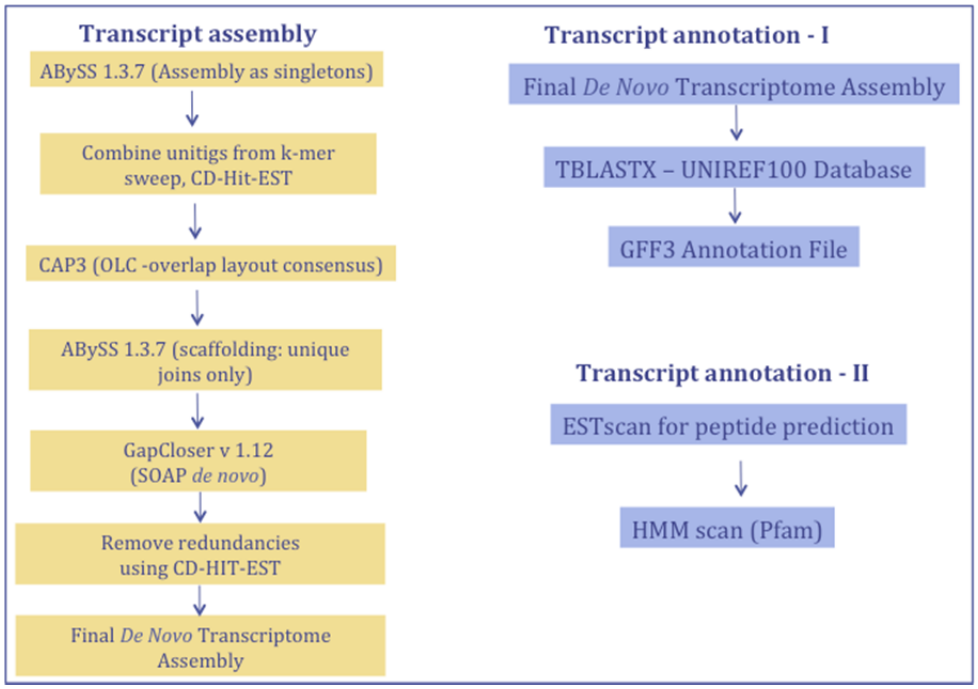

Figure L.1. *De novo* Assembly pipeline.

2. Differential Expression Analysis

Following *de novo* assembly of the Southern flounder transcriptome, differential expression analysis of flounder transcripts for experiments 904, 900, 113, and field-collected samples was performed using NCGR's Bioinformatics-in-a-Box (BiB) tool with default parameters (http://www.lumenogix.com/de-novo-rna-seq-expression-analysis). Differential expression analysis using BiB began with a quality check of the data using the FastQC program (Andrews, 2010), followed by mapping/alignment of the raw reads, in fastq format, to an annotated transcriptome using Bowtie (Langmead et al., 2009). After mapping of the raw reads to the transcriptome, the mapped/aligned reads were quantified using the RSEM (Li et al., 2011) algorithm that is built into BiB. The read counts generated by RSEM were used to perform differential expression analysis using the built-in EBSeq (Leng et al., 2013) algorithm. Differential expression results generated by EBSeq were filtered using BiB by a posterior probability of differential expression (PPDE) of greater than or equal to 0.95: transcripts with a PPDE > = 0.95 made up the list of differentially expressed transcripts, with a target false discovery rate (FDR) controlled at 5% (FDR < = 0.05).

3. Pathway Analysis of Differentially Expressed Transcripts

The filtered lists of differentially expressed transcripts (PPDE > = 0.95) contained uniref100 IDs that served as transcript identifiers. The uniref100 IDs were converted to Ensembl transcript IDs or associated gene names using the UniprotKB database (UniProt Consortium, 2015). The Ensembl transcript IDs and associated gene symbols were converted to Danio rerio (Zebrafish) Ensembl gene IDs using the Ensembl biomart website (Cunningham et al., 2015). The Danio rerio Ensembl gene IDs were used to perform pathway analysis (Biological Processes and Reactome or Molecular Functions) using the ClueGo app (Bindea et al., 2009) in Cytoscape (Shannon et al., 2003).

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4. Hopkins Marine Station of Stanford University and Northwest Fisheries Science Center General Laboratory Procedures and Practices

4.1 Methods

Tuna or mackerel or both were exposed to different concentrations of whole oil water accommodated fractions (WAFs) under laboratory conditions. Physiological data were generated from both whole organisms and tissue samples. Water chemistry analyses were carried out by ALS Environmental and Northwest Fisheries Science Center (NWFSC).

All organisms and tissues from organisms collected for and used during testing were archived according to the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3.

4.1.1 Test organism sources and husbandry

Pacific bluefin and yellowfin tuna (*Thunnus orientalis* and *Thunnus albacares*)

Pacific bluefin and yellowfin tuna were field collected by hook and line from off the coast of California and brought back to the Tuna Research and Conservation Center (TRCC) at Hopkins Marine Station of Stanford University (Hopkins), where they were housed in one of three holding tanks at the facility, each with its own life support system. Two of the tanks (T2 and T3) are 30,000 gal each (109 m^3) and the third (T1) is 90,000 gal (327 m^3). The tanks are matched to pumps, high-speed sand filters, aeration/degassing towers, and protein fractionator towers that provide high-quality seawater. The life support system supplies water to each tank at 100% oxygen saturation at a temperature range from 20 to 25°C, a pH range of 7.8–8.0, and unionized ammonia at less than 0.01 mg/L. In captivity, fish were typically fed three times weekly (30 kcal/kg) with a mix of squid, sardines, and an enriched gelatin diet.

Pacific mackerel (*Scomber japonicus*)

Pacific mackerel were field collected by hook and line, and brought back to the TRCC, where they were housed at 20°C in tanks T4 and T5. Another tank (T6) was the dedicated oil exposure tank for mackerel. The turnover rate for T4, which is 5,900 gal (22.3 m^3), was one volume per hour through two sand filters for biological filtration and then through a packed column for aeration and off-gassing any nitrogen. There was approximately 5 gpm of new seawater added to the tank continuously. There was a nightlight over the tank that was very dim but gave enough

light for the mackerel to see the tank walls. They were fed a mixture of chopped squid and sardines typically every other day.

Tanks T5 and T6 were each 1,000 gal (3.78 m^3), and each tank had its own dedicated life support system consisting of a sand filter for biofiltration. After filtration, the seawater returned to each tank through an aeration system consisting of a cascade arrangement of aeration biorings. Temperature was controlled by either immersion heaters or cooling coils; both temperature systems were controlled by thermostats. The turnover rate in both of these tanks can be adjusted to 1–1.5 volumes per hour. T5 was dedicated as the tank to hold mackerel that were trained to swim in the respirometer.

4.1.2 Exposure media preparations

All seawater used for exposure studies and used in the TRCC holding tanks was sterilized, filtered seawater from Monterey Bay and supplied to the TRCC by the Monterey Bay Aquarium.

WAFs for electrophysiological experiments were prepared in Ringer's solution (all mM: 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose, and 10 HEPES; and pH adjusted to 7.7 via NaOH), which is a normal physiological salt solution. Ringer's solution was made fresh for each experiment using Millipore filtered freshwater. All WAF preparations were filtered using a single 0.7-μm glass fiber filter as described in Appendix G of the QAPP.

High energy water accommodated fraction preparation

Note: This protocol was applicable only for mixing oil with volumes of water equal to 4 L or less.

High energy water accommodated fractions (HEWAFs) were made for tissue exposures with each of four oil types: Slick A (CTC02404-02), Slick B (GU2888-A0719-OE701), source oil (072610-03), and weathered source oil (072610-W-A). Control exposure solutions were made using the same protocols, but without any oil.

The protocol used for making the HEWAF is found in the QAPP with the following modifications:

- Ringer's solution, rather than seawater, was used for preparing all electrophysiology experiment HEWAFs.
- A Gilson Microman positive displacement pipette with positive displacement tips was used to transfer the liquid oil samples, rather than a gastight syringe.

- For Slick A and B oils, a small amount of the viscous oil was transferred to an amber vial that was then heated in a 65°C water bath for approximately 20 minutes, reducing the viscosity enough to allow the use of the Gilson Microman positive displacement pipette. Once this oil was pipetted onto the surface of the water in the blender, it was then gently pushed onto the blender blades with a metal spatula, ensuring a more uniform dispersion.

- Slick A and B HEWAF preparations were blended for 2 minutes, rather than 30 seconds, on the low setting in the Waring CB15 commercial blender.

- 4-L glass carboys with bottom spigots were used for the 1-hour separation step, rather than separatory funnels.

Globular high energy water accommodated fraction preparation

Note: This protocol was designed for mixing oil with volumes of water between 30 and 50 L. This concentrated globular high energy water accommodated fraction (GWAF) is then mixed with a larger volume of water (1,000–2,500 L) to reach the desired final concentration.

GWAFs were made for scombrid species exposures in dedicated exposure tanks in the TRCC for each of four oil types: Slick A, Slick B, source oil, and weathered source oil. A control without oil added was also made.

4.1.3 Testing methods

Electrophysiology experiments

A. Cardiac myocyte electrophysiology methods

The following procedures were used for these experiments and can be found in Hopkins and NWFSC General Laboratory Procedures and Practices (GLPP):

- *Testing Protocol 1: Calcium Transient Recording Using Confocal Microscopy*
- *Testing Protocol 2: Electrical Activity Recording Using the Patch Clamp Technique.*

For confocal experiments:

1. Cells were incubated in Ringer's solution plus oil (at various concentrations) for at least 1 hour. The maximum incubation time was 3 hours.

2. Measurements were taken after 1–2 hours of incubation, sometimes 2–3 hours if the cells were also incubated with a sarcoplasmic reticulum (SR) inhibitor.

3. Cells from at least two fish were used for each oil type tested. At least 10 cells (number of reps) were used for each oil type tested.

For the patch clamp experiments:

1. Each cell was in Ringer's solution for at least 5 minutes (control), then HEWAF was added to reach a HEWAF:Ringer's solution concentration of 1/20,000, followed by 2–3 minutes exposure to reach steady state before measurements were taken, then additional HEWAF was added to increase the nominal concentration to 1/10,000 followed by another 2–3 minutes exposure, then again HEWAF was added to increase the nominal concentration to 1/5,000 followed by 2–3 minutes exposure before final measurements were taken.

2. Measurements were taken the entire time, before and during oil exposure. Measurements were taken until a new steady state was reached, typically in 2–3 minutes.

3. Three replicate cells, from at least two different fish, were used for each oil type.

Respirometry experiments

The following protocols and test conditions were used to determine the impacts of crude oil or dispersant exposure or both on metabolic and behavioral responses of adult scombrid fish and to characterize the toxicity of crude oil on whole organism metabolic rates from Hopkins and NWFSC GLPP:

- *Testing Protocol 3: Fish Respirometry within 30-L Respirometer Chambers with or without Exposure to Oil.*

A. Respirometry methods

- Pacific mackerel were placed individually in a 30-L respirometer to measure baseline metabolic rate at 20°C water temperature, swimming at one body length per second (BL/s).

- Mackerel were exposed to specific concentrations of oil for specific durations (2–4 days) in a 1,000-gal exposure tank (Tank T6).

- Mackerel were then individually placed in a 30-L respirometer to measure metabolic rate during oil exposure at 20°C water temperature, swimming at 1 BL/s.

- Tailbeat frequencies were measured.

- Baseline metabolic rates were compared to post-exposure metabolic rates to determine if there is a metabolic response to oil exposure.

B. Respirometry water chemistry sampling

No WAF stock, archive water, or fluorescence samples were taken during respirometry exposures. Samples from the exposure tank after mixing in oil GWAF were taken at multiple time points during the 24-, 48-, 72-, and 96-hour exposures and analyzed as follows:

- All samples were analyzed for polycyclic aromatic hydrocarbons (PAHs)
- Source oil GWAF was analyzed for benzene, toluene, ethylbenzene, and xylenes (BTEX) in addition to PAHs.

Sample bottle, shipping, and handling requirements were performed as described in the QAPP.

4.1.4 Water quality monitoring

A. Water quality monitoring for electrophysiology experiments

Protocols in the Hopkins and NWFSC GLPP provide detailed descriptions of solutions used for the electrophysiological experiments. HEWAFs for electrophysiological experiments were prepared in Ringer's solution (made fresh daily), and pH was measured/recorded before cardiomyocyte exposures:

- *Testing Protocol 1: Calcium Transient Recording Using Confocal Microscopy*
- *Testing Protocol 2: Electrical Activity Recording Using the Patch Clamp Technique.*

B. Water quality monitoring for respirometry experiments

1. Temperature

Temperature in the exposure tank (T6) and in the respirometry reservoir was obtained by using a thermometer or a YSI Pro ODO meter with an oxygen and temperature probe. When this probe was used, it was calibrated prior to every test.

2. Salinity

Salinity was obtained using a refractometer. This instrument is commonly used in the laboratory and field. Accuracy of the refractometer is checked once monthly using salinity standards.

3. Dissolved oxygen (DO)

A YSI Pro series sonde with an optical DO probe was used to measure DO in the exposure tank (T6) and in the respirometry reservoir. The probe was calibrated according to manufacturer's specifications prior to every experiment.

4. pH

The pH was measured using litmus paper, within the exposure tank (T6) as well as in the respirometry reservoir.

5. Ammonia

Ammonia in the exposure tank (T6) and the respirometry reservoir was measured with a Hach Ammonia Test Kit (model NI-SA).

For definitive tests using *Deepwater Horizon* (DWH) oil, these water quality samples were taken and recorded at the beginning of each individual exposure to oil and each subsequent 24 hours until the end of the test.

4.1.5 Analytical chemistry

Analyses of PAHs and other compounds were conducted by ALS Environmental, with some samples analyzed at NWFSC. For the collection procedure, see the QAPP. Bile PAHs were measured at NWFSC.

4.2 Reporting and Testing Documentation

Reporting of the data followed procedures described in the QAPP.

4.3 General Testing Standard Operating Procedures

4.3.1 Preparation of GWAFs with DWH oils

This method was used for generating dispersed oil for large-scale (1,000-L) exposures of adult/subadult mackerel for respirometry studies.

Glassware/mixer decontamination procedure

Rinse all glassware and GWAF mixer components with three rinses of acetone, followed by three rinses of dichloromethane (DCM) before and in-between each prep. Allow sufficient time for full evaporation of final solvent rinse. Inspect the inside parts of the drywall paddle prongs for visible oil and scrub with solvent-soaked Kimwipes. Use gloved hands throughout all preparation steps, and use common-sense laboratory safety for handling solvents, especially DCM. Refer to appropriate material safety data sheets if necessary. All other decontamination efforts during GWAF preparation follow the *Decontamination SOP* found in the QAPP.

Recordkeeping

The *Water Accommodated Fraction Preparation and Sampling Table* bench sheet provided by Stratus Consulting in the QAPP was used to record data during GWAF preparations.

Procedure

A. Prepare negative control

1. Measure appropriate volume of seawater into pre-cleaned GWAF mixer (40 L).

2. Close mixer lid.

3. Blend for the same amount of time that the treatment WAF is blended.

4. After mixing, collect water samples immediately for analytical chemistry from below the surface using disposable glass pipettes. Store water samples and ship according to the QAPP.

B. Prepare oil GWAF

1. Measure appropriate volume of water into pre-cleaned GWAF mixer (40 L).

2. Measure desired volume of oil. The source oil and artificially weathered source oil samples are fluid enough to use a graduated cylinder. For the thick surface slick oils (i.e., Slick A and Slick B), warm a large-enough volume at 65°C in a water bath for 30 minutes to decrease the viscosity for measuring in a graduated cylinder.

   Common dilutions of oil include the following:

   - 1:10,000: 250 mL oil into 40 L of water, mixed, then added to 2,500 L
   - 1:100,000: 25 mL oil into 40 L of water, mixed, then added to 2,500 L.

3. Close GWAF mixer lid.

4. Blend 4 hours or until the oil and water looked sufficiently mixed.

5. Take appropriate water samples as indicated in #A.4 above.

6. Transfer remaining contents in GWAF mixer to the exposure tank, T6.

7. Note time of transfer. Details regarding water samples taken for analytical chemistry are in the *Water Accommodated Fraction Preparation and Sampling Table* bench sheet provided in the QAPP.

A. Testing Protocol 1: Calcium Transient Recording Using Confocal Microscopy

A.1 Isolated Myocyte Preparation

Myocytes from mackerel and tuna were obtained by adaptation of the isolation protocol (Shiels et al., 2004). Each fish was euthanized by pithing, and the heart was excised. The heart was perfused with isolating solution until it had stopped beating and was cleared of blood. This was accomplished by retrograde perfusion through the ventricular lumen for ~ 10 minutes in mackerel and ~ 20 minutes in tuna. In tuna, the coronary artery was additionally perfused to clear blood from the arteries and compact myocardial tissue. Proteolytic enzymes were then added to the isolating solution, and retrograde luminal perfusion continued for ~ 12 minutes in mackerel and ~ 40 minutes in tuna. After enzymatic treatment, the atrium and ventricle were placed in separate dishes containing fresh isolating solution. Tissues were cut into small pieces with scissors and then triturated through the opening of a Pasteur pipette to free individual myocytes. Myocytes were stored in fresh isolating solution for up to 8 hours at 20°C.

A.1.1 Solutions

All chemicals were obtained from Sigma unless otherwise indicated.

Laboratory notebooks were used to record solution preparation dates, parameters, and standard operating procedure (SOP) modifications.

The isolating solution contains (mM) 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, 50 taurine, 20 glucose, and 10 HEPES, with pH adjusted to 6.9 with NaOH at 20°C. For enzymatic digestion, collagenase (type IA), trypsin (type IX), and fatty acid-free Bovine Serum Albumin (BSA) were added to this solution (0.75 mg/mL, 0.25 mg/mL, and 0.75 mg/mL, respectively). For small mackerel (< 200 g), BSA was increased to 1.125 mg/mL. Isolating solution was made fresh daily.

The extracellular (Ringer's) solution used for recording myocyte Ca^{2+} transient contains (mM) 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose, and 10 HEPES, and pH is adjusted to 7.7 via NaOH. Ringer's solution was made fresh daily.

To assess the role of the SR, myocytes were incubated for at least 30 minutes with SR inhibitors (5 μm ryanodine and 2 μM thapsigargin) prior to experimentation.

In some experiments, a pulse of caffeine (20 mM, 5–10 seconds) was applied to the cell (via a local perfusion system) to assess SR Ca load. If a caffeine pulse was applied, it was noted in the designated laboratory notebook.

A.1.2 Oil incubation

HEWAFs were prepared using Ringer's solution, rather than seawater, following the *Protocols for Preparing Water Accommodated Fractions* in the QAPP. Myocytes were incubated for at least 1 hour with an oil preparation prepared with an equal volume of cell suspension and HEWAF (for example: 1 mL of cell suspension is added to 1 mL of HEWAF). The maximum incubation time was 3 hours. Exposure concentrations and exposure times were recorded in the designated laboratory notebook. All oil exposures were at 20°C.

A.1.3 Ca^{2+} transient recording

Measurements were taken after 1–2 hours of incubation, or sometimes 2–3 hours if the cells were also incubated with an SR inhibitor. All experiments were performed at 20°C. Myocytes were incubated with 5 µM Fluo-4 [stock solution 1 mM in with 20% Pluronic F127 in dimethyl sulfoxide (DMSO) solution] for 20–30 minutes. An Olympus 100x water-immersion objective was used in all measurements. The pin-hole aperture was set to the size of the Airy disk to optimize z-axis resolution. Images were collected using repetitive line scans (2,500 lines of 512 pixels) every 3 or 5 ms across the width of the cell. Start times of oil exposures, as well as oil exposure concentrations, were recorded in the designated laboratory notebook. The protocol is below.

- A sample (80–160 µL) of ventricular myocytes was added to the recording chamber (volume ~ 610 µL, filled with Ringer's solution or Ringer's solution with HEWAF oil preparation) and allowed to settle on the bottom.
- Ca^{2+} transients were elicited by field stimulation through a pair of platinum electrodes, at a frequency of 0.5 Hz.
- Line-scan images were acquired at a sampling rate of 3 or 5 ms to get the best signal-to-noise ratio.
- Cells were replaced by fresh ones, which were within either the control solution or an oil exposure solution, after several minutes of recording (< 15 minutes total). The chamber was cleaned with distilled water and 100% ethanol when changing cells from one experimental solution to the other.

B. Testing Protocol 2: Electrical Activity Recording Using the Patch Clamp Technique

B.1 Isolated Myocyte Preparation

Myocytes from mackerel and tuna were obtained by adaptation of the isolation protocol (Shiels et al., 2004). Each fish was euthanized by pithing, and the heart was excised. The heart was perfused with isolating solution until it had stopped beating and was cleared of blood. This was accomplished by retrograde perfusion through the ventricular lumen for ~ 10 minutes in mackerel and ~ 20 minutes in tuna. In tuna, the coronary artery was additionally perfused to clear blood from the arteries and compact myocardial tissue. Proteolytic enzymes were then added to the isolating solution, and retrograde luminal perfusion continued for ~ 12 minutes in mackerel and ~ 40 minutes in tuna. After enzymatic treatment, the atrium and ventricle were placed in separate dishes containing fresh isolating solution. Tissues were cut into small pieces with scissors and then triturated through the opening of a Pasteur pipette to free individual myocytes. Myocytes were stored in fresh isolating solution for up to 8 hours at 20°C.

B.2 Solutions

All chemicals were obtained from Sigma unless otherwise indicated.

Laboratory notebooks were used to record solution preparation dates, parameters, and SOP modifications.

The isolating solution contained (mM) 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, 50 taurine, 20 glucose, and 10 HEPES, with pH adjusted to 6.9 with NaOH at 20°C. For enzymatic digestion, collagenase (type IA), trypsin (type IX), and fatty acid-free BSA were added to this solution (0.75 mg/mL, 0.25 mg/mL, and 0.75 mg/mL, respectively). For small mackerel (< 200 g), BSA was increased to 1.125 mg/mL. Isolating solution was made fresh daily.

The experimental solutions designated for each specific recording are listed below. All experimental solutions were made fresh daily.

B.2.1 Action potential

When recording the action potential of myocytes, Ringer's was used as an extracellular solution (in mM): 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose, 10 HEPES, and pH adjusted to 7.7 via NaOH and the internal (pipette) solution contained (in mM): 10 NaCl, 140 KCl,

5 MgATP, 0.025 EGTA, 1 $MgCl_2$, and 10 HEPES. The pH was adjusted to 7.2 with KOH. A low concentration of EGTA was included to achieve near physiological Ca^{2+} buffering capacity.

B.2.2 K^+ current

To avoid contamination of overlapping currents, the external solution contained (in mM) 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 0.005 Tetrodotoxin (TTX), 0.010 nifedipine, 0.01 glibenclamide, 10 glucose, and 10 HEPES, and pH was adjusted to 7.7 via NaOH, and the internal (pipette) solution contained (in mM) 10 NaCl, 140 KCl, 5 MgATP, 5 EGTA, 1 $MgCl_2$, and 10 HEPES. The pH was adjusted to 7.2 with KOH. High concentration of EGTA was included to block Na-Ca exchanger current. TTX was included to block fast Na^+ current, nifedipine to block Ca^{2+} current, and glibenclamide to block ATP-sensitive K^+ current.

B.2.3 Ca^{2+} current

To avoid contamination of overlapping currents, the external solution contained (in mM) 150 NaCl, 5.4 CsCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose, and 10 HEPES, and pH was adjusted to 7.7 via NaOH; the internal (pipette) solution contained (in mM) 130 CsCl, 15 TEACl, 5 MgATP, 0.025 EGTA, 5 NA_2Phosphocreatine, 0.03 NaGTP, 1 $MgCl_2$, and 10 HEPES. The pH was adjusted to 7.2 with CsOH. Cs^+ was included to avoid contamination by K^+ currents. In some experiments, TTX was added to block I_{Na}; otherwise a pulse to -40 mV was used to inactivate this current. The EGTA concentration was designated to mimic physiological Ca^{2+} buffering properties of fish myocytes (see Shiels et al., 2004 for details).

B.2.4 Oil exposure

A single cardiac myocyte was exposed to control (external solution) until the recording had reached steady state (at least 5 minutes). Then external solution with oil (HEWAF prepared in Ringer's according to the QAPP) was applied to the same myocyte, via bath perfusion (~ 100 µL final volume) for typically 2–3 minutes, starting from the lowest concentration and then moving up to the highest concentration, in order to determine the dose response curve (all dilutions should reach steady state effect). In some cases (e.g., K^+ current recording), a specific blocker was included in the final solution to inhibit the specific current. All exposure times and concentrations were recorded in the designated laboratory notebook.

B.2.5 Electrophysiological recordings

Myocytes were studied in a chamber mounted on the stage of an inverted microscope (Nikon DIAPHOT 200, Japan). Cells were initially superfused with Ringer's solution. All experiments were performed at room temperature (~ 20°C).

The membrane potential and currents were recorded using the whole-cell configuration of the patch clamp technique (Hamill et al., 1981; Brette et al., 2006). An Axopatch 200B (Axon Instruments, Union City, California) amplifier was used, controlled by a Pentium PC connected via a Digidata 1322A A/D converter (Axon Instruments), which was also used for data acquisition and analysis using pClamp software (Axon Instruments). Signals were filtered at 2–10 kHz using an 8-pole Bessel low-pass filter before digitization at 10–20 kHz and storage. The resistance of the patch clamp pipettes was typically 1.5–2.5 MΩ when filled with intracellular solution (above).

In all experiments, at least 5 minutes was allowed for cell dialysis by the pipette solution before experiments were initiated. The cell membrane capacitance was measured by integrating the capacitance current recorded during a 10-mV hyperpolarizing pulse from -80 mV.

Action potentials were evoked by 2.5 ms subthreshold current steps. Trains of pulses were applied at 0.5 Hz.

In all voltage clamp experiments, cell capacitance and series resistance were compensated (> 60%) so that the maximum voltage error was < 2.5 mV. The holding potential was -80 mV, to be near to physiological resting membrane potential. I_K was elicited by a rectangular step to +40 mV (peak of activation of K^+ current in fish) (Galli et al., 2009) and then the tail current at -20 mV was analyzed (stimulation frequency 0.1 Hz). I_{Ca} was elicited by a rectangular step (300-ms pulse) to 0 mV (peak of Ca^{2+} current, current-voltage relationship; Brette and Cros, unpublished data) after a 100 ms prepulse to -40 mV to inactivate I_{Na}. Current-voltage relationship for I_K was obtained by a 4-second pulse starting from -40 mV up to +50 mV (10-mV step) and activation curve (tail current) at -20 mV (4 seconds). The current-voltage relationship for I_{Ca} was obtained by a 300 ms pulse from -40 mV up to +50 mV (10-mV step). Action potential clamp experiments were performed by averaging action potential from control conditions and one oil type. In this case TTX was added to the external solution to block I_{Na}.

B.2.6 Protocols

- A sample of ventricular myocytes was added to the recording chamber and allowed to settle on the bottom. Myocytes were initially perfused with Ringer's solution.
- Once gigaseal was achieved, the current or voltage stimulation was induced as described above.
- Once recording for the cell was achieved, the whole bath was changed after washing with pure ethanol.

C. Testing Protocol 3: Fish Respirometry within 30-L Respirometer Chambers with or without Exposure to Oil

C.1 Procedure

C.1.1 TRCC tank and respirometer setup

1. Tanks T4 and T5 were dedicated to holding untrained and respirometer-trained mackerel, respectively, and tank T6 was used solely for oil exposures of fish.

2. The TRCC holds two respirometers; R1 was used for respirometry related to non-oil-exposed fish, while R2 was used for respirometry related to oil-exposed fish and had oiled water from tank T6 running through the system.

3. Fish were trained to swim in respirometer R1 for 24–72 hours.

4. All fish swam for a baseline measurement. Baseline data were logged into a notebook and the video archives were kept on both the dedicated computer hard drive and the backup external hard drive.

C.1.2 Preparation of oil exposure tank (T6)

1. Prepare a GWAF according to the *Preparation of GWAFs with DWH oils* SOP in the Hopkins and NWFSC GLPP.

2. While the GWAF is mixing, fill exposure tank T6 with an appropriate volume of sterilized, filtered seawater (typically 2,500 L) from the Monterey Bay Aquarium seawater supply pumps.

3. Always wear dedicated elbow-length rubberized gloves, boots, and surgical apron when handling oil-exposed specimens or working with oil-contaminated water.

4. Check that the life support system is set to by-pass:

    a. Turn off pump #1
    b. Close valves #3 and #4
    c. Open valve #5
    d. Open valve #6
    e. Turn on pump #1 (check flow-rate and pressure gauge on top of the filter – it should read zero pressure).

5. After the GWAF has mixed for the appropriate amount of time and a sample has been collected for analytical chemistry (refer to the *Preparation of GWAFs with DWH oils* SOP in Hopkins and NWFSC GLPP), add the entire mixture to T6.

6. Once the exposure water in T6 has been thoroughly mixed (via pumping action) for 30 minutes, proceed to take water samples for analytical chemistry using a glass transfer pipette submerged below the surface. Store and ship water samples according to the QAPP.

7. Add the appropriate number of fish to T6 (typically 3) and record the water quality measurements on the TRCC *Water Quality Monitoring* bench sheet (see Section C.1.3 below).

C.1.3 Fish transfer to exposure tank (T6)

1. Ensure that the T6 environment is prepared according to the protocol above.

2. Use the crowder net to isolate specific fish (tagged with small Floy tags) in tank T4 or T5, depending on specimen location.

3. Catch fish with the rubberized net and carefully walk fish to T6. Typically three fish will be placed into T6.

4. Leave fish in T6 for 24, 48, or 72 hours, depending on the length of exposure that is being run.

5. After fish are exposed to oil in T6, a portion of the water will be used to fill the respirometer for the respirometry experiment (see Section C.1.4). After filling the respirometer and its reservoir, transfer the test fish to the respirometer (see Section C.1.5).

6. Record exposure times and concentrations in the TRCC in a dedicated notebook.

7. Obtain water quality data from T6 when fish are first moved into the tank, on a 24-hour cycle, and when fish are transferred to the respirometer (see *Water quality monitoring* in Hopkins and NWFSC GLPP) and recorded on the *Water Quality Monitoring* bench sheet.

8. Take exposure water samples for chemical analysis using a glass transfer pipette submerged below the surface every 24 hours. Store and ship water samples according to the QAPP.

9. Transfer any remaining oily seawater in the tank to the Baker Tank designated for oil waste.

10. Thoroughly wipe down T6 and rinse with seawater, and then transfer that water to the Baker Tank as well.

C.1.4 Respirometer preparation

1. Fill the reservoir to the 1,000-L line with water pumped from T6 (~ 20°C) for exposed fish or T5 for unexposed fish.

2. Plug in the heater and set to 20°C to maintain a constant temperature.

3. Plug the sump pump into the computer control unit to fill 30-L respirometer (ensure the return tube properly drains back to the reservoir).

4. Turn on the flume propeller and remove any air bubbles from the interior chamber of respirometer (the speed and direction may need to be varied to dislodge all trapped bubbles).

5. Set up the video camera to monitor (open circuit) the swim chamber.

6. Roll down the black-out tarp, turn off overhead lights, and turn on the clip-in light.

7. Set speed to 1 BL/s.

8. Place O_2 and temperature sensors in the swim chamber (visually inspect to ensure sensors are clean and no air bubbles remain in the chamber).

9. Press the "measure" button on the sensor computer to ensure the temperature (~ 20°C) and oxygen (~ 95% air sat.) are favorable.

10. Take water quality measurements from the swimming chamber (see *Water quality monitoring* in Hopkins and NWFSC GLPP).

11. Water samples to be sent for analysis must be taken according to the water sampling guidelines in the QAPP.

C.1.5 Fish transfer from tank to respirometer

1. Use the crowder net to isolate specific fish in the tank (either T5 or T6).

2. Catch fish with the rubberized net and carefully walk fish to the respirometer.

3. Place fish in the swim chamber and close the lid (do not screw shut at this time).

4. Once the fish swims unaided in the correct location (forepart of the chamber), seal the lid closed.

5. Use the Excel spreadsheet that calculates speed versus length (file location and name: \\Mola\topp\TRCC\TRCC\Calibrations\Respirometers\TRCC_30L_OilSpill_respo_callibration_0411011.xls) to determine the correct motor setting and adjust it so that the speed is 1.0 BL/s.

6. On the data logging computer, press the "measure and data" button and give the file a name, including "year month day – fish identification (ID) – type of run – flume – species" (e.g., 2011 0711 – fish024 – exposure X – oil 30L – PacMack).

7. Set the oxygen drop-down button to record in mg/L, if not already set.

8. Go to the computer control box and set the interval for desired on/off flush in seconds (610 for flush, 605 for closed, if cycle is for 10 minutes).

9. Start the flush/closed cycles on a 5-minute interval (e.g., 01:05, 01:10, 01:15).

10. Record the following in a dedicated notebook: time fish entered the flume, electric speed setting used, and time flush/closed cycles were started. Also record any deviations from SOP or other relevant details (e.g., observations concerning fish swimming patterns, difficulty catching while in holding tank).

11. Record all digital data (water quality monitoring, video footage, etc.) on the dedicated oil spill computer. Data will also be backed up on the dedicated TRCC external hard drive.

12. Run the fish for 24 hours in the flume.

C.1.6 During run

1. Turn oxygen from the compressed oxygen tank on and off in reservoir as needed (maintain DO between 5.9 and 6.5 mg/L). Use O_2 flow settings "1" or "2" for up to 20 minutes to increase DO concentration.

2. Conduct tailbeat frequency counts hourly by watching the live video feed and counting how many times the tail beats in 1 minute during the closed cycle. Record these data into the dedicated notebook.

3. Record the time of any disturbances in the Tuna Center (e.g., slammed doors, accidental changes in lighting), including the time at which they occur and any changes in the fish's swimming patterns.

4. Record any changes in the swimming behavior of the fish.

5. Record any entrances or disturbances to the respirometry black-out area.

6. At the end of the respirometer run, wipe down and rinse the reservoir and respirometer, transferring all oily wastewater to the Baker Tank for disposal.

C.1.7 Fish removal

1. Slowly enter the black-out area.

2. Quickly unseal the chamber.

3. Remove the fish with a small net and return it to the appropriate holding tank or, if tissue sampling is to be performed, sample appropriate tissue (Section C.1.8). If bile is to be collected, follow the instructions in Section C.1.9.

4. Take water quality sample from swimming chamber.

5. Turn off the data logging on the data logging computer.

6. Upload the file to a secure network location.

7. Copy all digital files onto the TRCC external hard drive.

C.1.8 Tissue collection

After the fish was euthanized by pithing, the body cavity was opened with a clean scalpel. Using a new scalpel blade the internal organs were severed at the esophagus and the entire internal organ mass was removed and placed on aluminum foil.

C.1.9 Fish bile collection and storage

1. Euthanize/sacrifice animal.

2. Open the body cavity using scissors and forceps, or a clean knife if necessary on large fish. Use one set of tools to cut open the animal and a separate set for cutting tissue sections inside the animal.

3. Note the gender of the fish, if possible, based on the presence of testes or ovaries. Disconnect the esophagus and pull the entire internal organ mass gently away from the fish cavity. Place the excised internal organ mass on a clean sheet of aluminum foil on the cutting board.

4. If there is blood on the outside of the gall bladder, rinse the gall bladder with distilled water (contained in squirt bottle).

5. Separate the gall bladder (sac-like organ that is a green to yellow color, and either bulbous or elongate depending on species) from the liver, being sure to grip it by the bile duct or nearby connective tissue to prevent bile from flowing out of the bladder.

6. Hold the gall bladder at the mouth of the amber vial, and puncture the bladder with the scalpel blade, thus directing the bile fluid into the vial.

    a. The volume of bile collected into the individual 4-mL vial should be > 50 µL, but as much bile as possible should be collected, leaving some space at the top of the 4-mL vial to allow for expansion during freezing.

        i. With small fish that do not yield 50 µL, form a composite sample from fishes for up to 200 µL. These composites should be collected into inserts provided with the amber vials. Document in the notebook how many fish were used to make the composite sample and each fish's weight and length.

        ii. For large gall bladders (> 1.5 cm diameter), use a 1-cc tuberculin syringe to draw bile from the gall bladder; high-volume samples may be stored in 20-mL scintillation vials; however, those samples must be wrapped in foil

to avoid any exposure to light. Do not reuse syringes, and when disposing of them, do not re-cap the needles.

7. Verify the proper labeling of the sample vial, and place on ice in a covered cooler. Samples must be transferred to -20°C for longer-term storage.

8. Replace the viscera into the body cavity. Wrap fish in aluminum foil, and place in a Ziploc freezer bag that is labeled with a unique Stratus Consulting Sample ID (example: HS-B0926-TA-451-101).

9. Rinse the scalpel blade and dissecting tools with isopropanol and shake dry between each fish. If tools become fouled with tissue, wash them with soap and water, and rinse with isopropanol. Replace scalpel blades instead of washing them.

10. Change scalpel blades when switching to a different species of fish, or between sites or treatment groups.

    a. Hold the scalpel by the handle with the blade pointing away from any person, and ensure that the blade and blade lock face upward with the slanted blade end facing your hand.

    b. Grip the slanted edge with forceps, ensuring a secure hold to avoid slipping.

    c. Lift the blade from the slanted end until the lock hole of the blade separates from the lock. The blade should be loose at this time.

    d. Pull blade away from the tip to remove. Dispose of blade in a sharps container.

Appendix References

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5. Miami University of Ohio General Laboratory Procedures and Practices

5.1 Methods

Miami University of Ohio (MUO) conducted laboratory studies with low levels of simulated sunlight under constant controlled conditions. Laboratory tests simulated the diminished levels of ultraviolet (UV) radiation present at water depths up to 10 m. These experiments were used to establish the lower range of photo-induced toxicity. In addition, holding other environmental variables constant (e.g., intensity of light over time, temperature, photoperiod) provided greater control of test conditions.

5.1.1 Test organism sources and husbandry

This section describes the sources and husbandry for sheepshead minnow (*Cyprindon variegatus*) and mahi-mahi (*Coryphaena hippurus*) used in the toxicity tests. The test organisms were provided by independent culture facilities and transported to test laboratories at MUO.

Sheepshead minnow

Sheepshead minnow adults were obtained from Aquatic Biosystems Inc. in Ft. Collins, Colorado. The MUO protocol for maintenance and spawning of sheepshead minnow can be found in Section 5.3.1.

Mahi-mahi

Mahi-mahi embryos used in MUO testes were shipped from the University of Miami Rosenstiel School of Marine and Atmospheric Science (RSMAS). The RSMAS General Laboratory Procedures and Practices (GLPP; Section 7.1.1, *Test organism sources and husbandry – cobia and mahi-mahi*) contains information regarding the source and husbandry of these organisms.

5.1.2 Exposure media preparations

Test media were prepared according to established protocols. See *Protocols for Preparing Water Accommodated Fractions* in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. The synthetic saltwater used in all exposure studies was prepared with Fritz's Sea Salt or Instant Ocean mixed with dechlorinated water (DCW) from MUO.

The experimental design was factorial and included two oil types [Slick A (CTC02404-02) and weathered source oil (072610-W-A)], two water accommodated fraction (WAF) preparation methods [high-energy (HEWAF) and chemically enhanced (CEWAF)], and two UV radiation treatments. Each test included a control and four WAF concentrations.

5.1.3 Whole organism exposures

Test procedures

Details regarding test procedures and testing apparatuses are outlined in the testing protocols in the appendix of the MUO GLPP. In short, organisms were exposed to treatment media in glass crystallizing dishes (exposure chambers). Loading of organisms into exposure chambers never exceeded 0.5 g/L. Each test used different WAF concentrations, as well as negative (i.e., no oil) controls [specific concentrations can be found in test-specific test conditions tables (TCTs)]. Exposures were carried out as static renewals with daily water changes. Treatments included different WAF concentrations and two UV treatments, with or without UV. Information regarding the light intensity used during each test can be found in the test-specific TCTs. Concentrations of oil, determined in consultation with Stratus Consulting and the National Oceanic and Atmospheric Administration, can be found in the test-specific TCTs.

Each test included WAF stock samples, archive water samples, and fluorescence water samples. WAF stock water samples were sent to ALS Environmental for chemical analysis. The types of analyses were recorded on Chain of Custody (COC) forms when samples were sent to ALS, and depended on the WAF preparation method used. If the same stock solution in which samples were previously drawn was used to renew test media, then WAF stock water sampling was not repeated on this solution. This would only occur with WAF stock solutions that were less than 24 hours old and had been securely stored in a cool, dry, and dark location. See the QAPP for more details.

Fluorescence water samples were collected from each WAF stock and dilution series. These samples were used to generate standard curves for analyzing dilution series samples. See the QAPP for more details.

When possible, all organisms used in toxicity tests were retained as archive tissue samples. These samples included dead organisms removed from test chambers when making daily observations or renewals, and any remaining organisms left at the end of each test. Archive tissue samples were placed in sample containers with as little water as possible; this sometimes required blotting the sample on a clean Kimwipe prior to placing it in a sample container.

Equipment used in processing archive tissue samples was made of stainless steel, anodized aluminum, borosilicate glass, polytetrafluoroethylene, ceramic, or quartz (U.S. EPA, 2000). New, certified pre-cleaned borosilicate glass or polytetrafluoroethylene bottles were used to store tissue. Some tissue samples were wrapped in aluminum foil and placed into a properly labeled sample containers or plastic bags. All retained samples were securely stored at -20°C. Sample labeling, storage, and shipping methods followed requirements outlined in the QAPP.

5.1.4 Phototoxicity model development

A global model of polycyclic aromatic hydrocarbons (PAHs) phototoxicity was developed over the past 25 years (Jeffries et al., 2013). Photo-enhanced toxicity is a function of (1) a particular PAH's ability to generate reactive oxygen species upon absorption of actinic UV radiation, (2) the extent to which a PAH is taken up by an organism (i.e., combination of bioavailability and bioaccumulation potential – aka fugacity), and (3) the intensity of actinic UV exposure. Model input is thus a combination of structure-activity data on compound-specific levels of phototoxicity, bioaccumulation data, and quantitative information on intensity and duration of UV exposure. Model output is the predicted time-to-death for an organism exposed to combinations of PAH levels that are taken up by an organism, and UV intensity and duration. This output can be used to estimate site-, time-, season-, or depth-specific phototoxic injury for specific mixtures of PAH. The model takes the form of:

$$TTD = f\left\{\sum_{i=1\text{-}n}([PAH]_i * RPA_i) * (UV)\right\}, \qquad \text{Equation 1}$$

where TTD = predicted time to death; $[PAH]_i$ = molar body burden of PAH "i" over PAH "i" to "n;" RPA_i = relative photodynamic action of PAH_i (ratio of phototoxicity intensity of PAHi and anthracene – a PAH with a median level of phototoxicity); and UV = dose of actinic UV radiation exposure (typically expressed as broad-band UV-A radiation in the range of 320–400 nm).

This model can be used to predict time-to-death under specific conditions or can be used to determine predicted no-effect levels of PAH and UV exposure on a site-specific basis. Data from tests conducted under the MUO GLPP were modeled using these concepts to predict levels of toxicity and areas of injury within the *Deepwater Horizon* spill zone. In addition, the data were incorporated into the global model of phototoxicity, and model comparisons served as one form of validation of both the model and its predictions.

5.1.5 Water quality monitoring

Water quality was monitored as described in the QAPP and using the *MUO protocol for water-quality monitoring during Deepwater Horizon toxicity testing standard operating procedures*

(SOP) in Section 5.3.4. Dissolved oxygen (DO), pH, conductivity, salinity, ammonia, and temperature were measured once daily from each test medium stock. Temperature, DO, salinity, and ammonia were also measured once over the course of the test in each of five randomly selected test chambers prior to renewal. In addition, solar radiation was measured using a calibrated biospherical radiometer, which takes continuous measurements of UV during the entire test period. Calibration was performed and certified by Biospherical Instruments in San Diego, California.

5.1.6 Analytical chemistry

Analytical samples were taken as described in the QAPP.

Analytical chemistry was performed on WAF stock water samples by ALS Environmental. A set of water samples was collected from each WAF stock solution. Sample type, bottle type, and requested analyses were specific to the test oil type and WAF preparation method as described in the QAPP. Sample collection, labeling, shipping, and custody procedures followed guidelines specified in the QAPP.

All archive water samples were unfiltered and collected in 250-mL amber glass bottles and stored at 4°C. When taken, all archive samples were sent to ALS Environmental for extraction and preservation. COC forms indicated that these samples were to be extracted and archived by marking the "Extract and archive only" box for each sample.

Archive tissue samples were collected and preserved according to methodologies required for organic analysis outlined in the QAPP. The samples were securely stored at -20°C at the testing laboratory. COC forms were used to document the transfer of archive tissue samples to the storage facility.

5.2 Reporting and Testing Documentation

All documentation of test procedures, results, etc., was carried out as described in the QAPP. Documentation was provided to Stratus Consulting via test-specific bench sheets and electronic and hard copies were stored at MUO.

5.3 General Testing SOPs

5.3.1 Laboratory culture and maintenance of the sheepshead minnow SOP

Purpose

This section provides an outline of procedures for the successful culture of sheepshead minnow.

Procedures

A. Source of organisms

Sheepshead minnows were obtained from Aquatic Biosystems Inc. An in-house laboratory culture facility served as the source for eggs and other organism developmental stages for use in toxicity tests, or to increase the broodstock supply.

B. Laboratory culture facility

Sheepshead minnows were maintained in aquaria in the MUO Animal Facility. The culture facility comprises the following main components:

1. Water supply
2. Holding aquaria in which young or adult broodstock were held
3. Egg incubation units in which fertilized eggs were placed to hatch
4. Water baths and culture containers for the care of fry.

Water

A. Synthetic saltwater was mixed from Instant Ocean or Fritz's Sea Salt and DCW from the MUO Animal Facility. The DCW was City of Oxford (Ohio) well water conditioned by carbon filtration and blended with reverse-osmosis deionized water to a hardness of 150 mg Ca/L, stored and aerated in a 7,600-L recirculating [closed, high-efficiency particulate absorption (HEPA)-filtered, UV-sterilized] tank.

The synthetic saltwater was held in a recirculating 400-L polyethylene holding tank with UV sterilization. Salinity was generally maintained between 20 and 25 ppt.

B. Adult broodstock were maintained in static tanks. Water was continuously aerated and biologically filtered with recirculating power filters (e.g., Penguin Biowheel and Aqueon Quiet Flow models), and a 20–30% water change was effected twice weekly. DCW was added as needed to compensate for evaporation.

Water quality parameters (i.e., salinity, pH, temperature, and DO) were regularly measured and recorded.

General culture conditions

A. Adult fish were generally maintained at a density of approximately 0.5 g/L on a 16-hour light:8-hour dark photoperiod, at a temperature of 21–25°C, and a salinity of 15 ppt in 20-gal aquaria (Figure 5.1).

B. Adult fish were fed *Artemia* nauplii and Tetramin dry-flake food twice daily.

Figure 5.1. Broodstock tanks for adult sheepshead minnows in Room 47B of the MUO Animal Facility.

Spawning

Individuals selected for use in egg production were those that were actively feeding, exhibited normal swimming patterns, and reflected no outward indications of stress or abnormality. Brood fish ranged in age from 3–24 months. Adult broodstock obtained from Aquatic Biosystems were generally 7–8 months old.

A. Natural egg production

Females produced 10–25 eggs per day. At culture temperatures (~ 25°C), fertilized eggs hatched in 5–6 days. At times embryo production was enhanced by maintaining the temperature in the broodstock tanks at closer to 30°C. Supplemental feedings were also used to improve embryo production.

The egg collection method follows:

1. When embryos were required, broodstock were transferred from their holding tanks to spawning tanks prepared with "spawning rings" (generally 5–7 days prior to the initiation of a test). The spawning tanks were 20-gal glass aquaria equipped with recirculating power filters. Generally, six females and four males were placed in each tank. The males selected a territory (i.e., spawning ring) and attracted females to spawn over them.

2. The spawning rings were a modified "Schesny ring" design consisting of a 4" diameter polyvinyl chloride (PVC) ring fitted with a coarse (3–5 mm) Nitex mesh above and a fine (200 micron) mesh below (Figures 5.2 and 5.3). Two to three spawning rings were placed in each tank (Figure 5.4). Spawning tanks were generally 1°C warmer than holding tanks (~ 25°C). Spawning generally began within 24 hours or less. Embryos fell through the coarse mesh and onto the collecting screen. They were collected for 24 hours and then transferred to crystallizing dishes for incubation. Spawning rings were replaced in the spawning tanks until a sufficient number of embryos had been collected. To help keep the embryos clean, the adults were fed while the spawning rings were removed.

Figure 5.2. Spawning ring for inducing natural spawning in sheepshead minnows.

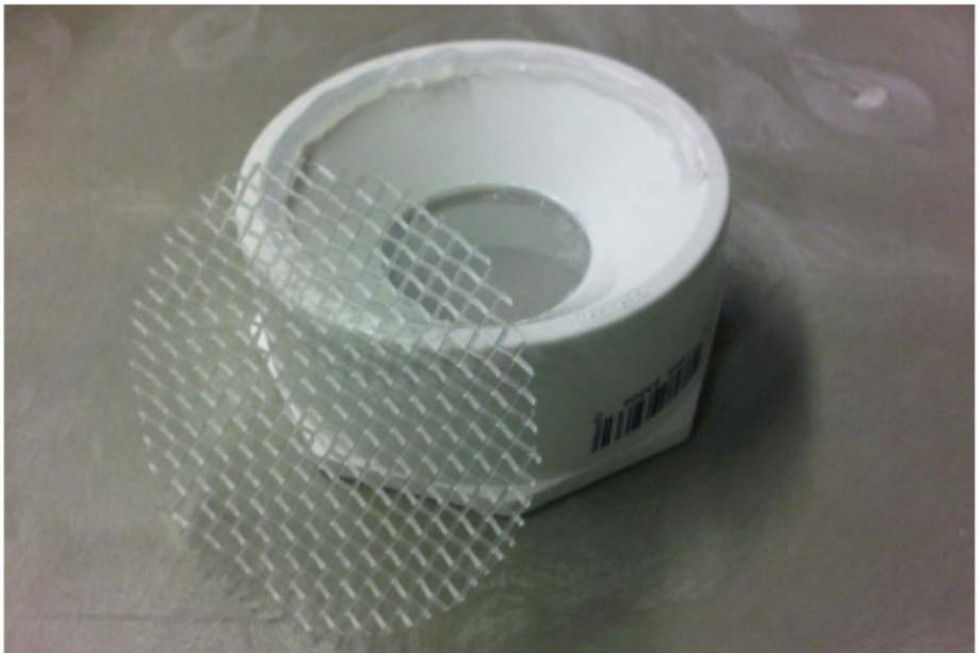

Figure 5.3. Spawning ring with coarse mesh top removed.

Figure 5.4. Spawning tank with spawning rings in place.

3. Alternatively, spawning chambers placed into designated tanks were used. The spawning chambers were rectangular boxes, approximately 20 × 35 × 22-cm high, with sides made of polycarbonate. Windows on two sides of the box increased water circulation through the chamber. If parents were selected, male and female fish were isolated in spawning chambers placed into designated tanks or raceways. Generally, three females and two males were sequestered in each spawning chamber that met these specifications.

4. During the egg collection period, each spawning chamber sat atop an egg collection tray, which consisted of a Nitex screen attached to a polycarbonate frame.

Embryo/fry care

A. Egg collection

Each spawning ring was checked daily for the presence of eggs (prior to cleaning or feeding).

1. If eggs were present, they were quickly transferred to glass crystallizing dishes (700-mL capacity) for incubation. Care was taken to keep the eggs submerged in water whenever possible.

    a. A crystallizing dish was filled with ~ 250 mL of synthetic saltwater from the recirculating synthetic saltwater holding tank.

    b. The spawning ring was inverted over the crystallizing dish and the underside of the spawning ring was sprayed vigorously (using a squirt bottle with synthetic saltwater) to transfer the eggs into the crystallizing dish. The mesh and the area around the silicone bead on the top of the spawning ring were checked carefully for eggs, and any remaining eggs were sprayed into the crystallizing dish.

    c. Eggs were transferred at a density of 1–4 per 10 mL of water (i.e., ≤ 200 eggs per dish).

2. When eggs were used for toxicity testing, an initial count of the eggs was required. The crystallizing dish was placed on a light table and the embryos were counted.

3. Incubation dishes were labeled with the DATE, the TANK # from which the eggs were collected, and the # of EGGS in the dish. The water level was also marked on the side of the dish as a reference for water renewal.

4. The dishes were placed on a shaker table at a moderate speed to promote aeration and minimize fungal growth.

B. Egg care (through hatching)

1. Egg incubation units were checked daily on a light table or with a dissecting scope. Unfertilized eggs and eggs that had become infected by fungus were removed and discarded.

2. Hatched fry were removed daily, placed in larval containers (generally, ~ 10-L square plastic containers) with approximately 6 L of synthetic saltwater, and maintained in a water bath at 25°C. Fry were maintained at a density not exceeding 100 per 2 L.

   a. Larvae were transferred with a wide bore plastic transfer pipette. Larvae were counted during transfer and containers were labeled with the HATCH DATE and # OF LARVAE.

3. Containers were aerated using a disposable glass pipette attached to the air supply line with silicone tubing. The air flow was high enough to generate a constant stream of air bubbles, but not high enough to cause ripples on the water surface.

C. Larval care

1. A 75% water change was effected daily in each bowl or tank, and water quality (i.e., temperature, salinity, pH, DO) was monitored daily. Water was poured from the culture vessel into a second container. In this way, any larvae accidentally poured out were recovered and placed back into the culture vessel. Synthetic saltwater (from the holding tank) was added to reach the 6-L mark, and containers were returned to the water bath and aerated.

2. During their first week post-hatch, fry were fed *Artemia* nauplii twice daily. During the third week post-hatch, a dry-flake food feeding was introduced. Two or three weeks post-hatch, fry were transferred to a larger aquarium or chamber (10-gal).

Post-hatch fry care and growth

Post-hatch fry were maintained in aquaria until their use in testing or their release into the broodstock pool. When fry reached an average length of approximately 15- to 20-mm (standard length), 50 fish were loaded into 10-gal aquaria, which offered rapid growth potential. After reaching an average size of 28- to 30-mm (standard length), the tanks were thinned to approximately 25 fish. Optimal growth was attained at a temperature of 25 to 30°C and a 16-hour light:8-hour dark photoperiod.

5.3.2 Protocol for the culture and collection of *Artemia* for sheepshead minnow SOPs

1. The air supply was removed from the appropriate *Artemia* hatching chamber. The eggs (cysts) and hatched *Artemia* were allowed to separate, which took 5–10 minutes.

2. A 1-L glass beaker was filled with approximately 100 mL of saltwater from the 20-L carboy in the sheepshead minnow fish culture area.

3. Hatched *Artemia* were captured by pouring the hatching jar contents (excluding the very top layer of unhatched eggs) into a fine-mesh round sieve. *Note:* The round sieve was suspended inside a square plastic collecting container so that the water that passed through the sieve could be returned to the hatching jar after the *Artemia* were filtered out.

4. Using a squirt bottle filled with DCW, the sieve was sprayed to concentrate the *Artemia*. The *Artemia* were then poured into the 1-L beaker containing 100 mL of synthetic saltwater, and the sieve was thoroughly rinsed in the beaker using the squirt bottle and spraying vigorously.

5. The 1-L beaker (filled with *Artemia* and some remaining cysts) was placed near a light source for 3–5 minutes.

6. Water in the square plastic collecting container was poured back into the hatching jar. It was poured down the edges of the hatching jar to rinse *Artemia* eggs caught on the sides back into the solution.

7. After 3–5 minutes, the *Artemia* in the 1-L beaker had congregated near the light source. They were then separated from the layer of cysts on the bottom of the beaker. A plastic transfer pipette was used to carefully siphon out the *Artemia* nauplii. The nauplii were then placed into a 100-mL glass beaker.

8. The remaining cysts in the 1-L beaker were poured back into the hatching jar.

9. The air hose was then returned to the chamber.

10. Using a graduated disposable glass pipette, the *Artemia* were thoroughly stirred to distribute them evenly in the water. Sheepshead minnow larvae were fed 0.5 mL per test dish. The remaining nauplii were fed to adults.

11. The collecting container, the sieve, and the 100-mL and 1-L glass beakers were thoroughly rinsed with tap water.

5.3.3 Protocol for the daily care of sheepshead minnow SOPs

Daily care overview

1. The temperature and salinity of each tank were measured and recorded on the International Animal Care and Use Committee (IACUC) #838 daily care log. Temperature was between 21 and 25°C and salinity was between 20 and 25 ppt.

2. Fish were fed twice daily at 7–8 a.m. and 3–4 p.m. (see details below).

3. Aquaria were cleaned if necessary. Larval tanks received a minimum 50% water change once daily. Adult tanks were changed once weekly (see details below).

4. Any abnormal changes of tank systems or fish health/behavior were reported. Dead fish were removed and recorded on the daily care log.

Detailed instructions

Feeding: Each tank received food as follows:

Tank #	Food
1–4 (adult broodstock)	***2 large pinches of Tetramin fish flakes*** – Males in tanks 1 and 2 may have taken 3 pinches but fish were fed no more than what would be consumed in 5 minutes. ***Artemia*** – Adults were fed whatever remained after feeding larvae evenly across tanks with a plastic transfer pipette.
1 (larva)	***Artemia*** – Fed 4 full pipettes.
2 (larvae)	***Artemia*** – Fed 2 full pipettes.

Cleaning

A. Larval tanks – once daily

1. After water quality was checked, at least 50% of the tank water (~ 1.5 L) was poured into a separate container. Larvae that were accidentally poured out of the culture tank were retrieved.

2. The culture tank was refilled to the 3-L mark with saltwater from the 20-L carboy in the sheepshead culture area.

B. Adult tanks – once weekly

1. Water was siphoned from the tank to remove any remaining fish waste and food. Siphoning generally removed approximately 25% of the water from each tank (~ 15 L).

2. Tanks were refilled with water from the synthetic saltwater holding tank.

5.3.4 MUO protocol for water-quality monitoring during *Deepwater Horizon* toxicity testing SOPs

Temperature, pH, and conductivity/salinity were measured with a portable VWR sympHony multimeter (model SP90M5) and probes. DO was measured with the Mettler Toledo SevenGo pro optical DO meter. Total ammonia was measured with a Hann Instruments 93700 photometer.

pH

Test solution pH was measured with the VWR sympHony multimeter and a gel-filled 3-in-1 pH/ATC electrode with an epoxy body (Model 14002-860).

A. Electrode calibration

1. pH calibration with two buffers (performed once monthly or daily whenever one-point calibration checks were outside the acceptable range as described below)

   a. Press the power key to turn on the meter.
   b. Select two buffers that bracket the expected sample pH and are one to four pH units apart (e.g., 7.00 and 10.01).
   c. Press the calibrate key.
   d. Rinse the electrode with deionized water and blot dry with a lint-free tissue. To avoid static buildup, do not wipe or rub the electrode.
   e. Insert the electrode into the first buffer and gently stir.

f. Wait for the pH icon to stop flashing and the pH value to appear in the meter window. The meter should display the temperature-corrected pH buffer value. If the displayed buffer value is incorrect, enter the value by pressing the up/down arrow keys to adjust each digit and the digits key to move to the next digit.
g. Press the calibrate key.
h. Rinse the electrode with deionized water and blot dry with a lint-free tissue.
i. Insert the electrode into the second buffer and gently stir.
j. Wait for the pH icon to stop flashing and the pH value to appear in the meter window. The meter should display the temperature-corrected pH buffer value. If not, enter the correct value as above.
k. Press the calibrate key and then press the measure key to save and end the calibration. The slope will be displayed and the meter will proceed to the measurement mode.

2. pH one-buffer calibration check (to be performed daily prior to measurements)

a. Choose a buffer near the expected sample pH (e.g., 7.00).
b. Rinse the electrode first with distilled water and place it into the buffer.
c. When the reading is stable, observe the measured pH. If the measured value is within 5% of the pH value of the buffer, proceed with measurements. If the reading is greater than 5% ± of the expected pH, proceed with the two-buffer pH calibration described above.

B. pH measurements

1. Calibrate the electrode as described in the "Electrode calibration" section below.

2. Rinse the electrode with deionized water, blot dry with a lint-free tissue, and insert the electrode into the sample.

3. With the meter in AUTO-READ mode, press the measure key to start the reading. The auto-read icon will flash until the reading is stable. Once the reading is stable, record the pH value on the appropriate data form.

4. Remove the electrode from the sample, rinse three times with distilled water, spray with a mild detergent (warm water and liquid household detergent), gently brush the surface of the probe with a soft brush, and rinse three times with distilled water. Proceed to the next test solution.

C. Electrode storage

Soak the electrode in a pH electrode storage solution (Model 14002-828).

Conductivity/salinity

Test solution conductivity and salinity were measured with the VWR sympHony multimeter and a VWR epoxy body two-cell platinum conductivity probe (Model 11388-372).

A. Electrode calibration

1. Conductivity calibration was performed daily prior to measuring test solutions.

    a. Press the power key to turn on the meter. Use the line select key to move the arrow icon to the conductivity measurement line.
    b. Select the VWR conductivity standard that has the closest conductivity to the expected sample value (e.g., 12.9 mS/cm).
    c. Enter the nominal cell constant value for the conductivity probe as follows:

        i. Press the setup key.
        ii. Press the up arrow key until COND is displayed on the top line.
        iii. Press the line select key to move the arrow icon to the middle line. Press the up arrow key until CELL is displayed.
        iv. Press the line select key to move the arrow icon to the bottom line. Enter the nominal cell constant by pressing the up/down arrow keys to adjust each digit and the digits key to move to the next digit. 0.475 cm-1 is the default setting.

    d. Rinse the conductivity probe with deionized water and blot dry with a lint-free tissue.
    e. Insert the conductivity probe into the conductivity standard and gently stir.
    f. Press the calibrate key. The meter will show the manual calibration display for about five seconds. During this time, do not press any keys.
    g. After about 5 seconds, the meter will proceed to the autocalibration display. Wait for the µS/cm or mS/cm icon to stop flashing and the arrow icon to start flashing. The meter should display the conductivity standard value at 25°C.
    h. Press the measure key to save and end the calibration.

B. Conductivity/salinity measurements

1. Calibrate the electrode as described in the "Electrode calibration" section in the DO section below.

2. Rinse the conductivity probe with deionized water, blot dry with a lint-free tissue, and insert the probe into the sample.

3. With the meter in AUTO-READ mode, press the measure key to start the reading. The auto-read icon will flash until the reading is stable. Once the reading is stable, record the conductivity and salinity values on the appropriate data form.

4. Remove the electrode from the sample, rinse three times with distilled water, spray with a mild detergent (warm water and liquid household detergent), gently brush the surface of the probe with a soft brush, and rinse three times with distilled water. Proceed to the next test solution.

C. Electrode storage

The conductivity electrode should be stored clean and dry.

DO

DO in test solutions was measured with the Mettler Toledo SevenGo pro optical DO meter.

A. Electrode calibration

Calibration was performed weekly. The first point of a DO calibration was always done in water-saturated air (100% O_2).

1. Remove the OptiOx calibration tube cap and then the sponge from the cap.

2. Saturate the sponge with distilled water and squeeze the excess water out of the sponge.

3. Reassemble the OptiOx calibration tube.

4. Ensure that no water droplets are on the surface of the OptiOx sensor cap.

5. Slide the calibration tube over the front of the sensor until the calibration tube is firmly connected to the sensor.

6. Allow at least 5 minutes for the temperature to stabilize prior to calibration.

7. Press and hold MODE for 3 seconds to switch to the single-channel measurement screen when in dual-channel measurement.

8. Press CAL. Cal 1 will appear on the display. The meter calibrates according to the preselected endpoint mode automatically after the signal has stabilized or after pressing READ. The standard value is shown on the display.

9. Press End to accept the calibration and return to sample measurement. The calibration result is shown on the display.

10. Press Exit to reject the calibration.

B. DO measurements

1. Place the sensor in the sample. The InLab OptiOx must be immersed in a minimum 3.5-cm solution so that the temperature sensor is covered.

2. Press READ to start a measurement. As soon as the measurement is stable according to the selected stability criterion, the Stability icon will appear.

3. Press READ to manually stop the measurement and record the DO value.

4. Remove the electrode from the sample, rinse three times with distilled water, spray with a mild detergent (warm water and liquid household detergent), gently brush the surface of the probe with a soft-bristled brush, and rinse three times with distilled water. Proceed to the next test solution.

C. Electrode storage

Place the DO sensor in the calibration tube, making sure to wet the sponge with distilled water.

Ammonia

Total ammonia was measured using a Hanna Instruments 93700 photometer. This instrument uses an adaptation of the ASTM International Manual of Water and Environmental Technology, D1426-92, Nessler method.

A. Measurement procedure

1. Turn the meter ON.

2. Remove the cuvette cap. Fill the cuvette to 1.5 cm below the rim with 10 mL of unreacted sample and replace the cuvette cap.

3. Place the cuvette into the photometer and ensure that the notch on the cap is positioned securely into the groove.

4. Press ZERO and "SIP" will appear on the display.

5. Wait for 5 seconds and the display will show "-0.0-," which means that the meter is zeroed and ready for measurement.

6. Remove the cuvette.

7. Remove the cuvette cap and add 6 drops of Reagent #1. Replace the cap and swirl the solution for 5 seconds.

8. Remove the cap and add 10 drops of Reagent #2.

9. Replace the cap and swirl the solution for 5 seconds.

10. Reinsert the cuvette into the instrument.

11. Press READ TIMED and the display will show the countdown prior to the measurement (e.g., 3 minutes and 30 seconds).

12. The instrument directly displays the concentration in mg/L of ammonia nitrogen (NH_3-N). To convert the reading to mg/L of ammonia, multiply the display reading by a factor of 1.214.

13. *Note*: The cuvette should be cleaned between each test solution as follows: Rinse the cuvette with a 1% Alconox solution, gently clean with a cotton-tipped applicator, and follow with three rinses of distilled water.

5.4 MUO Test Media Disposal Guidelines

1. All oil-containing solutions were disposed of by draining through an activated charcoal bed, with outflow leading into a sink drain.

2. Any solid pieces (e.g., weathered oil) were removed from the charcoal bed after use and placed in a waste vessel stored in a fume hood.

3. At the completion of the project, both the activated charcoal bed and solid waste were disposed of under the chemical safety and disposal regulations of the Environmental Health and Safety Office at MUO.

References

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A. Testing Protocol 1: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Mahi-Mahi (*Coryphaena hippurus*)

A.1 Testing Apparatus

The indoor testing apparatus consists of a bank of solar-simulating light bulbs and a water table covered in either UV transparent (Aclar) or UV opaque (Courtgard) plastics. Courtgard (CP Films, Inc., Martinsville, VA, USA) is a long-wave-pass plastic that in water transmits photosynthetically active radiation (PAR; 95% 400–800 nm) but blocks most UV radiation (transmits no UV-B 295–319 nm, and only 9% of UV-A 320–400 nm, with a sharp wavelength cutoff and 50% transmittance at 400 nm). Aclar (Honeywell International, Morristown, NJ, USA; http://www.honeywell.com/) is a long-wave-pass plastic that in water transmits both photosynthetically active radiation (PAR; 100% 400–800 nm) and most UV radiation (98% of UV-B 295–319 nm, 99% UV-A 320–399 nm, with a sharp wavelength cutoff and 50% transmittance at 212 nm). The water table acts simply as a cooling bath and organisms are never in contact with cooling water.

Feed water into the water table and vary the rate of flow to maintain a constant temperature during the test. Provide simulated sunlight by a bank of 84 solar-simulating fluorescent bulbs (Durotest Vitalite, 40W, and fluorescent blacklight bulbs), suspended from a grid above the laboratory, with the height above tanks adjustable between 0.5 and 2.5 m. This setup provides a spectrum that is > 90% equivalent to natural sunlight at an intensity approaching 10% of natural sunlight.

A.2 Test Procedure

A.2.1 Prior to test initiation

1. Prepare WAF according to the *Protocols for Preparing Water Accommodated Fractions* SOP as described in the QAPP. Retain samples as specified in the *Analytical Sample Shipping and COC* SOP as described in the QAPP.

2. Bench sheets should be filled out as described in the QAPP (e.g., ”WAF preparation”).

A.2.2 ~ 12 hours prior to test initiation

1. Turn on water to water tables to cool to desired temperature.

2. Prepare appropriate dilutions of test media from stock WAF. The dilution series should include a synthetic saltwater blank and span the working range of WAF to be used in the test (see test-specific TCTs). Dilutions should be mixed in clean, large (≥ 4 L) glass flasks using the same synthetic saltwater source used for WAF preparations. Mix well between each serial dilution. Retain samples as described in the QAPP.

3. Sample water quality in test media dilutions (as described in the MUO GLPP).

4. Obtain clean, glass crystallizing dishes. Label each dish with the test treatment and tank # (replicate number) according to QAPP guidelines.

5. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately (as described in the MUO GLPP).

6. Place organisms into each replicate dish (see test-specific TCTs for the total number of organisms to add to each replicate).

7. Place test dishes randomly on the water table under appropriate plastic according to UV + or UV- treatment.

8. Start UV monitor. UV will be monitored continuously using a Biospherical Instruments multi-wavelength radiometer. The radiometer measures wavelengths in the UV-B, UV-A, and visible light spectra.

9. Bench sheets should be filled out as described in the QAPP.

10. Leave dishes in a secure laboratory area in the dark until test initiation.

11. After approximately a 12-hour uptake period, turn on UV lights (48-hour test period begins).

12. After 14 hours of exposure to artificial sunlight, turn off UV lights.

13. Measure temperature and DO content of five randomly selected dishes from each UV treatment.

14. Prepare WAF and retain sample according to protocols described in step 2. Prepare appropriate dilutions of test media from WAF. Retain samples as described in the QAPP.

15. Sample water quality in test media dilutions (as described in the MUO GLPP).

16. Remove at least 90% of the test medium (~ 180 mL) from each replicate dish using a syringe. Dispose of test media appropriately (as described in the MUO GLPP).

17. Using a glass graduated cylinder and the appropriate WAF dilution, replace the test media in each replicate dish with a volume equal to the volume extracted.

18. Return test dishes on the water table to respective location under appropriate plastic according to UV + or UV- treatment.

19. After 10 hours of "dark," turn on UV lights. Repeat steps 12 and 13.

20. At the conclusion of the test, count the dead and living organisms. All dead test organisms should be sampled and retained according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

B. Testing Protocol 2: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Embryonic and Larval Sheepshead Minnow (*Cyprinodon variegatus*)

B.1 Testing Apparatus

The indoor testing apparatus consists of a bank of solar-simulating light bulbs and a water table covered in either UV transparent (Aclar) or UV opaque (Courtgard) plastics. Courtgard (CP Films, Inc., Martinsville, VA, USA) is a long-wave-pass plastic that in water transmits PAR (95% 400–800 nm) but blocks most UV radiation (transmits no UV-B 295–319 nm, and only 9% of UV-A 320–400 nm with a sharp wavelength cutoff and 50% transmittance at 400 nm). Aclar (Honeywell International, Morristown, NJ, USA; http://www.honeywell.com/) is a long-wave-pass plastic that in water transmits both PAR (100% 400–800 nm) and most UV radiation (98% of UV-B 295–319 nm, 99% UV-A 320–399 nm, with a sharp wavelength cutoff and 50% transmittance at 212 nm). The water table acts simply as a cooling bath and organisms are never in contact with cooling water.

Feed water into the water table and vary the flow rate to maintain a constant temperature during the test. Simulated sunlight is provided by a bank of 84 solar-simulating fluorescent bulbs (Durotest Vitalite, 40W, and fluorescent blacklight bulbs), suspended from a grid above the laboratory, with the height above tanks adjustable between 0.5 and 2.5 m. This setup provides a spectrum that is > 90% equivalent to natural sunlight at an intensity approaching 10% of natural sunlight.

B.2 Test Procedure

B.2.1 Prior to test initiation

1. Prepare WAF according to the *Protocols for Preparing Water Accommodated Fractions* SOP as described in the QAPP. Retain samples as specified in the *Analytical Sample Shipping and COC SOP* as described in the QAPP. Prepare WAF daily for subsequent renewals.

2. Bench sheets should be filled out as described in the QAPP (e.g., “WAF preparation”).

B.2.2 ~ 12 hours prior to test initiation

1. Turn on water to water tables to cool to desired temperature.

2. Prepare appropriate dilutions of test media from stock WAF. The dilution series should include a synthetic saltwater blank and span the working range of WAF to be used in the test (see test-specific TCTs). Dilutions should be mixed in clean, large (≥ 4 L) glass flasks using the same synthetic saltwater source used for WAF preparations. Mix well between each serial dilution. Retain samples as described in the QAPP.

3. Sample water quality in test media dilutions (as described in the MUO GLPP).

4. Obtain clean, glass crystallizing dishes. Label each dish with the test treatment and tank # (replicate number) according to QAPP guidelines.

5. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately (as described in the MUO GLPP).

6. Place fertilized embryos or larvae into each replicate dish (see test-specific TCTs for total number of organisms to add to per replicate). Ensure that embryos are less than 24 hours post-fertilization at test start.

7. Place test dishes randomly on the water table under appropriate plastic according to UV + or UV- treatment.

8. Start UV monitor. UV will be monitored continuously using a Biospherical Instruments multi-wavelength radiometer. The radiometer measures wavelengths in the UV-B, UV-A, and visible light spectra.

9. Fill out bench sheets as described in the QAPP.

10. Leave dishes in a secure laboratory area in the dark until test initiation.

B.2.3 After ~ 12 hour uptake period (early morning of day 1)

1. Prepare appropriate dilutions of test media from stock WAF. Retain samples as described in the QAPP.

2. Sample water quality in test media dilutions (as described in the MUO GLPP).

3. Count the dead and living organisms in each replicate dish. Remove dead organisms and retain according to the QAPP guidelines. Do not replace organisms. Fill out bench sheets as described in the QAPP.

4. Measure temperature and DO in five randomly selected dishes from each UV treatment (as described in the MUO GLPP).

5. Remove at least 90% of the test medium (~ 180 mL) from each replicate dish using a syringe. Dispose of test media appropriately (as described in the MUO GLPP).

6. Using a glass graduated cylinder and the appropriate WAF dilution, replace the test media in each replicate dish with a volume equal to the volume extracted.

7. Return test dishes on the water table to respective location under appropriate plastic according to UV + or UV- treatment.

8. Feed Artemia nauplii (larvae only) ad libitum (as described in the MUO GLPP).

9. Turn on UV lights (larvae-only 96-hour test period begins).

B.2.4 After 14 hours of exposure to artificial light

1. Turn off UV lights.

B.2.5 Early morning of day 2

1. Repeat procedures from Section B.2.3. Note that WAF is prepared daily (as in Section B.2.1) for subsequent test solution renewals.

Carry out test until the last organism in the lowest WAF concentration has died or for a maximum of 7 days. Retain all remaining test organisms as archive tissue samples following the methodology described in the Status Consulting QAPP. At the conclusion of the test, count the dead and living organisms. All dead test organisms will be sampled and retained according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

6. Mote Marine Laboratory General Laboratory Procedures and Practices

6.1 Introduction

Mote Marine Laboratory (Mote) conducted toxicity tests on two fish species [red drum (*Sciaenops ocellatus*) and inland silverside (*Menidia beryllina*)] to identify the toxicological impacts of the 2010 *Deepwater Horizon* oil. This chapter describes the General Laboratory Procedures and Practices (GLPP) used at Mote.

6.2 Methods

This section describes sources and respective husbandry/maintenance procedures for test organisms that were used for toxicity evaluations.

6.2.1 Fish culturing

Fish that were produced for the toxicity studies were obtained from appropriate fish culturing facilities for each species. Each species had its own set of rearing conditions, which are described below. Juvenile fish were produced in 3,300-L nursery tanks to the desired size for the trials.

Red drum

Red drum were obtained from the Florida Fish and Wildlife Conservation Commission Stock Enhancement Research Facility. Red drum were provided to Mote as fertilized embryos at about 12 hours before hatching and were collected within a few hours of fertilization. These embryos were used for toxicity trials or reared to older early life stages used for larval or juvenile trials. Larvae were grown to the desired size (see Testing Protocols) for the trials at 26°C in either 100-L or 129-L rearing tanks maintained using dedicated recirculating filtration systems. Larval feeding, using enriched rotifers, was initiated at 3 days after hatch (DAH) and continued until 11 DAH. Algae paste was added to the rotifer culture vessels to ensure that the rotifers met the nutritional needs of the larvae. Live food (rotifers) to feed the larval fish was produced either in a high-density rotifer production system or in a batch culture system. A dry diet was initially introduced with the rotifers at 3 DAH. Larvae were fully weaned off of rotifers and fed *Artemia* nauplii and a commercially prepared dry diet by 12 DAH. Larvae began to metamorphose to juvenile red drums at 24 DAH.

Inland silverside

Silversides were obtained from Marinco Bioassay, Sarasota, Florida. Both larvae (7 DAH) and juveniles (12 DAH) were transported to the Mote Aquaculture Park for 24 hours of acclimation before toxicity tests began. Larvae were placed in acclimation beakers (1 L) in 28 ppt salinity, 7.5–8.0 pH, 26°C water for a 24-hour acclimation period. Silversides were fed concentrated *Artemia* nauplii.

6.2.2 Toxicity test exposure media preparations

Media preparation

Protocols for test solution preparation are found in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. The types of toxicity testing solutions are as follows:

- Two different water accommodated fraction (WAF) preparations, high energy and chemically enhanced WAFs (HEWAF and CEWAF, respectively) using two oil types: Slick A (CTC02404-02) and weathered source oil (072610-W-A). Oils were provided to Mote by Stratus Consulting.
- Corexit-only dispersant exposures – as a definitive test (dose-response) without the addition of oil. Corexit was provided to Mote by Stratus Consulting.
- Control water – prepared similarly as WAFs but without the addition of oil.

Seawater used for all exposure studies was obtained from Mote Aquarium's supply of natural seawater from the Gulf of Mexico. It was sand-filtered, purified by ozonation, and monitored for proper dissolved oxygen (DO), pH, temperature, and salinity.

Mote ozonation process for seawater purification

Mote's seawater ozonation process included seawater intake at the New Pass (Sarasota Bay, Florida) dock that collected water from the coastal Gulf of Mexico, average salinity 34 to 36 ppt. The water was pumped to one of four treatment tanks (ranging in volume from 8,000 to 30,000 gal), where it was ozonated for 1 to 2 hours to attain an oxidation-reduction potential (ORP) of 650 mV. Ozone was produced from compressed air with a PCI WedCo ozonation unit, 7 lb O_3/hour capacity, and injected through a Mazzei induction venturi. Following ozonation, air continued to be injected for 24 hours to complete degassing and ORP reduction to attain seawater background levels of less than 300 mV. The stored water was continuously passed through a sand filter at ~ 150 gpm to clean and polish the water. Each storage tank had a

dedicated venturi, pump, and filter system for complete isolation and redundancy. The treated water was continuously monitored for ORP and temperature. The water was sampled and tested for bromine residue before putting the tank on line for use. Cleaned seawater was transported to the aquarium through a dedicated one-way directional pipe. The salinity was tested and adjusted with reverse osmosis (RO) water as needed for specific uses.

6.2.3 Water quality monitoring

Water quality measurements (temperature, pH, DO, salinity, ammonia) were taken as follows (see the QAPP and Table 6.1).

Table 6.1. Water quality constituents and monitoring schedule

Temperature	At initial preparation of stock solution and daily in water bath
pH	At initial preparation of stock solution and from two random test chambers at the end of each 48-hour period
DO	At initial preparation of stock solution and then daily from each test chamber
Salinity	At initial preparation of stock solution and from two random test chambers at the end of each 48-hour period
Total ammonia	Initially for source water for preparation of stock solution and then from two random test chambers at the end of each 48-hour period

6.2.4 Analytical chemistry

Final confirmatory analytical chemistry was conducted offsite at ALS Environmental as described in the QAPP.

6.2.5 Standard operating procedure: Mote standard operating procedures for pH, DO, temperature, ammonia, and salinity

Meters were bench-calibrated or their accuracy was verified against standards or alternative methodologies prior to use, and the accuracy was assessed. Bench-calibration (verification) records were maintained in a laboratory file specific to instrument type and traceable to individual units by serial number. Calibration or verification protocols were based on the manufacturer's methods. Calibrations and calibration verifications were performed prior to sampling. For measurements using probes in the test chambers, measurements began with deionized (DI) water and proceeded to the highest concentrations. This limited the possibility of cross-contamination of chambers. The analyses for pH and DO took place directly in the

exposure chambers. Ammonia was collected from subsamples of test solution during the solution renewal at 48 hours or at the end of the test for analysis. Similarly, drops of test solution from selected chambers were collected with a pipette for the refractometer measurements. Temperature measurements were taken within the water bath table and also during the in-chamber DO measurements.

pH: VWR sympHony meter

1. Record serial number of meter and probe

2. Verify that the batteries are not low

3. Turn on pH meter, then press CALIBRATE to go to CAL 1

4. Rinse probe with DI water; shake and put in either pH 7 or 10 buffer solution

5. When pH display stops flashing, record values of pH and temperature (e.g., 7.00 pH and 24.2°C)

6. Press CALIBRATE while the triangle on right side of screen is still blinking; rinse probe with DI water; shake probe to shake off water and place in next buffer

7. With display of CAL 2, record values of pH and temperature (e.g., 10.02 pH and 24.4°C), and record slope while triangle is still blinking (e.g., SLP 95.4%)

8. On the screen of the pH meter, both 7.00 and 10.00 buffers should be displayed

9. Rinse probe with DI water, shake and place into water sample, and press the measure button

10. Record the value of the pH after the pH display stops flashing

11. Rinse the probe between each of the three triplicate test samples, and record readings from lower concentrations to higher (e.g., C1-3, T4-6)

12. After all the pH parameters are recorded, clean probe with warm water and Simple Green cleaning solution, rinse in DI water, then store in the electrode storage solution until the next sampling.

DO and temperature: YSI ProODO

A. DO

To accurately air calibrate, ensure that the altitude setting is set to the approximate altitude of the region in which the meter will be used and the approximate salinity of the water being analyzed.

For both ease of use and accuracy, YSI recommends performing a DO % water-saturated air calibration as described below prior to sample analysis.

B. Calibrating DO % in water-saturated air; air calibrate (prior to sample analysis)

Note: Air calibration must take place within ± 10°C of the sample temperature.

1. Moisten the sponge in the storage sleeve with a small amount of clean water. (*Note*: the sponge must be clean and free of bacterial growth, which may consume oxygen and interfere with calibration.)

2. Make sure that there are no water droplets on the sensor cap and temperature sensor, then install the storage sleeve over the probe.

3. Wait 15 minutes for the storage container to become completely saturated and to allow the temperature and DO sensors to stabilize.

4. Press CAL (calibration).

5. Highlight DO % and press ENTER to confirm.

6. The instrument will use the value from the internal barometer during calibration, and will display this value in brackets at the top of the display.

7. Wait for the temperature and DO % values under “Actual readings” to stabilize, then highlight Accept Calibration and press ENTER to calibrate.

8. Press CAL to complete the calibration.

9. Press ESC to cancel calibration.

C. DO measurement

Always analyze starting with the lowest concentration of oil or dispersant (control) and work up to the highest concentration.

To take readings, insert the probe into the sample. The DO and temperature sensors should be immersed in the sample.

Allow the temperature readings to stabilize, and wait approximately 20–25 seconds for the DO readings to stabilize.

1. Record each sample's DO directly from the meter display
2. Rinse probe in DI water and repeat
3. To clean probe, wash with warm water and Simple Green cleaning solution.

D. Temperature

All ProODO probes have built-in temperature sensors. Temperature calibration is not required nor is it available. To set the units, press the Probe key on the ProODO instrument and select Display. Highlight temperature and press ENTER. Highlight the desired temperature units of °F, °C, or K and press ENTER to confirm the selection. Record each sample's temperature directly from the meter display as you read the DO measurement.

Ammonia: Hach DR2800 using Method 8155 salicylate method

- Reagents
  - Ammonia Cyanurate Reagent Powder Pillows
  - Ammonia Salicylate Reagent Powder Pillows
  - Sample Cells, 1-in. Square, 10 mL.

A. Calibration verification

Calibration is incorporated into the software and is not adjusted by the user. In lieu of a calibration, a weekly "Accuracy Check" is performed to verify that the instrument is working properly.

1. Create a sample blank as described in the procedure or use sample blank from previous test.

2. Place the blank in the cell holder with the fill line facing right.

3. Press OPTIONS > MORE. Press STANDARD ADDITIONS. A summary of the standard additions procedure will appear.

4. Press OK to accept the default values for standard concentration, sample volume, and spike volumes.

5. After values are accepted, the unspiked (BLANK) sample reading will appear in the top row.

6. Open an Ammonia Nitrogen Standard Solution, 10 mg/L as NH_3N provided by Hach.

7. Prepare three sample spikes by filling three mixing cylinders with 25 mL of sample water.

8. Use a calibrated pipette to dispense 0.2 mL, 0.4 mL, and 0.6 mL of standard to the three respective cylinders and mix each thoroughly.

9. Analyze each sample spike as described in the procedure above, starting with the 0.2-mL sample spike.

10. Accept each standard addition reading by pressing READ.

11. Each addition should reflect approximately 100% recovery.

12. After completing the sequence, press GRAPH to view the best-fit line through the standard additions data points accounting for matrix interferences.

13. Press IDEAL LINE to view relationships between the sample spikes and the “Ideal Line” of 100% recovery.

B. Ammonia measurement

1. Turn on instrument. Press STORED PROGRAMS.

2. Select the test (385 N. Ammonia, Salic).

3. Prepare the sample by filling a 1-in. square sample cell to the 10-mL mark.

4. Prepare the blank by filling a second square sample cell with RO water to the 10-mL mark.

5. Add the contents of one Ammonia Salicylate Reagent Powder Pillow to each cell.

6. Stopper each cell and shake to dissolve.

7. Press TIMER > OK, and a 3-minute reaction period will begin.

8. When the timer expires, add the contents of one Ammonia Cyanurate Reagent Powder Pillow to each cell.

9. Stopper each cell and shake to dissolve.

10. Press TIMER > OK, and a 15-minute reaction will begin. (A green color will develop if ammonia-nitrogen is present.)

11. When the timer expires, insert the BLANK into the cell holder with the fill line facing right.

12. Press ZERO. The display will show 0.00 mg/L NH_3-N.

13. Wipe the sample cell and insert it into the cell holder with the fill line facing right.

14. Press READ. Results are given in mg/L NH_3-N.

Salinity: VEE GEE STX-3

The VEE GEE STX-3 has an automatic temperature range of 10°C to 30°C. It has a range of 0–100 ppt salinity with an accuracy of ± 1 ppt.

A. Zero verification

1. Prior to use, place two drops of RO water on the prism and "zero" the unit using the ZERO RING directly behind the prism. It is best to zero at or near the temperature of the sample.

2. Record time and location of zeroing.

B. Salinity measurement

1. Rinse prism with DI water and blot dry between each sample
2. Place two drops of sample directly on the center of the prism (hold prism level)
3. Close the clear plastic prism cover
4. Read the salinity in ppt from the view scope to the nearest ppt
5. Record salinity
6. Rinse with DI water, clean with Simple Green solution, rinse with DI water again, and blot dry prior to storage.

Dispersant measurement spectrophotometer

The instrument used for the analysis of the absorbance of the Corexit 9500 samples is a Perkin Elmer Lamda 35, UV/VIS Spectrometer with double-beam operation. All samples were recorded at 240 nm wavelength in the absorbance mode with the 1-nm slit using the UV WinLab software.

A five-point calibration curve was developed appropriate for the range of concentrations to be measured in the samples. Samples were then analyzed relative to the calibration curve for Corexit concentration confirmation.

A. Testing Protocol 1: Aquatic Toxicity Procedure

A.1 General Considerations

Decontamination of all glassware, spatulas, etc., followed the QAPP.

WAFs were prepared with the appropriate treatment oil [see test-specific test conditions tables (TCTs)] according to *Protocols for Preparing Water Accommodated Fractions* in the QAPP, Appendix A. WAFs were prepared daily for subsequent renewals.

A.2 Experiment

Static and static-renewal exposures were conducted using standard guidance and the basic testing protocols of the QAPP and the Mote GLPP.

Static and static-renewal tests were carried out in clean decontaminated glass beakers for up to 96 hours. After preparation, the test solutions were added to the exposure containers holding the proper number of test organisms. An air tube was placed in each container, and air was bubbled at a rate of approximately 1–2 bubbles/second for aeration. Containers were covered by aluminum foil. Test organisms, with the exception of embryos, were fed during exposures.

All controls and the various concentrations of test solutions for each exposure test were in triplicate. Control treatments were prepared using the same methods as exposure treatments, but did not contain any contaminants. All sample and control preparations, as well as basic laboratory chemistry and standard cleaning procedures, followed the QAPP and the Mote GLPP for Toxicity Testing Protocols.

1. Set up water bath with an aquarium heater to achieve appropriate temperature for test organisms.

2. Transfer test organisms into test beakers. Test organisms should be transferred in seawater to each container for a species-specific acclimation period prior to the addition of test solution. The organisms must remain suspended in water during the transfer and be allowed to acclimate to minimize stress. This acclimation period is specific to each organism; e.g., red drum larvae have an acclimation period of 3 days, which begins at 15 DAH of the test species. Silversides have an acclimation period of 24 hours that begins at 8 DAH for larvae and 15 DAH for juveniles. There is no minimum acclimation period for red drum embryos. The number of test organisms placed in the beakers for acclimation exceeds the final number used for the test. Remove organisms that have deceased during this acclimation period from the beakers daily by siphon. On day 1 of the

test, remove excess organisms. Only the required number of test organisms for each exposure remains in the exposure chambers, and the test commences.

3. Keep beakers in water bath to maintain constant temperature.

4. Attach aeration stones to tubing attached to aquarium air pumps.

5. Place one air stone in each beaker, bubbling at a rate of approximately 1–2 bubbles/ second.

6. Cover beakers with foil.

7. After the acclimation period, add WAF at t = 0 by gently removing approximately 95% of the seawater from the container and replacing it with appropriate test solutions.

8. Collect and archive required samples for analytical chemistry according to the QAPP.

9. Measure water quality parameters (temperature, salinity, DO, ammonia, and pH) as described in the Mote GLPP document.

10. Feed test organisms with the appropriate food type and frequency determined for the organism's life stage. Remove unconsumed food daily from the bottom of beakers by a minimal-flow, small-diameter siphon.

11. Remove dead organisms from each test chamber each day, and record and archive according to the QAPP.

12. After 48 hours, repeat steps #7–10. Note: This step pertains only to tests that require a WAF renewal (see test-specific TCTs).

13. At the end of the exposure study, count the number of live organisms remaining in each exposure chamber. Record and archive all live and dead organisms according to the QAPP.

A.3 Analytical Testing Methods

All analytical testing methods followed the QAPP.

All sample and control preparations, as well as basic laboratory chemistry and standard cleaning procedures, followed the QAPP and the Mote GLPP for Toxicity Testing Protocols.

B. Testing Protocol 2: Red Drum Juvenile Behavioral Oil Toxicity Studies

B.1 General Considerations

Decontamination of all glassware, spatulas, etc., followed the QAPP.

WAFs and dispersant-only test solutions were prepared with the appropriate treatment oil (see test-specific TCTs) according to *Protocols for Preparing Water Accommodated Fractions* in the QAPP, Appendix A.

B.2 Experimental Design

The study used a before-after control-impact (BACI) experimental design to measure pre-stimulus and post-stimulus reactions of juvenile red drum contained in experimental chambers filled with combinations of treatment water as follows:

1. Control tanks that contained seawater (no oil or dispersant) before and after alarm pheromone stimulus.

2. Tanks that contained CEWAF exposures before and after an alarm pheromone stimulus.

3. Tanks that contained dispersant (DISP) exposures before and after alarm pheromone stimulus.

Behavioral tests were carried out in decontaminated 1.9-L glass aquaria with blacked-out backgrounds for up to 16 hours. One to five experimental replicates were set up for each treatment. For a specific test, all of the experimental replicates from a specific treatment were conducted on the same day in combination with paired controls. See test-specific TCTs for treatment designations and number of replicates used for each treatment.

The behavioral responses of juvenile red drum were assessed just prior to and after the addition of an alarm pheromone cue to the exposure chambers. Cue additions and response observations were conducted at approximately 2- and 16-hours post-oil/dispersant exposure (see test-specific TCTs). Responses were video-recorded and focal observations quantified using computerized event recorder software (JWatcher). All trials were conducted between 0900 and 1300 hours on each exposure day. Observations consisted of a 10-minute pre-stimulus and a 10-minute post-stimulus observational period. Each behavioral experiment used 30-day-old red drum. Each trial was stocked with new fish because the fish were sacrificed and preserved for every trial. No fish

was ever used more than once. All experimental tanks were custom-made from inert window glass. Aquaria sealant was used to seal joints.

B.3 Pre-trial Preparations

- Randomly arrange the chambers and place labels on each chamber appropriately. Insert visual barriers around and between the tanks so that the tank inhabitants cannot see any other tanks or researchers in the area.
- Arrange individual chamber air lines and cue addition lines with respective labeling.
- Hang a tarp barrier around the exposure chamber.
- Set up the overhead light timer appropriately (12 hours light, 12 hours dark).
- Prepare video cameras; stands, data storage disks, mobile table for cameras, charge batteries in all cameras, have A/C power chargers setup.
- Print appropriate bench sheets for data collection.

B.4 Test Setup

1. Prepare test exposure water and collect analytical chemistry samples according to the QAPP.
2. Fill chambers with 1,500 mL of the appropriate treatment.
3. Insert air stones and adjust aeration to a gentle rate so that juveniles do not have to swim against currents.
4. Calibrate water quality instruments (pH meter, DO meter) and measure pH, salinity, DO, and temperature. Record measurements on the “Water Quality Monitoring” bench sheet (QAPP).
5. Record all exposure data on the appropriate bench sheets.
6. Harvest red drum juveniles from Mote rearing tanks as follows:
    a. Do not feed juveniles on harvest day.
    b. Set up the harvester bucket.

c. Secure 3/4” silicone siphon hose to tank outflow and into harvester bucket.

d. Turn off bypass for drain water allowing all water to enter harvester bucket.

e. Open the spare tank to capture any extra water and prevent sump overflow.

f. Pull the standpipe from the source tank and drain into the harvester bucket. Flush thoroughly until all of the juvenile red drum are in the harvester bucket.

g. Collect approximately 50 individuals using a small nylon aquarium net.

h. Place fish into a bucket and transport them to the behavior room.

7. Stock the appropriate number of juvenile drum per individual chamber (see test-specific TCTs). Make sure to select healthy and vigorous-looking individuals for all chambers; however, do not select by size. Aerate the bucket while transferring fish to exposure chambers.

8. Record the harvest and stocking times on the appropriate bench sheet.

B.5 Initial Behavior and Acclimation

1. Using a hand-held video camera, record approximately 2 minutes of video per chamber and make initial observations of fish performance. Record start and stop times on the appropriate bench sheet.

2. Drop a tarp barrier around the exposure chambers.

3. Set up five video cameras with tripods focused on the first round of tanks to be recorded. Record tank/camera combinations on appropriate bench sheet.

4. Leave the behavior room for 2 hours to allow red drum to acclimate to the tanks.

B.6 Chemical Alarm Cue and Behavioral Response

1. After 2 hours and 16 hours, return to the behavior room and start filming with the 5 cameras 10 minutes prior to the addition of alarm cue.

2. Record the respective video start times on the appropriate bench sheets.

3. Leave the room and prepare the alarm cue solution from a donor fish as follows:

    a. Harvest a large a red drum individual (approximately 100–160 mm total length).

    b. Record the donor fish's length, weight, source tank, and condition at harvesting on the appropriate bench sheet.

    c. Lightly dab the fish with a paper towel to remove any excess water or slime.

    d. Sever the spine and homogenize the brain.

    e. Using a sharp tip scalpel (#11 blade), make 10 superficial and parallel cuts into the skin of each side of the donor fish. Make sure urine does not contaminate the work area.

    f. Pour 60 mL of seawater over the red drum and collect wash water in a 250-mL beaker.

    g. Fill a second beaker with an additional 60 mL of clean source water.

    h. Draw 10 mL of cue water into a pre-cleaned 60-mL syringe (cue syringe).

    i. Draw 10 mL of seawater water into a second 60-mL syringe (wash syringe).

4. After 10 minutes of filming, return to the behavior room and quietly connect the cue syringe to the respective delivery hose and slowly add the entire contents of the syringe into the delivery hose. Remove the cue syringe, connect the wash syringe, and deliver the contents into the delivery hose.

5. Fill the wash syringe with air and slowly purge the line to ensure that all the cue water has entered the tank.

6. Record the time the chemical alarm cue was added on the appropriate bench sheets.

7. Repeat the steps listed above for each replicate tank where cameras are set up.

8. Keep filming for 10 minutes post exposure to cue.

B.7 Post-behavioral Observational Period

1. Calibrate the water quality instruments.

2. Collect water quality measurements in each chamber according to the Mote GLPP: Test for pH, salinity, DO, and temperature.

3. Record the water quality measurements on the "Water Quality Monitoring" bench sheet found in the QAPP.

4. Collect water for ammonia analysis and place the water in a pre-labeled storage container and refrigerate.

5. Collect water for PAH analysis and place in pre-labeled storage container and ship to ALS Environmental.

6. Harvest all fish from each tank and record state (alive or dead) at harvest.

7. Place dead fish in labeled weigh dish. If fish are alive, euthanize fish by placing them in 200 ppm of MS-222 anesthesia. Then place each fish in a labeled weigh dish.

8. Dab the fish dry with paper towels and individually weigh (± 0.01 g) and measure total length (mm) using calipers. Record the data on the appropriate bench sheets.

9. Prepare archive tissue samples according the QAPP by placing all fish from one treatment group into a single sample container. Record sample information in the *Analytical Sample Inventory Bench Sheet* in the QAPP.

7. University of Miami Rosenstiel School of Marine and Atmospheric Science General Laboratory Procedures and Practices

7.1 Methods

All fish experiments, except for ones performed on yellowfin tuna, were conducted with embryos or larvae from broodstocks of fish maintained at the Rosenstiel School of Marine and Atmospheric Science (RSMAS), University of Miami, Miami, Florida. Copepod experiments were performed with copepod cultures purchased from AlgaGen (Vero Beach, FL), and maintained at RSMAS.

7.1.1 Test organism sources and husbandry – cobia, mahi-mahi, and yellowfin tuna

This section describes the sources and husbandry for test organisms used for toxicity tests, which were conducted with cobia, mahi-mahi, and tuna. Currently, the University of Miami Experimental Hatchery (UMEH) maintains broodstock populations of cobia stocked in semi-recirculating maturation tanks at the UMEH facility on Virginia Key, Florida.

Cobia

The primary group of F1 broodstock cobia (*Rachycentron canadum*) were stocked in an 80-ton semi-recirculating maturation system at the UMEH facility (Benetti et al., 2008b). Additional broodstock fish were maintained in 15-ton flow-through holding tanks. For cobia, a sex ratio of 1:1 (male:female) was maintained.

Fertilized eggs were obtained by allowing the fish to spawn in the tanks. When natural spawns were obtained from conditioned fish, fertilized eggs were harvested from the egg collectors outside the maturation tanks and incubated in 1-ton cylindrical-conical tanks with flow-through filtered seawater and constant aeration. Eggs were disinfected using 100 ppm formalin (Paracide) for 1 hour during the first cleavages. After hatching, yolk-sac larvae were transferred to the larval rearing tanks using 15-L buckets with water to avoid the use of nets.

Larval rearing: The intensive methods used for larval rearing described previously (Benetti, 1997; Benetti et al., 1998; Feeley and Benetti, 1999; Watanabe et al., 2005) have been refined and implemented at the UMEH (Benetti et al., 2007, 2008a, 2008b). These larval rearing techniques used ultraviolet (UV)-sterilized flow-through seawater ranging in temperature from 24°C to 32°C and a daily turnover rate of 100–800%. Newly hatched larvae were stocked at 5–

10/L. The standard protocol consisted of live feeds added 3–5 times a day as needed to maintain proper concentrations of microalgae (*Isochrysis galbana* at 10,000 cells/mL), rotifers (*Brachionus plicatilis* at 5/mL), and *Artemia* (at 0.1–1.0/mL). Cobia larvae were fed *ad libitum* using a pulse feeding technique. Microalgae and rotifers were added between 2 and 10 days post-hatch (DPH), with *Artemia* added from 7 DPH through 22 DPH. From 16 to 23 DPH, the post-larvae were weaned onto starting diets. Larvae were sampled daily and dissected under a microscope to look for any signs of pathogenic organisms. For information about acceptable control performance and other test success criteria, refer to the specific testing protocols for individual tests.

Mahi-mahi

Mahi-mahi (*Coryphaena hippurus*) broodstock were collected from coastal waters off Miami using hook and line fishing equipment. Once caught, fish were held in 250-gal cylindrical transport tanks mounted on the fishing vessel, and water quality was maintained by pumping in fresh seawater.

Upon arrival at UMEH, fish were quarantined for 5 to 7 days. As part of this process, the fish were weighed, measured, tagged for identification (ID) purposes, and sampled for assessment of sexual maturity (Benetti et al., 2007). All fish were injected with 1.5 mL of 100 mg/mL oxytetracycline to mark otoliths for posterior age and growth ID purposes, and to prevent infections that may occur as a result of bruising and stress during capture, transport, and handling (Wexler et al., 2003). Selected broodfish received prophylactic treatments according to standard quarantine protocols used at the UMEH for a variety of pelagic fish (Benetti et al., 2008a). The fish were treated for 1 hour each in a solution of 100 ppm formalin, or treated in a 200 ppm formalin bath for 2–5 minutes followed by a freshwater bath for 5–10 minutes. The freshwater/formalin treatments removed any ectoparasites from the gills and skin of the fish that may have accompanied the fish from the wild and that could later persist and proliferate after transfer to the maturation tank (Benetti et al., 2007). At times, a low dose of clove oil or MS-222 (10 ppm) was used during the freshwater bath to anesthetize the fish and ensure easier handling (Benetti et al., 2007, 2008a). After completion of the 5–7 day quarantine, the fish were stocked in maturation tanks.

Sex ratios (male:female) in nearly all mahi-mahi broodstock tanks were maintained between 1:1 and 1:4. In general, the mahi-mahi spawned in the early morning; fertilized embryos were collected by 8:00–10:00 a.m. and were ready to use by 12:00 p.m. The approximate developmental stage of embryos was the late blastula period or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

When natural spawns were obtained from conditioned fish, fertilized eggs were harvested from the egg collectors outside the maturation tanks and incubated in 1-ton cylindrical-conical tanks with flow-through filtered seawater and constant aeration. Eggs were disinfected using 100 ppm

formalin (Paracide) for 1 hour during the first cleavages. After hatching, yolk-sac larvae were transferred to the larval rearing tanks using 15-L buckets with water to avoid the use of nets.

Yellowfin tuna

Yellowfin tuna (*Thunnus albacares*) embryos were obtained from the standing broodstock of the Achotines Laboratory in Las Tablas, Panama. Embryos were collected within an hour of spawning and were treated in formalin for 1 hour followed by a 15-minute rinse before being placed in exposure chambers. Tests were conducted on-site at Achotines.

7.1.2 Test organism sources and husbandry – *Acartia tonsa* copepods

In addition to pelagic fish, the calanoid copepods *Acartia tonsa,* (*A. tonsa*) were used to investigate the toxicity of *Deepwater Horizon* (DWH) oil. Live adult *A. tonsa* were purchased from AlgaGen (Vero Beach, FL) and transferred to 1-gal glass jars filled with 0.45-µm filtered seawater at the same temperature and salinity as the water the copepods were shipped in (typically 25 ppt and 24°C); the jars were gently aerated at approximately 1 bubble per second. Copepods were held at a density of one animal per 10 mL of water (Gentile et al., 1976). Salinity was adjusted in the jars at a rate of 4 ppt/d (Gentile et al., 1976) until all jars were at 35 ppt. Copepods were fed 1 x 10^3 cells/mL (Gentile et al., 1976) of *Isochrysis galbana* (TISO) and *Chaetocerous muelleri* (CHGRA). To set up synchronous cultures, mature adults were collected and transferred to a 2-L dish in which a generation cage was submerged (Gentile et al., 1976). The generation cage used a 100-µm filter screen that allowed copepod eggs to pass through, while retaining cannibalistic adults. After 24 hours, the eggs were collected and used to start a synchronous culture where all animals were the same age. For mass production, *A. tonsa* were grown in three 3,000-L static semi-continuous cultures in uncovered tanks outside and fed live TISO at concentrations of 40,000 to 100,000/cells per mL. Water temperature was maintained between 24°C and 30°C, and salinity was typically maintained at 20 ppt; these conditions assisted with better egg production: 17–40 eggs/day/female or 4–9 million eggs/day, assuming a 1:1 male to female ratio. For the oil exposures, a salinity of 30 ppt was used, so before tests began, the culture was slowly acclimated to this higher salinity. Two 250-L tanks were used as the hatching tanks for nauplii production. Live algae (TISO and CHGRA) were added to reach a concentration of 50,000 cells/mL at a 1:1 ratio, providing a stronger nutritional profile in the nauplii.

To maintain live algae cultures for feeding to copepods, primary microalgae inocula were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Working stocks were aseptically transferred weekly to fresh f/2 media (Guillard, 1975) and prepared with autoclaved 32 ppt ambient seawater. Stocks were aerated and housed at 22°C under continuous light from a single, cool-white fluorescent bulb. Cell concentrations were determined by hemocytometer/fluorometer.

7.1.3 Test organism sources and husbandry – Gulf toadfish

Finally, Gulf toadfish (*Opsanus beta*) were used to investigate the effects of DWH oil on adrenal function and stress response. Toadfish were collected from Biscayne Bay, Florida, using commercial shrimp trawlers (Florida Fish and Wildlife Conservation Commission Special Activity License #SAL-12-0729-SR). Upon arrival, toadfish were treated with 10 L of distilled water and 500 mL of seawater for 5 minutes, followed by a treatment of formalin (15 mg/L in seawater. Toadfish were held in 60-L glass aquaria with recirculated, aerated seawater (32 ppt) and maintained at approximately 20–25°C for at least seven days before the start of experimentation. Toadfish were fed raw squid weekly.

7.1.4 Exposure media preparations

Seawater used for all exposure studies at RSMAS was obtained from RSMAS' supply of natural seawater from Biscayne Bay, which has been sand-filtered and monitored for proper dissolved oxygen (DO), pH, temperature, and salinity.

Exposures took place in accord with the testing protocols and test conditions tables (TCTs) specific for each species and test. In all cases, water accommodated fractions (WAFs) of oil were prepared as outlined in the standard operating procedure (SOP) *Protocols for Preparing Water Accommodated Fractions* found in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3.

Generally, three different WAF preparations were tested: high-energy, low-energy, and chemically enhanced WAFs (HEWAF, LEWAF, and CEWAF, respectively) using the following four oil types: Slick A (CTC02404-02), Slick B (GU2888-A0719-OE701), source oil (072610-03), and weathered source oil (072610-W-A), see test-specific TCTs for details. In addition, Corexit 9500-only exposures were performed as a definitive test (dose-response). In all cases, control waters were prepared as the WAFs but without the addition of oil.

7.1.5 Water quality monitoring

See the QAPP and TCTs for required monitoring.

7.1.6 Analytical chemistry sampling

See the QAPP, related testing protocols, and TCTs for sampling and processing of water and tissue collected for chemical analyses.

7.2 Reporting and Testing Documentation

Reporting and testing documentation was done as outlined in the QAPP.

7.3 Water Quality Protocols – General Laboratory SOPs

7.3.1 Fluorescence measurements

Measuring oil in water using fluorescence spectroscopy SOP

This protocol is adapted from the *Standard Operating Procedure – Fluorescence Spectroscopy to Verify Dilutions of Water Accommodated Fraction for Toxicity Testing* available in the QAPP. The modifications herein are due to the difference of equipment between RSMAS and Queen's University, Kingston, Ontario. *Note:* Prior to any definitive testing, the emission and excitation settings were established for source and weathered source oil, with identical settings being optimal. The excitation wavelengths were typically 224 and 258 nm, with emission ranges of 275–425 nm and 275–475 nm, respectively. Definitive scans were still performed to verify these ranges for all definitive tests.

Determining excitation and emission wavelengths of sample oil

1. Turn on the LS-45 fluorescence spectrometer at least 1 hour prior to use. The lamp for the LS-45 turns on automatically with the machine. Turn on the computer and open the FL Winlab software package.

2. To deduce the excitation and emission wavelengths, run an excitation scan. This scan monitors the emission spectrum over a range of excitation wavelengths. To run an excitation scan, click on the scan.mth method on the startup page of FL Winlab. Once inside the scan method, click the multiple scan icon on the top toolbar (rainbow-colored spectral icon). Under setup parameters, select the excitation tab. Set the scan range from 200–500 nm and set the initial emission value to 200 nm. Under the 3D scan range heading, set the number of scans to 15 and the emission increment to 20 nm, which will result in an emission range of 200–500 nm. Input the results file name at the bottom. Name the file with the test ID# and the letters "es" (emission scans).

Note: The resulting files consisted of one .sp3 file, which is a 3D representation of all 15 scans. The software automatically adds a #01 to the end of the file name. Each individual scan was also saved as a .sp file, starting with the number #01 and ending at #15.

3. Load sample (see sample preparation section below) and press the start scan button (traffic light icon with the green light highlighted). Perform this scan with an intermediate-high WAF dilution. The scan takes approximately 30–45 minutes.

4. To evaluate the excitation and emission spectrums, open the main FL Winlab window and click the data handling tab on the toolbar. Select 3D view and open the test .sp3 file. Two background emission spectra are apparent on the 3D view, which are the result of the excitation light and not the sample. Typical scans have shown two dose-dependent peaks at approximately 224 and 258 nm. To identify the exact wavelength of the respective peaks, click on the excitation cut icon (last icon in the toolbar), and place the red line over the center of the peak. The vertical cut will appear in a new window with the excitation wavelength. To determine the emission wavelength range, look at the range of emission peaks in the excitation vertical cut window. Do not incorporate any of the background peaks into the emission range.

5. Once the excitation and emission wavelengths have been verified, proceed to standard curve and sample analysis.

Standard preparation and standard curve

Note: The procedures regarding sample and standard storage are also described in the QAPP, testing protocols, and RSMAS General Laboratory Procedures and Practices (GLPP).

1. Preserve all samples and standards immediately in 50% ethanol, and store at 4°C until analyzed. It is important that the curve be made in duplicate, with one set for analysis and the other for archive. Label samples as describe in the QAPP.

2. Make the standard curve during the test setup.

3. Perform standards curve dilutions in bulk test seawater to a 10 mL volume, after which add 10 mL of ethanol (ex. 10 ppt standard = 10 µL of HEWAF, 9.99 mL of seawater, 10 mL ethanol). It is best practice to serially dilute the following series: 100 ppt, 50 ppt, 10 ppt, 5 ppt, 1 ppt, 0.5 ppt, 0.1 ppt, and 0.05 ppt. Vortex the WAF/standard prior to proceeding to the subsequent dilution step. Not all standards are necessary, depending on the working range of the test.

4. The working range of the LS-45 is relatively small. For example, the detectable working range for preliminary weathered source HEWAF is 10 to 0.1 ppt. It is possible to change the detection sensitivity by changing the emission attenuator; however, a standard curve and samples must be performed using the same attenuator. Usually only 4–6 samples of the serial dilution curve can be measured. Any test samples that do not fit into this standard curve must be diluted appropriately.

5. To analyze standards, first sonicate at low intensity for 3 minutes. Make sure to sonicate on ice, as the sample will get extremely hot if not on ice. Following this, transfer 3 mL of the sample to two 1.5 mL microcentrifuge tubes and spin at 10,000 RPM for 10 minutes, which will remove salts. Carefully pipette the clear water layer from both microcentrifuged tubes into a pre-cleaned quartz cuvette. Do not pipette any of the precipitated salt.

6. Place the standard into the LS-45 and open a scan method in FL Winlab. Under the scan method, make sure the single wavelength scan is highlighted (this is the default setting). Click the single excitation wavelength tab and enter the appropriate excitation wavelength and emission wavelength range (as previously determined by the user). Also enter the desired file name. Name the file for standard scans with the test ID# followed by "sc" (standard curve), followed by the dilution (i.e., 333sc10). Scan all standards as well as the seawater blank. Between replicates, clean the quartz cuvette with Simple Green and distilled water, and dry with a Kimwipe.

7. To analyze the data, go to the main page of FL Winlab and click the data handling tab and select area. Multiple files can be opened at once with this tool, so open all standards and the blank. The tool allows for optimizing the analysis wavelengths within this window; however, the conditions should be optimized so the entire read will be analyzed. Ensure that the emission wavelengths at the bottom of the window reflect this. Click analyze; a new text window will open with the areas under the curve within the respective windows. Record the values on the run data sheet. This file does not need to be saved, as it is readily reproducible from the saved raw data file.

8. To calculate the standard curve, first subtract the blank value from all standards (using Excel). Plot the values as an x-y scatter graph, with the peak fluorescence area values on the x-axis and the WAF concentration (ppt) on the y-axis. After plotting the graph, click on one data point and then right-click. Select add trend-line from the menu. Ensure that the trend-line is linear and that the options to show R^2 and equation are selected. Record both the R^2 and curve equation on the data sheet. Save this Excel spreadsheet (test ID# followed by data analysis). Ensure that the raw input numbers are double-checked against the data sheet by a second individual.

9. If the R^2 value is below 0.95, the procedure should be repeated from the beginning.

Test replicate analysis

1. The steps for analyzing unknowns are the same as steps 5–7 of the standards, including sonicating and centrifugation procedures. The standard curve is valid only over the working range of the serial dilutions, so if values come out higher than the curve, they must be diluted. If a value comes in lower than the curve, the curve must be extended on

the low end. A sample that comes in lower than the standard curve in practice cannot be differentiated from zero.

2. All unknowns files (.sp files) must be saved in duplicate, with one as an unopened archive file and one as a working file in the test folder. Name all files with the test ID#, replicate chamber number, and day (e.g., 333.T7.4 = test 333, test chamber 7, day 4).

3. Use the same procedure as for the standards to analyze the area under the curve. Record all values on the data sheets.

4. To calculate the actual value of oil (WAF) in the test replicate, open the test data analysis spreadsheet and input the raw unknown values. Using Excel, input the calculated area into the standard curve calculation (x-value) and solve the equation. Record all these values on the data sheet. Make sure that all numbers and the standard curve equation are double-checked by another individual. Once the final analysis is complete, save a second copy of the spreadsheet in the archive folder.

5. The final values are in ppt WAF, but this will be converted to units of total polycyclic aromatic hydrocarbon (PAH) by RSMAS personnel after the mass spectroscopy analysis is provided by ALS Environmental.

Note: At the end of the test, all data sheet files were checked by a third person against the raw data. This included checking the data sheet values against the area calculation .txt file, and checking that all values in Excel were input on the data sheets and that the standard curve calculation in Excel was correct. After the data were triple-checked, a second copy of the Excel spreadsheet was saved in the archive folder. This file was not accessed after this point.

7.3.2 Temperature measurement SOP

1. Measure temperature using a standard laboratory thermometer (VWR; 61028-080).
2. Place thermometer in solution and wait for 1 minute. Record value.
3. Clean the thermometer with absolute ethanol and distilled water between measurements.

7.3.3 Measurement of salinity SOP

1. Measure salinity using a refractometer (VWR SW series, #12777-992). Using a glass Pasteur pipette, place one or two drops of test solution on the measurement window and close the lid. Point toward the light, keep the refractometer level.

2. Record value.

7.3.4 pH measurement SOP

1. Measure pH using a combination glass electrode coupled to a PHM220 pH meter (Radiometer). Calibrate the meter each day according to the automated manufacturer procedures and using IUPAC buffers for pH 7 and 10 (expected sample pH of approximately 8.1).

2. Perform calibration in the environmental chamber and with temperature-acclimated buffer solutions.

3. Record the calibration performance on the log, with acceptable calibration at values at or above 95%.

4. For testing, immerse the electrode into the center of the test beaker and allow it to equilibrate for 1 minute, record the value. Clean the electrode between samples using Simple Green cleaning solution and distilled water.

7.3.5 Measurement of total ammonia SOP

Note: All samples that were tested for total ammonia were stored at -20°C until assayed. All water samples from a given toxicity test were tested for ammonia at the same time using the same standards. If multiple plates were needed, the same diluted standards were used; however, each plate was treated as a distinct assay that contained a standard curve. This took into account variation in development time between plates.

1. Measure total ammonia using the colorimetric assay described by Verdouw et al. (1978).

2. Run the assay on a flat-bottomed polystyrene 96 well plate (Model # 9017; Corning), with each sample/standard run in triplicate.

3. Prepare the standard curve from an acidified 50 mM stock solution of ammonium sulfate. Make a 0, 10, 25, 50, and 100 uM dilution series using seawater.

4. Add 160 µL of each standard/unknown to the respective wells.

5. Using a repeat pipette, add 20 µL of sodium salicylate solution to each well. Sodium salicylate solution = 40 g of sodium salicylate/80 mL Milli-Q. Make fresh daily.

6. Using a repeat pipette (different tip), add 20 µL of catalyst citrate solution to each well. Catalyst citrate solution = 0.02 g, sodium nitroprusside, 35 g sodium citrate, volume to 100 mL using Milli-Q water. Protect the solution from light, and store in the refrigerator.

7. Using a repeat pipette (different tip), add 20 µL of alkaline hypochlorite solution to each well. Alkaline hypochlorite solution = 4 g NaOH, 14 mL sodium hypochlorite, volume to 100 mL using Milli-Q. Store solution in the refrigerator.

8. Allow to develop for at least 1 hour and no more than 24 hours in a dark place at room temperature.

9. Read samples at 650 nm using the microplate spectrophotometer. Standard curve should have an $R^2 \geq 0.95$. Because the assay is run in triplicate, it is acceptable to remove obvious outlying assay replicates, but make careful notes of any removed outliers. Keep all raw data files, and also record all mean replicate ammonia values on data sheets, as well as the standard curve R^2 value.

7.3.6 Measurement of DO SOP

1. Measure DO using a hand-held YSI ProODO DO meter and probe (#626279).

2. Calibrate the oxygen meter daily. Fill out the calibration logs daily.

3. Calibration:

   a. *Salinity*: Because the solubility of oxygen in water decreases as salinity increases, it is important to input the test salinity into the meter prior to calibration and use. To input the salinity value into the meter, push the probe button and select salinity from the menu. Enter the appropriate value. Input the mean value of the measured salinity for the test replicates (salinity should not vary much between replicates). Record the calibration salinity used in the calibration log.

   b. *Temperature*: This meter does not need temperature calibration, although the meter reading should be recorded on the calibration log.

   c. *One-point calibration in water-saturated air*: This procedure is as described in the manufacturer's manual. Moisten the calibration sponge with a small amount of water, and ensure that no water droplets are present on the sensor/temperature caps; do not immerse the sensors in water. Place sensors in calibration sleeve and allow to equilibrate for 5 minutes. Press calibration button, highlight DO%, and press enter. Highlight the barometer value and press enter. (Although the value can be changed, the value measured by the meter should be used. If the barometer value is off, then a barometric pressure calibration should be performed.) Record the barometric pressure value on the calibration log. Wait for temperature and DO% values to stabilize, highlight accept calibration, and press enter. No

calibration efficiency rating is provided, but the user should check off on the calibration log that the meter was calibrated.

d. *Barometric pressure*: This value does not need to be routinely calibrated. If the value appears to be inaccurate, press the calibrate button and select barometer. Input an acceptable value and select calibration.

4. To test the DO in the test replicates, first set the meter to auto-stabilize by pressing probe and selecting auto-stable (the sensitivity should be set to the middle value, which allows a data variance of 1.275%). Place probe in the sample and briefly move to release air bubbles from the sensor. Continued movement is not necessary due to the optical luminescent measurement. Allow value to stabilize and record value on the data sheet.

5. Between replicates, clean the probe using Simple Green cleaning solution followed by distilled water.

7.3.7 WAF and toxicity test water disposal procedures SOP

1. Dispose of all oil-containing solutions by draining through an activated charcoal bed, with outflow leading into a sink drain at the University of Miami.

2. Remove any solid pieces (weathered oil) from the charcoal bed after use and place in a waste vessel stored in a fume hood.

3. At the completion of the project, dispose of both the activated charcoal bed and solid waste under the chemical safety and disposal regulations of the University of Miami.

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A. Testing Protocol 1: Yellowfin Tuna (*Thunnus albacares*) Embryo Acute Toxicity Test – Static Exposure

Note: In general, the animals spawn in the evening and are available for testing a few hours after fertilization. The approximate developmental stage of these eggs is the late blastula or early gastrula period. Embryos that have entered the shield stage were deemed too old for embryonic testing.

Prior to beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test seawater collected at the Achotines Laboratory was filtered down to 0.35 µm, UV-sterilized, and temperature-matched to the water in which the eggs were collected prior to use.

A.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the evening of embryo collection, while CEWAFs should be prepared 24 hours prior and allowed to settle the evening of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C, record all necessary sample numbers, and prepare necessary chain-of-custody (COC) documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

3. Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. The test medium is UV-sterilized seawater. Seawater volume should be measured using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add WAF. Preform dilution of WAF to treatment concentrations in bulk, with enough volume for all treatment replicates and ALS Environmental sample analysis (see test-specific TCTs). Add both water and WAF volumes to 5-L aspirator bottle(s) equipped with a closed-valve Tygon tubing outflow. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels either through the tubing outflow or by decanting, as the

user deems appropriate. Note that at least 50 mL of volume should be allowed to drain from the outflow tubing before sampling. Collect 250 mL of each bulk solution, acidify with hydrochloric acid (HCl), and ship on ice to ALS Environmental, as described in the QAPP.

4. Take initial measurements of water temperature, pH, DO, and salinity, within each test chamber, as outlined in the *Temperature measurement SOP*, *pH measurement SOP*, *Measurement of DO SOP***,** and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Also remove a 5-mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

5. Collect embryos as soon as possible after a spawn, recording the time.

6. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo's quality. At a 45x magnification, the developing embryonic morphology should be clearly discernible. Discard any embryos that look unfertilized or malformed. Since more embryos are obtained than the number needed for a test, it is best to remove any embryos that are at all questionable.

7. Transfer 20 embryos into each test replicate in random order. Because embryos are too large for Pasteur pipettes, use a glass eye dropper to count the embryos. Count the embryos both as they come out of the eye dropper and after they have all been transferred. Between test replicates, rinse the eye dropper briefly with Simple Green and distilled water.

8. After all replicates are set up, cover exposure chambers with glass panes or inverted glass petri dishes to limit evaporation.

9. Observe and record survival, mortality, missing, and non-treatment mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Remove and archive dead animals unless they have decomposed and ensure that all COC documentation is filled out as described in the QAPP. Retain all dead animals to the extent possible and archive according to the QAPP. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP. Make the final survival count after 72 hours. Retain all remaining live and dead animals, to the extent possible, and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue samples, tissue samples were sometimes stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that all dead animals were not counted or collected. An animal was considered dead if absent from the test chamber unless there was evidence of non-treatment mortality, such as jumping from the tank.

Note: Hatching mortality was recorded, and was defined as the number of animals that died before hatch or immediately post-hatch.

10. Take final measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Take a 5-mL sample for final PAH quantification by fluorescence spectroscopy as described in the RSMAS GLPP. Also remove a 5 mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out.

11. Remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anaesthetize all surviving animals with sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers drop-wise until the animals stop moving. Collect animals and archive as outlined in the QAPP.

12. Discard remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

13. The test will be said to pass test criteria if the average control survival exceeds 70% across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

B. Testing Protocol 2: Yellowfin Tuna (*Thunnus albacares*) Embryo Acute Toxicity Test – Static Recirculating

Note: In general, the animals spawn in the evening and eggs are collected within 2 hours of the spawning event. The approximate developmental stage of the eggs at the initiation of testing is the late blastula or early gastrula period.

Prior to beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test seawater collected at the Achotines Laboratory was filtered down to 0.35 µm, UV-sterilized, and temperature-matched to the water in which the eggs were collected prior to use.

B.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the evening of embryo collection, while CEWAFs should be prepared 24 hours prior and allowed to settle the afternoon of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Acidify each sample with several drops of concentrated HCl to increase holding time. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

B.1.1 Embryo exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone

using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Seawater volume should be measured using a graduated cylinder. Because oil/PAHs can adhere to plastic, only glass graduated cylinders or Hamilton syringes should be used to add WAF. Dilution of WAF to treatment concentrations should be performed in bulk, with enough volume for all replicates and ALS Environmental sample analysis (see test-specific TCTs).

Add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times. Dispense an equal volume from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8-L total). Collect 250 mL of each bulk solution and ship on ice to ALS Environmental for extraction and archiving, as described in the QAPP. Discard unused WAF according to the procedures in the *WAF and toxicity test water disposal procedures SOP* in the RSMAS GLPP.

B.1.2 Initial water quality measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber as outlined in the *Temperature measurement SOP*, *pH measurement SOP*, *Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

B.1.3 Embryo collection and experimental setup

1. Collect embryos the evening of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35 µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish

and improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation levels throughout the treatment period. Following the 1 hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the exposure room (set to 27°C) at the Achotines Laboratory for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 20–60 embryos into each test replicate using a large-bore Pasteur pipette (see test-specific TCTs).

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Replicates are maintained in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP and RSMAS GLPP.

B.1.4 Larvae collection and measurements

1. Following hatch [approximately 24–36 hours post-fertilization (hpf)], collect larvae for imaging or preserve in RNAlater for potential future RNA extraction. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, then quickly turning the pump off. Disconnect tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining. Proceed with imaging as outlined in Testing Protocol 4 of the RSMAS GLPP, *Assessment of Crude Oil Cardiotoxicity in Yellowfin Tuna* (Thunnus albacares) *Yolk-sac Larvae – Static Recirculating Exposure*. Observe mortality visually and verify by prodding the animal with a pipette. Remove and archive numbers of dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality. Yellowfin tuna were not large enough for cannibalism at this life stage, and the static test setup made this a valid conclusion.

2. Take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

3. Anesthetize all surviving animals with clove oil by adding a concentrated solution to the exposure beakers. Collect and archive the animals as outlined in the QAPP.

4. Discard remaining test solutions as outlined in the *WAF and the toxicity test water disposal procedures SOP* in the RSMAS GLPP.

5. The test will be said to pass the test criteria if the average control survival meets or exceeds 70% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

C. Testing Protocol 3: Assessment of Crude Oil Cardiotoxicity in Yellowfin Tuna (*Thunnus albacares*) Yolk-Sac Larvae – Static Exposure

These tests were performed in collaboration with the Northwest Fisheries Science Center/University of Miami RSMAS/Hopkins Marine Station of Stanford University.

C.1 WAF Preparation

1. Prepare the WAFs in advance according to *the Protocols for Preparing Water Accommodated Fractions* SOP, as described in the RSMAS GLPP. HEWAFs should be made the evening of embryo collection, while CEWAFs should be prepared 24 hours prior and allowed to settle the evening of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

C.2 Embryo Exposure

Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. Measure seawater/WAF treatment volume using a graduated cylinder. Decant seawater/WAF into all replicate beakers for each treatment (see test-specific TCTs for number of replicates).

1. Transfer 20 fertilized embryos, screened at 2–3 hpf (~ 64-cell stage), into each exposure vessel using wide-bore glass pipettes.

2. Place vessels on a reciprocating shaker in a temperature controlled room set at 27°C.

3. Expose embryos until hatch (~ 24 hours at 27°C).

4. Monitor water quality and record before hatch. Water quality monitoring includes measurements of pH, DO, salinity, ammonia, and temperature.

C.3 Day 2: Morphological Assessment after Hatch

Image and process larvae in a temperature controlled room set to the same temperature as the exposure room (27°C).

A. Digital videomicroscopy

1. Randomly select a beaker (replicate) from one of the treatment groups to be processed via Steps 2–8 below. Once finished, randomly select another beaker from one of the remaining treatment groups and process. Repeat, until one replicate from each treatment group has been processed, then start with the second replicate from each treatment group, and so on until all beakers have been processed.

2. To anesthetize the fish, add 10 µL of clove oil to 10 mL of seawater in a 15-mL conical tube and shake vigorously by hand for 45 to 60 seconds. Add clove oil water to the treatment vessel and allow to sit for 5 minutes, and then gently and briefly stir with a glass pipette.

3. Capture two or three larvae at a time using a wide-bore glass pipette.

4. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

5. Image all the larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

6. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Unibrain Fire-i400 1394 camera connected via firewire to a laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame.

    a. Video capture – take a 10-second video at the highest magnification (6.3x). Focus on cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

    b. Image capture – capture composite images of the entire larvae for three fish per beaker (5x magnification).

7. After imaging, use a wide-bore glass pipette to transfer larvae from methyl cellulose to clean seawater with 10-ppm clove oil. Repeat process of capturing and mounting two or three larvae at a time until all larvae from the treatment vessel have been imaged, then move on to next beaker.

8. Save and copy files to two back-up hard drives.

9. Process images according to the NWFSC GLPP.

C.4 RNA Preservation (RNAlater)

1. Label tubes using standardized nomenclature.

2. Post-imaging, transfer larvae from methyl cellulose into a petri dish of clean seawater with 10-ppm clove oil.

3. Using a wide-bore glass pipette, collect larvae from a replicate into a microcentrifuge tube.

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Add 1 mL of RNAlater.

6. Store sample tubes at 4°C.

7. Record samples on bench sheet and fill out COC form.

8. Pack samples for shipment and store at 4°C until shipping.

9. Follow shipping guides written in the QAPP.

Day 2 alternative: Collecting the majority of the replicates for RNA preservation

A. Digital videomicroscopy

1. Randomly select one replicate per treatment group.

2. To anesthetize the fish, add 10 µL of clove oil to 10 mL of seawater and shake vigorously by hand for 45 to 60 seconds. Add clove oil water to the treatment vessel and allow to sit for 5 minutes, then gently and briefly stir with a glass pipette.

3. Capture two or three larvae at a time using a wide-bore glass pipette.

4. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

5. Image all larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

6. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Fire-i400 1394 camera connected via firewire to a laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame.

    a. Video capture – take a 10-second video at the highest magnification (6.3x). Focus on cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

    b. Image capture – capture composite images of the entire larvae for three fish per beaker (5x magnification).

7. After imaging, discard larvae.

8. Save and copy files to two back-up hard drives.

9. Process images according to the NWFSC GLPP.

B. RNA preservation (RNAlater)

1. Label tubes using standardized nomenclature.

2. For the remaining replicates that were not imaged, anesthetize the fish using the same protocol above.

3. Using a wide bore glass pipette, collect anesthetized larvae from a replicate into a microcentrifuge tube.

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Add 1 mL of RNAlater.

6. Store sample tubes at 4°C.

7. Record samples on bench sheet and fill out COC form.

8. Pack samples for shipment and store at 4°C until shipping.

9. Follow shipping guides written in the QAPP.

C.5 RNA Sequencing Analysis[1] – Performed at the National Center for Genomic Research

RNA sequencing was performed at the National Center for Genome Research in Santa Fe, New Mexico. Total RNA samples were processed by taking an aliquot for quality control (QC) analysis to determine the amount sent and the integrity of the RNA using Qubit and Bioanalyzer, respectively. All samples passed QC and were made into sequencing libraries using the Illumina TruSeq RNA Sample Preparation Kit. Total RNA went through poly-A selection reaction in which the mRNA is pulled down using poly-T oligo attached to magnetic beads. The pulled-down mRNA is fragmented and randomly primed in a one-step reaction. The randomly primed mRNA is then taken through first strand synthesis using reverse transcriptase enzyme. The product then undergoes second strand synthesis using a second strand master mix that contains DNA polymerase I and RNase H. The synthesized second strand is end repaired using End Repair Mix (converts overhangs generated from fragmentation into blunt ends), follow by the addition of an A-base on the 3' end of the double-stranded cDNA molecule. The addition of A-base prepares it for the ligation of Illumina adapters that has a T-base on its 3' end. After the ligation of sample with uniquely barcoded adapters, the resulting product was taken through 15 cycles of PCR amplification. A QC is then performed on the Nanodrop for the amount and the Bioanalyzer for fragment size determination of the library, and also to check for any adapter dimers.

1. Protocol applies to definitive test 543 only.

D. Testing Protocol 4: Assessment of Crude Oil Cardiotoxicity in Yellowfin Tuna (*Thunnus albacares*) Yolk-Sac Larvae – Static Recirculating Exposure

These tests were performed in collaboration with the Northwest Fisheries Science Center/ University of Miami RSMAS/Hopkins Marine Station of Stanford University.

D.1 WAF Preparation

1. Prepare the WAFs in advance according to *the Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the evening of embryo collection, while CEWAFs should be prepared 24 hours prior and allowed to settle the evening of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental, as specified in the QAPP. Acidify each sample with several drops of concentrated HCl to increase holding time. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C, record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

D.1.1 Embryo exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, only glass graduated cylinders or Hamilton syringes should be used to add WAF. Perform dilution of WAF to

treatment concentrations in bulk, with enough volume for all replicates and ALS Environmental sample analysis (see test-specific TCTs).

Add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times. Dispense an equal aliquot from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8-L total). Collect 250 mL of each bulk solution and ship on ice to ALS Environmental, as described in the QAPP. Discard unused WAF according to the procedures in the *WAF and toxicity test water disposal procedures SOP* in the RSMAS GLPP.

1. Collect embryos the evening of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone, and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35 µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation levels throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, and remove them using a soft mesh net and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the exposure room (set to 27°C) at the Achotines Laboratory for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate using a large-bore Pasteur pipette.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Replicates are maintained in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

D.1.2 Larvae collection and measurements

Following hatch (approximately 24–36 hpf), collect larvae for imaging or preserved in RNAlater for potential future RNA extraction. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening the stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining.

D.2 Day 2: Morphological Assessment after Hatch

Image and process larvae in a temperature-controlled room set to the same temperature as the exposure room (27°C).

A. Digital videomicroscopy

1. Randomly select a beaker (replicate) from one of the treatment groups to be processed by steps 2–8 below. Once finished, randomly select another beaker from one of the remaining treatment groups and process. Repeat, until one replicate from each treatment group has been processed, then start with the second replicate from each treatment group, and so on until all beakers have been processed.

2. To anesthetize the fish, add 10 µL of clove oil to 10 mL of seawater in a 15-mL conical tube and shake vigorously by hand for 45 to 60 seconds. Add clove oil water to the treatment vessel and allow to sit for 5 minutes, and then gently and briefly stir with a glass pipette.

3. Capture two or three larvae at a time using a wide-bore glass pipette.

4. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

5. Image all larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

6. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Unibrain Fire-i400 1394 camera connected via firewire to a laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame.

    a. Video capture – take a 10-second video at the highest magnification (6.3x). Focus on the cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

    b. Image capture – capture composite images of the entire larvae for three fish per beaker (5x magnification).

7. After imaging, use a wide-bore glass pipette to transfer larvae from methyl cellulose to clean seawater with 10 ppm clove oil. Repeat process of capturing and mounting two or three larvae at a time until all larvae from the treatment vessel have been imaged.

8. Save and copy files to two back-up hard drives.

9. Process images according to the NWFSC GLPP.

D.3 RNA Preservation (RNAlater)

1. Label tubes using standardized nomenclature described in the QAPP.

2. Post-imaging, transfer larvae from methyl cellulose into a petri dish of clean seawater with 10 ppm clove oil.

3. Using a wide-bore glass pipette, collect larvae from a replicate into a microcentrifuge tube.

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Add 1 mL of RNAlater.

6. Store sample tubes at 4°C.

7. Record samples on the bench sheet and fill out the COC form.

8. Pack samples for shipment and store at 4°C until shipping.

9. Follow shipping guides written in the QAPP.

Day 2 alternative: Collecting the majority of the replicates for RNA preservation

A. Digital videomicroscopy

1. Randomly select one replicate per treatment group.

2. To anesthetize the fish, add 10 µL of clove oil to 10 mL of seawater and shake vigorously by hand for 45 to 60 seconds. Add clove oil water to the treatment vessel and allow to sit for 5 minutes, and then gently and briefly stir with a glass pipette.

3. Capture two or three larvae at a time using a wide-bore glass pipette.

4. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

5. Image all larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

6. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Fire-i400 1394 camera connected via firewire to a laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and the dorsal to the top of the frame.

    a. Video capture – take a 10-second video at the highest magnification (6.3x). Focus on cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

    b. Image capture – capture composite images of the entire larvae for three fish per beaker (5x magnification).

7. After imaging, discard larvae.

8. Save and copy files to two back-up hard drives.

9. Process images according to the NWFSC GLPP.

B. RNA preservation (RNAlater)

1. Label tubes using standardized nomenclature described in the QAPP.
2. For the remaining replicates that were not imaged, anesthetize the fish using the same protocol above.
3. Using a wide-bore glass pipette, collect anesthetized larvae from a replicate into a microcentrifuge tube.
4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.
5. Add 1 mL of RNAlater.
6. Store sample tubes at 4°C.
7. Record samples on the bench sheet and fill out the COC form.
8. Pack samples for shipment and store at 4°C until shipping.
9. Follow shipping guides written in the QAPP.

E. Testing Protocol 5: Embryo Acute Toxicity Test: Mahi-Mahi (*Coryphaena hippurus*) and Cobia (*Rachycentron canadum*)

Note: In general, mahi-mahi spawn in the early morning, and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. In general, cobia spawn in the early evening and eggs are collected by 6:00–8:00 p.m. and are ready to use by 12:00 a.m. The approximate developmental stage of these eggs is the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Prior to beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test water should be obtained from the UV-sterilized seawater system at UMEH.

E.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the morning or evening of embryo collection, while LEWAFs and CEWAFs should be prepared 18-24 hours prior and the latter allowed to settle the morning or evening of embryo collection.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples overnight on ice to ALS Environmental as soon as possible.

3. Use the bulk WAF solution to make the standard curve dilution series described in the RSMAS GLPP. This dilution series should include a seawater blank and span the working range of WAF to be used in the test. Ensure that all documentation is complete.

4. Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add WAF. Perform dilution of WAF to treatment

concentrations in bulk, with enough volume for all treatment replicates and ALS Environmental sample analysis. Add both water and WAF volumes to a 5-L aspirator bottle equipped with a closed-valve Tygon tubing outflow. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels either through the tubing outflow or by decanting, as the user deems appropriate. Note that at least 50 mL of volume should be allowed to drain from the outflow tubing before sampling. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental as described in the QAPP. Discard unused WAF according to the procedures in the *WAF and toxicity test water disposal procedures SOP* in the RSMAS GLPP.

E.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature measurement SOP*, *pH measurement SOP*, *Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Take 5 mL for initial PAH quantification, as described in the RSMAS GLPP. Also remove a 1.5-mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

E.3 Embryo Collection and Experimental Setup

1. Cobia – collect embryos the evening of the spawn (within 2 hpf; approximately 8:00 p.m.) and place in 100 ppm formalin for 1 hour.

   Mahi-mahi – collect embryos the morning of a spawn and place in 100 ppm formalin for 1 hour.

   Formalin acts as an antifungal/antibacterial agent increasing embryo survival. Rinse the embryos with three chamber volumes of UV-treated seawater (approximately 30 minutes) and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryos' quality. At 45x magnification, the differentiating cells should be discernible. Discard any embryos that look unfertilized or malformed. Because the embryos are obtained well in excess of what is needed for a test, it is best to remove any embryos that are at all questionable.

3. Transfer 20 embryos into each test replicate in random order. Because embryos are too large for Pasteur pipettes, use a glass eye dropper to count out the embryos. Count the embryos both as they come out of the eye dropper and after all have been transferred. Between test replicates, rinse the eye dropper briefly with Simple Green and distilled water.

4. After all replicates are set up, cover exposure chambers with glass panes to limit evaporation. Replicates are maintained in the environmental control chamber at 27°C with 16:8 light/dark photoperiod.

E.4 Measurements

1. Observe and record survival, mortality, missing individuals, and non-treatment mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Remove and archive dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP. Perform daily measurements of water chemistry and physical parameters, as outlined in QAPP and RSMAS GLPP. Make the final survival count after 96 hours. Retain all remaining live and dead animals to the extent possible and archive according the QAPP.

Note: Because attempts to separate water and dead animals most likely resulted in destruction of the tissue sample, tissue samples were sometimes stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that all of the dead animals were not counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality, such as jumping from the tank. Cobia and mahi-mahi are not large enough for cannibalism at this life stage, and the static test setup made this a valid conclusion.

Note: Hatching mortality was recorded, and was defined as the number of animals that died before or immediately post-hatch.

2. Take final measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the RSMAS GLPP. Take 5 mL for final PAH quantification by fluorescence spectroscopy, as described in the RSMAS GLPP. Also remove 5 mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out.

3. Remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anesthetize all surviving animals with sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers drop-wise until the animals stop moving. Collect and archive the animals as outlined in the QAPP.

4. Discard remaining test solutions as outlined in *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

5. The test was said to pass the test criteria if the average control survival exceeded 70% across the four replicates, and if there was no unacceptable deviation in water quality among replicates, as described in the QAPP and RSMAS GLPP.

F. Testing Protocol 6: Morphological Assessment of Crude Oil Cardiotoxicity in Mahi-Mahi (*Coryphaena hippurus*) Yolk-Sac Larvae

These tests were performed in collaboration with the Northwest Fisheries Science Center/ University of Miami RSMAS/Hopkins Marine Station of Stanford University.

F.1 Day 0: Exposure

1. Prepare WAF and dilutions according to the QAPP.
2. Transfer 1 L of each WAF dose into a 1-L glass beaker.
3. Transfer 20 or 40 fertilized eggs into the 1-L glass beaker.
4. Expose embryos for 48 hours.

F.2 Day 2: Morphological Assessment after Hatch

Digital video microscopy:

1. Randomly select a beaker (replicate) from one of the treatment groups to be processed in Steps 2–8 below. Once finished, randomly select another beaker from one of the remaining treatment groups and process. Repeat, until one replicate from each treatment group has been processed, then start with the second replicate from each treatment group, and so on until all beakers have been processed.

2. For each beaker, without anesthetic capture two or three larvae at a time using a plastic transfer pipette or a wide-bore glass Pasteur pipette.

3. Mount larvae in 2% methyl cellulose in seawater (~ 35 ppt).

4. Image all larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

5. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereomicroscope fitted with a phototube and Unibrain Fire-i400 1394 camera connected via firewire to laptop with BTV Pro. Magnifications for imaging will be identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame. In some earlier tests, iMovie was used for image acquisition at one microscope station due to issues with the operating system on the available MacBook Pro laptop.

a. Image capture

   i. At 3x, focus on cardiac/pericardial region and frame it from the head to the posterior of the yolk sac.
   ii. At 3x, for two or three larvae per beaker, take composite shots of the entire larvae.

b. Video capture – 10-second video clips of the heart and pericardial area are captured for each larva at the highest magnification (6.3x).

6. After imaging, use a wide-bore glass pipette to transfer larvae from methyl cellulose to clean seawater. Repeat procedure until all larvae from the beaker have been imaged. Once all larvae from a replicate have been imaged and transferred to clean water, fix larvae in paraformaldehyde (PFA) or snap freeze (see below).

7. Save and copy files to two back-up hard drives.

8. Process image according to the NWFSC GLPP.

F.3 Fixation

1. Label 1.7-mL microcentrifuge tubes using standardized nomenclature.

2. Post-imaging, transfer larvae from methyl cellulose into a petri dish of clean seawater.

3. Using a plastic transfer pipette or a wide-bore glass pipette, transfer and pool larvae of one replicate into a microcentrifuge tube (i.e., one microcentrifuge tube per beaker).

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Add 1.5 mL 4% Millonig's buffered PFA to tube and cap tube.

6. Fix overnight at 4°C or at room temperature for 4 hours.

7. After fixation, wash embryos into phosphate buffered saline (PBS) with one fast wash and then several longer washes (minimum three); store at 4°C.

8. Wash larvae into methanol with 10-minute washes of methanol/PBS.

   a. 25% methanol
   b. 50% methanol
   c. 75% methanol

d. 100% methanol
e. Store at -20°C.

9. Record samples on bench sheet and fill out COC form.

10. Pack samples for shipment and store at 4°C until shipping.

11. Follow shipping guides written in the QAPP.

F.4 Cryopreservation

1. Label vials using standardized nomenclature described in the QAPP.

2. Post-imaging, transfer larvae from methyl cellulose into a petri dish of clean seawater.

3. Using a plastic transfer pipette or a wide-bore glass pipette, transfer and pool larvae of one beaker into a cryo vial (i.e., one cryo vial tube per beaker).

4. Remove excess liquid using a plastic transfer pipette fitted with a 20-µL pipette tip.

5. Immediately place sample tube in liquid nitrogen.

6. Store samples at -80°C.

7. Record samples on bench sheet and fill out COC form.

8. Pack samples for shipment and store at 4°C until shipping.

9. Follow shipping guides written in the QAPP.

G. Testing Protocol 7: Juvenile (~ 30 - 45 DPH) Mahi-Mahi (*Coryphaena hippurus*) Swim Performance Following Acute Embryonic Exposure

Prior to beginning exposures, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP.

G.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs are made the morning of embryo collection, while CEWAFs are prepared 24 hours prior and allowed to settle the morning of embryo collection.

2. Perform acute embryo exposures in 2,500-L cylindrical fiberglass tanks at UMEH with a total test solution volume of approximately 2,200 L. The test medium is natural, filtered, and UV-treated seawater. Because oil/PAHs can adhere to plastic, use glass graduated cylinders to add WAF. Fill treatment tank with seawater and add WAF under inflow at approximately one-half to two-thirds final volume to achieve adequate mixing, and continue to fill to final volume. Use a single exposure chamber for each concentration.

3. After exposure chambers are filled and all WAF has been added, collect an initial water sample from the control and treatment tanks for chemical analysis by ALS Environmental as specified in the QAPP. Collect a water sample from each tank at 24 and 48 hours after WAF addition. Collect samples taken 48 hours after WAF preparation just prior to initiating flow-through conditions. Collect additional water at 24 and 48 hours following initiation of flow-through conditions from each treatment tank. Send all water samples for chemical analysis by ALS Environmental. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Ship samples overnight on ice to ALS Environmental as soon as possible.

G.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature measurement SOP, pH measurement SOP, Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in RSMAS GLPP. See respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

G.3 Embryo Exposure

1. Expose embryos for 48 hours under static conditions with light aeration (see test-specific TCTs for details). Make initial egg counts using a volumetric method, whereby eggs are allowed to settle in oxygen-saturated water under static conditions in a graduated cylinder or beaker. Non-viable eggs will sink out under these conditions and be removed, so as to ensure that all embryos used in the trials are viable. From the mass of floating (i.e., viable) eggs, remove three 1-mL subsamples and count to obtain an egg-per-mL count. Obtain the final number of embryos using the mean egg-per-mL count and multiplying by the total volume (mL) of floating eggs. Thus each tank will be stocked with a specific volume of eggs to obtain the desired stocking density (see test-specific TCTs for details). Make effort to stock similar densities of embryos in control and treatment tank. Following the 48-hour exposure period, return flow of clean seawater to all tanks and maintain under flow-through conditions with gentle aeration for the remainder of the experiment until the fish reach adequate size for swimming (approximately 30–45 days, depending on rearing temperature). During this growout period, rear fish at UMEH using hatchery protocols.

2. During the 48-hour oil exposure, perform daily measurements of water quality and physical parameters, as outlined in RSMAS GLPP and test-specific TCTs. Remove 1.5 mL water sample for ammonia analysis daily for first 48 hours, and store at -20°C until assayed.

3. Keep notes throughout the larval rearing process, indicating any major drops in the number of larvae in each tank and being careful to note the time and date when these drops occurred. Additionally, note any signs of health impairment or distress should these conditions occur during the growout period.

G.4 Swimming Performance

1. Once fish are approximately 30–45 DPH, select fish to be used for swimming experiments at random using a slow scooping motion from below the fish in a way that avoids detection and stress. Transfer collected fish in buckets filled with seawater from the UMEH to RSMAS and hold in an environmental chamber overnight without access to food until ready to initiate experiments.

2. Every morning prior to swimming experiments, calibrate each swim tunnel setup for oxygen concentration and flow velocity. Open the AutoResp2 software. Oxygen is calibrated by locking a "high" value using vigorously aerated water (typically 1 hour) and by locking a "low" value using a 10 g/L solution of sodium sulfite. Calibrate flow velocity by adjusting the voltage using the speed-dial on the motor controller and entering the predetermined corresponding "high" and "low" velocity values for each voltage. Adjust water temperature to 27°C using a submersible aquarium heater. Record calibration values on Swim Tunnel Calibration and Data Collection Table.

3. Collect each fish by gently corralling into a small transfer vessel (e.g., 500 mL beaker) and place into one of four swim chambers (every attempt should be made to randomize selection of swim chamber). Measure mass and total body length (BL) post-swimming to minimize handling stress.

4. Once placed in the swim tunnel, monitor the fish using a small video camera connected to a computer, as well as manual adjustments to swim speed remotely in an area partitioned off from the respirometer to avoid disturbing the fish. Record and measure oxygen concentrations continuously with a computer using a Pt100 fiber-optic probe connected to the Fibox 3 minisensor oxygen meter. Collect temperature readings simultaneously using a separate probe.

5. Perform automated intermittent flow respirometry using up to four miniature Blazka-type variable speed respirometers with a DAQ-M control device and the AutoResp2 software.

6. Initiate a low flow velocity within the swim tunnel (~ 1 cm/second) to maintain mixing within the chamber without inducing exercise, and allow the fish to acclimate for a minimum of 4 hours.

7. To measure *U*crit, exercise fish at 20-minute intervals beginning with a flow rate of approximately 4 cm/second followed by consistent increments in flow every interval (~0.5 BL/second) until the fish is exhausted. Exhaustion is designated as when the fish either rests on its caudal fin against the back screen of the tunnel or becomes pinned against the back screen and does not regain activity after briefly decreasing flow and then

returning to the last speed achieved. Record the duration (T, in seconds) at the final swim speed. Calculate the *U*crit (in cm/second) using the following equation: *U*crit = [Vf + (T/t)dV]/cm, where *t* is the time interval (20 minute), *dV* is the increment in swim speed, and Vf is the final velocity maintained for a full interval. Upon completion of the swimming experiment, remove and weigh the fish and measure total BL to transform *U*crit values to BL per second.

8. Euthanize fish according to the University of Miami animal care protocol using an overdose of MS-222. Record mass and length.

9. Archive each animal at completion of swim trial and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP.

10. Clean each swim tunnel system in its entirety daily with a mix of mild detergent (Simple Green) and 10% bleach and rinse thoroughly two or three times with deionized water.

11. Discard remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

H. Testing Protocol 8: Sub-Adult Mahi-Mahi (*Coryphaena hippurus*) Swim Performance Following Acute Sub-Adult Stage Exposure

Prior to beginning exposures, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP.

H.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the evening prior to sub-adult fish exposure.

2. Perform sub-adult fish exposures in 2,500-L cylindrical fiberglass tanks at UMEH with a total test solution volume of approximately 360–900 L. The test medium is natural, filtered, and UV-sterilized seawater. Fill treatment tank with seawater. After treatment tank is filled, turn on the circulation pump to generate a directional current within the treatment tank for the ram ventilating fish species. Add WAF to treatment tank (do not expose control fish to WAF but otherwise treat as the exposed fish). Because oil/PAHs can adhere to plastic, use glass graduated cylinders to add WAF. Use a single treatment tank for each concentration.

3. Following a short period of circulation (~ 5 minutes) to achieve adequate mixing, collect an initial water sample from the control and treatment tanks for chemical analysis by ALS Environmental as specified in the QAPP. Collect additional water samples at 24 hours after WAF addition from each treatment tank. Send all water samples for chemical analysis by ALS Environmental. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

H.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature measurement SOP, pH measurement SOP, Measurement of DO*

SOP, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. See respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

H.3 Sub-Adult Exposure

1. Expose one sub-adult fish (0.15–1 kg) for 24 hours under static conditions (i.e., no new water inflow) with light aeration. Generate a directional water flow in the treatment and control chambers using a small pump to recycle the treatment media. Following the 24-hour exposure period, transfer the fish to the swim tunnel.

2. During the 24-hour oil exposure, perform measurements of water quality and physical parameters, as outlined in the QAPP and RSMAS GLPP. Remove a 1.5-mL water sample for ammonia analysis at the start and end of the 24-hour exposure period and store at -20°C until assayed.

3. Keep notes throughout the exposure period indicating any loss of fish in control and treatment tanks. Additionally, note any signs of health impairment or distress should these conditions occur during the exposure period.

H.4 Swimming Performance

1. Sub-adult fish used for swimming experiments are either hatchery-raised or from wild populations. Use only fish that are deemed to be in good health for swimming experiments, and select individuals randomly from the available fish at the RSMAS aquaculture facility. Feed fish on the day of their removal from their holding tanks, and withhold food from individuals during the exposure period.

2. Remove fish from their holding tanks (2,500–8,000 L each) using soft mesh nets, a water-filled vinyl sling, or water-filled plastic bags depending on the size being captured. Select fish randomly using a slow scooping motion from below the fish in a way that avoids detection and stress.

3. Calibrate the swim tunnel setup for oxygen concentration and flow velocity every morning prior to swimming experiments. Open the AutoResp2 software; oxygen is calibrated by locking a “high” value using vigorously aerated water (typically 20 minutes) and by locking a “low” value using a 10 g/L solution of sodium sulfite. Flow velocity is calibrated by adjusting the voltage/RPM using the speed-dial on the motor

controller and entering the predetermined corresponding "high" and "low" velocity values for each voltage. Record water temperature (ambient) for calibration purposes and throughout the experiment. The swim tunnel will have filtered and UV-sterilized water flowing through the buffer chamber for the duration of the experiment to eliminate build-up of metabolic wastes. Water temperature will either be the ambient temperature of the RSMAS aquaculture facility or will be controlled by an inline heat pump to maintain a specific temperature (see test-specific TCTs). Record calibration values on the Swim Tunnel Calibration and Data Collection Table.

4. Transfer collected fish to the swim tunnel following treatment using the aforementioned methods (nets, sling, or bags). Generate a slow water current in the swim tunnel to replicate the water flow in the treatment tank from which the fish came. Place fish into the test section of the swim tunnel and secure the lid. Keep the test section on a "flush" cycle until fish become acclimated to the swim tunnel, during which time the fish will be swimming at a slow and steady speed. Confirm acclimation by the occurrence of two consecutive routine oxygen consumption (MO_2) readings of similar value at a level known to be indicative of low-exertion swimming.

5. Measure mass, total BL, and fork BL post-swimming to minimize handling stress.

6. Once placed in the swim tunnel, monitor the fish using two small video cameras connected to a computer, as well as manual adjustments to swim speed remotely in an area partitioned off from the respirometer to avoid disturbing the fish. Record and measure oxygen concentrations continuously with a computer using a Pt100 fiber-optic probe connected to the Fibox 3 minisensor oxygen meter. Collect temperature readings simultaneously using a separate probe.

7. Perform automated intermittent flow respirometry using a 90-L swim tunnel variable-speed respirometer with a DAQ-M control device and the AutoResp2 software.

8. Initiate a low flow velocity within the swim tunnel (~ 1–3 BL/second) to maintain mixing within the chamber without inducing exercise, and allow the fish to acclimate for a minimum of 1 hour or longer if necessary until two consecutive 20-minute measurements of MO_2 are approximately the same.

9. To measure *U*crit, exercise fish at 20-minute intervals beginning with a flow rate of approximately 80 cm/second (depending on size of test organisms) followed by consistent increments in flow every interval until the fish is exhausted. Exhaustion is designated as when the fish either rests on its caudal fin against the back screen of the tunnel or becomes pinned against the back screen and does not regain activity after briefly decreasing flow and then returning to the last speed achieved. Record the duration

(T, in seconds) at the final swim speed. Calculate the *U*crit (in cm/second) using the following equation: *U*crit = [Vf + (T/t)dV]/cm, where *t* is the time interval (20 minute), *dV* is the increment in swim speed and Vf is the final velocity maintained for a full interval. Upon completion of the swimming experiment, remove and weigh the fish and measure total BL to transform *U*crit values to BL per second.

10. At the conclusion of the test, either sedate using MS-222 and transfer to a recovery tank or euthanize according to the University of Miami animal care protocol using an overdose of MS-222; mass and length should be recorded.

11. Archive each animal at completion of swim trial and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP.

12. Clean the swim tunnel system in entirety after each testing period using a 10% bleach solution and rinse thoroughly two or three times with clean tap water. Drain all water from the swim tunnel system following each testing period to allow for drying of system components.

13. Discard remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

I. Testing Protocol 9: Juvenile Mahi-Mahi (*Coryphaena hippurus*) Swim Performance Following Acute Juvenile Exposure

Prior to beginning exposures, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP.

I.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. HEWAFs should be made the morning of the juvenile fish exposure.

2. Perform juvenile fish exposures in 20-L glass jars with a total test solution volume of approximately 12 L. The test medium is natural, filtered, and UV-sterilized seawater. Because oil/PAHs can adhere to plastic, use glass graduated cylinders or Hamilton syringes to add WAF. After adding WAF to the seawater, mix by gently stirring the solution using a Teflon coated stir rod.

I.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature measurement SOP, pH measurement SOP, Measurement of DO SOP*, and *Measurement of salinity SOP*, respectively, after filling and prior to adding test organisms. Also, collect initial samples for total PAH analysis by ALS Environmental and store samples at 4°C. All SOPs can be found in the RSMAS GLPP. See respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

I.3 Juvenile Exposure

1. Collect juveniles just after first feeding so that the animals are starved for at least 24 hours prior to swimming, and immediately transfer to the University of Miami environmental chamber (set to 27°C or an alternative temperature, as specified in the test-specific TCT) for exposure set-up. Juveniles should be transferred in at least 15 L of pre-aerated saltwater.

2. Transfer juveniles into each replicate chamber in random order 24 hours before transfer to swim tunnels (see test-specific TCTs for total number of individuals). Note that exposure initiation may be staggered to accommodate the lag time (timing effect) associated with swim performance tests.

3. After all replicates are set up, cover exposure chambers with a glass lid to limit evaporation. Maintain exposures in the environmental control chamber at 27°C with 16:8 light dark photoperiod and gentle aeration with an air stone.

4. Note that some WAF exposure durations may be less than the actual 24 hour holding time (e.g., the second 12 of the 24 hours). In such cases, fish will be held in a chamber containing clean seawater for a given time period and then transferred to another chamber containing the desired WAF dilution for the remaining time period. Initial and final measurements for water temperature, pH, salinity, and DO will be collected from the WAF dilution exposures, as outlined in the QAPP and RSMAS GLPP. Initial and final samples for total PAH analysis by ALS Environmental will also be collected and stored at 4°C. Alternatively, exposures may occur within the actual swim tunnel apparatus following a 24 hour holding period in clean seawater in the environmental chamber as described above. Such exposures will be initiated just following the introduction of the fish to the swim tunnel by adding the appropriate volume of WAF directly to the surrounding swim tunnel reservoir and mixed well with a glass stir rod to obtain the desired WAF dilution. Exposures will proceed during the acclimation period (typically 4 hours) and continue through the swim trial itself. Initial and final measurements for pH and salinity will be collected from the swim tunnel reservoir, as outlined in the QAPP and RSMAS GLPP (temperature and DO are measured continuously in the swim tunnel using the Fibox system used for measuring respiration). Initial and final samples for total PAH analysis by ALS Environmental will also be collected from the swim tunnel reservoir and stored at 4°C. Total PAH samples should be shipped on ice to ALS Environmental as soon as possible.

5. Observe and record mortality daily. Observe mortality visually and verify by prodding the animal with a Teflon coated stir rod. Remove and archive dead animals and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP.

6. Take final measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Also remove 1.5-ml water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out. Collect final samples for total PAH analysis by ALS Environmental and store at 4°C. Ship samples on ice to ALS Environmental as soon as possible.

I.4 Swimming Performance

1. Every morning prior to swimming experiments, calibrate each swim tunnel setup for oxygen concentration and flow velocity. Open the AutoResp2 software. Oxygen is calibrated by locking a "high" value using vigorously aerated water (typically 1 hour) and by locking a "low" value using a 10 g/L solution of sodium sulfite. Calibrate flow velocity by adjusting the voltage using the speed-dial on the motor controller and entering the predetermined corresponding "high" and "low" velocity values for each voltage. Adjust water temperature to 27°C using a submersible aquarium heater. Record calibration values on Swim Tunnel Calibration and Data Collection Table.

2. Collect each fish by gently corralling into a small transfer vessel (e.g., 500 mL beaker) and place into one of four swim chambers (every attempt should be made to randomize selection of swim chamber). Measure mass and total BL post-swimming to minimize handling stress.

3. Once placed in the swim tunnel, monitor the fish using a small video camera connected to a computer, as well as manual adjustments to swim speed remotely in an area partitioned off from the respirometer to avoid disturbing the fish. Record and measure oxygen concentrations continuously with a computer using a Pt100 fiber-optic probe connected to the Fibox 3 minisensor oxygen meter. Collect temperature readings simultaneously using a separate probe. For hypoxia exposures (see test-specific TCTs), only measure oxygen concentrations during the acclimation period. Because the oxygen levels are likely very near the survival threshold, and measuring oxygen consumption requires a closed interval during which ambient oxygen is depleted by the fish, conduct the hypoxia swim trials with a continuous flush cycle and no additional oxygen measurements.

4. Perform automated intermittent flow respirometry using up to four miniature Blazka-type variable speed respirometers with a DAQ-M control device and the AutoResp2 software.

5. Initiate a low flow velocity within the swim tunnel (~ 1 cm/second) to maintain mixing within the chamber without inducing exercise, and allow the fish to acclimate for a minimum of 4 hours. For hypoxia exposures, slowly decrease oxygen levels to the desired level (~ 37.5% O_2 saturation) during the acclimation period by gently bubbling nitrogen gas into the reservoir water surrounding the swim chamber (see test-specific TCTs).

6. To measure *U*crit, exercise fish at 20-minute intervals beginning with a flow rate of approximately 4 cm/second followed by consistent increments in flow (~0.5 BL/second) every interval until the fish is exhausted. Exhaustion is designated as when the fish either rests on its caudal fin against the back screen of the tunnel or becomes pinned against the

back screen and does not regain activity after briefly decreasing flow and then returning to the last speed achieved. Record the duration (T, in seconds) at the final swim speed. Calculate the *U*crit (in cm/second) using the following equation: *U*crit = [Vf + (T/t)dV]/cm, where *t* is the time interval (20 minute), *dV* is the increment in swim speed and Vf is the final velocity maintained for a full interval. Upon completion of the swimming experiment, remove and weigh the fish and measure total BL to transform *U*crit values to BL per second.

7. Euthanize fish according to the University of Miami animal care protocol using an overdose of MS-222. Record mass and length.

8. Archive each animal at completion of swim trial and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP.

9. Clean each swim tunnel system in its entirety daily with a mix of mild detergent (Simple Green) and 10% bleach and rinse thoroughly two or three times with deionized water.

Discard remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

J. Testing Protocol 10: Mahi-Mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Exposure to WAF – Static Recirculating

Note: In general, mahi-mahi spawn in the early morning and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. The approximate developmental stage of these eggs is the late blastula or early gastrula period.

Prior to beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP. All test water should be obtained from the UV sterilized seawater system at UMEH.

J.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP and RSMAS GLPP. For tests designated as an "unsettled HEWAF exposure" make the following modifications concerning Step C (Separation) in the *Protocols for Preparing Water Accommodated Fractions* SOP in the QAPP. For all other WAF exposures proceed to Step 2.

    a. Make HEWAFs the morning of the exposure initiation and do not allow them to settle before use. Instead, maintain the HEWAF under agitation for 1 hour on the stir plate before use.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice to ALS Environmental as soon as possible.

3. Use the bulk WAF solution to make the standard curve dilution series described in the RSMAS GLPP. This dilution series should include a seawater blank and span the working range of WAF to be used in the test. Ensure that all documentation is complete.

J.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

J.3 Embryo Collection and Experimental Setup

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour (Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions). Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate in random order using a large-bore Pasteur pipette. Embryos should be added 1 cm below the water surface to avoid the oil slick forming on surface.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Maintain replicates in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP and RSMAS GLPP.

6. For each treatment, at 96 hours (or final time point; see test-specific TCT), collect an equal volume sub-sample from each replicate beaker and composite. The composite sample volume must be at least 250 mL to ensure enough solution is available to fill the sample bottle for PAH analysis. Ship samples on ice overnight to ALS Environmental, as described in the QAPP.

J.4 Embryo Exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. The total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, only use glass graduated cylinders or Hamilton syringes to add WAF. Perform the dilution of WAF to treatment concentrations in bulk, with enough volume for all treatment replicates and ALS Environmental sample analysis (see test-specific TCTs).

Add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times. Dispense an equal volume from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8-L total). Collect 250 mL of each bulk solution and ship on ice to ALS Environmental, as described

in the QAPP. Discard unused WAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

J.5 Larvae Collection and Measurements

1. At end of test, take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

2. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, then quickly turning the pump off. Disconnect tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Record survival, mortality, missing individuals, and non-treatment mortality. Remove and archive numbers of dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP. All surviving animals should then be anaesthetized with sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222) and retained and archived according to the QAPP or preserved for possible histology analysis using a histological fixative (e.g., Zfix).

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality. Mahi-mahi were not large enough for cannibalism at this life stage, and the closed test setup made this a valid conclusion.

3. Discard remaining test solutions as outlined in the *WAF and the Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

4. The test will pass the test criteria if the average control survival meets or exceeds 80% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

K. Testing Protocol 11: Mahi-Mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Differential Exposure Intervals – Static Recirculating

Note: In general, mahi-mahi spawn in the early morning and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. The approximate developmental stage of these eggs is the late blastula or early gastrula period.

Prior to beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP. All test water should be obtained from the UV sterilized seawater system at the UMEH.

K.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP and RSMAS GLPP. HEWAFs should be made the morning of exposure initiation.

2. Use the bulk WAF solution to make the standard curve dilution series described in the RSMAS GLPP. This dilution series should include a seawater blank and span the working range of WAF to be used in the test. Ensure that all documentation is complete.

K.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

Additional temperature, pH, DO, and salinity measurements should be taken whenever WAF is switched to clean seawater or clean seawater to WAF. These measurements should be taken just following the washout or mixing period.

K.3 Embryo Collection and Experimental Setup

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour (Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions). Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate in random order using a large-bore Pasteur pipette.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Maintain replicates in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP and RSMAS GLPP.

K.4 Embryo Exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add WAF. Perform dilution of WAF to treatment concentrations should be performed in bulk, with enough volume for all treatment replicates and ALS Environmental sample analysis.

To prepare WAF dilutions, add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times (see test-specific TCT).

1. Depending on the test-specific conditions, different exposure intervals (e.g., 2, 6, 24 hours) are initiated at different time points during 96-hour tests (e.g., 0-, 24-, 48-. and 72-hour time points). Thus, tests are either started with WAF in the exposure chamber or they are started with clean seawater in the exposure chamber. See test-specific TCTs for details on exposure interval length and exposure starting point for each individual test.

2. If the WAF exposure interval is at the start of the test, bulk WAF dilutions should be prepared at the desired nominal treatment concentrations (i.e., 1x) per description above. Once dilutions are prepared, dispense an equal volume from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8 L total). After the specified exposure interval, replace the WAF with clean seawater by briefly stopping flow to the Imhoff cone. Once flow is stopped, connect tubing from the 1-L beaker to a pre-filled intake tube drawing from a large reservoir of fresh seawater. Place a second larger tube on the outflow spout to drain into a bucket for disposal. Return flow to the Imhoff cone and allow fresh seawater to flush the Imhoff cone for 0.5 hour. During this time, discard the water in the beaker, rinse and wipe the beaker clean, and add 1 L of fresh seawater. After the 0.5-hour flush period, return tubing from the Imhoff to the 1-L beaker and remove the outflow tubing to allow for over flow to the 1-L beaker as it was set up before. Replace water in the control replicates to ensure that handling stress does not impact survival.

3. Tests with exposures initiated at the later time points begin with embryos placed in control seawater for each replicate at 0 hour. At the specified time, add WAF to the exposure chamber by replacing the 1-L beaker of clean seawater with 1 L of WAF that is 1.8x the desired WAF treatment concentration. Pause the flow from the peristaltic pump and the stopcock closed (simultaneously) during the exchange. Once the beaker is replaced, reinitiate flow to the Imhoff cone, allowing the 1.8x WAF in the beaker to mix with the clean seawater in the Imhoff cone. It is expected that the WAF in the beaker will be fully mixed with the clean seawater in the Imhoff cone within 1 hour of initiating flow.

4. Collect 250 mL from each initial bulk solution (the 1x or the 1.8x WAF) as well as a 250-mL composite sample for each dilution at the end of the exposure period. For the tests where clean seawater is replaced with WAF, also collect a 250-mL composite sample for each dilution after a 1-hour mixing period (i.e., the period after which the beaker water and Imhoff cone water are expected to be completely mixed). Ship all samples on ice overnight to ALS Environmental for total PAH analysis as described in the QAPP. Discard all WAF solutions according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP*.

5. In addition, take a 10-mL composite sample from each treatment either after the 1-hour mixing period (when switching from clean seawater to WAF) or after the 0.5-hour washout period (when switching from WAF to clean seawater) for fluorescence measurements. Immediately dilute the 10-mL composite sample with 10 mL of ethanol for total PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and the *RSMAS GLPP*.

K.5 Larvae Collection and Measurements

1. At the end of the test, take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

2. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect the tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Record survival, mortality, missing individuals, and non-treatment mortality. Remove and

archive numbers of dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP. Euthanize all surviving animals with an overdose of sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222) and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. The volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality. Mahi-mahi were not large enough for cannibalism at this life stage, and the closed test setup made this a valid conclusion.

3. Discard remaining test solutions as outlined in the *WAF and the Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

4. The test will pass the test criteria if the average control survival meets or exceeds 80% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

L. Testing Protocol 12: 48-Hour Exposure for RNA Analysis: Mahi-Mahi (*Coryphaena hippurus*)

Note: In general, mahi-mahi spawn in the early morning, and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. The approximate developmental stage of these eggs is the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Prior to beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP. All test water should be obtained from the UV-sterilized seawater system at UMEH.

L.1 WAF Preparation

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP, as described in the QAPP. Prepare HEWAFs the morning of exposure initiation.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples on ice overnight to ALS Environmental as soon as possible.

3. Use the bulk WAF solution to make the standard curve dilution series described in the RSMAS GLPP. This dilution series should include a seawater blank and span the working range of WAF to be used in the test. Ensure that all documentation is complete.

L.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

L.3 Embryo Collection and Experimental Setup

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour (Formalin is a commonly used paracide to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions). Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation levels throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate using a large-bore Pasteur pipette.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Maintain replicates in a temperature-controlled room at 27°C with 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP and RSMAS GLPP.

L.4 Embryo Exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add WAF. Perform dilution of WAF to treatment concentrations in bulk, with enough volume for all replicates and ALS Environmental sample analysis.

Add both water and WAF volumes to 5-L glass aspirator bottles. Spin the solutions for 5 minutes on a stir plate with a 50% vortex. If more than 5 L of diluted WAF is needed for all the replicates of a treatment, dilute stock WAF into multiple 5-L glass aspirator bottles and mix solutions together by decanting back and forth between each bottle several times. Dispense an equal volume from each aspirator bottle into each replicate Imhoff cone/beaker combination (1.8-L total). Collect a composite 250-mL sample from each treatment and ship on ice to ALS Environmental as described in the QAPP. Discard unused WAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

L.5 Larvae Collection and Cryopreservation

1. Take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

2. Following hatch [approximately 48 hpf], collect larvae for cryopreservation for future RNA extraction. Halt pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining.

3. Concentrate larvae by gently pouring through a fine-mesh filter and then transfer to a petri dish by squirting seawater through the opposite side. Score survival. Collect all live larvae with a large bore transfer pipette and transfer to a 2-mL cryotube. Remove as much excess water as possible with a Pasteur pipette, close tube, and immediately transfer to a dewar filled with liquid nitrogen. Once all animals are collected, transfer tubes immediately from liquid nitrogen to -80ºC freezer. Ship samples to the National Oceanic and Atmospheric Administration's Northwest Fisheries Science Center overnight on dry ice.

4. Retain all dead animals to the extent possible and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. When possible, the volume was limited.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if absent from the test chamber, unless there was evidence of non-treatment mortality. Mahi-mahi are not large enough for cannibalism at this life stage, and the closed test setup made this a valid conclusion.

5. Discard remaining test solutions as outlined in the *WAF and the Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

6. The test will pass the test criteria if the average control survival meets or exceeds 70% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates, as described in the QAPP.

M. Testing Protocol 13: Mahi-Mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Differential Exposure Intervals – Static

Generally, mahi-mahi spawned in the early morning; eggs were collected by 8:00–10:00 a.m. and were ready to use by 12:00 p.m. The approximate developmental stage of these eggs was the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test water was filtered using UV-sterilized seawater.

M.1 LEWAF Preparation

1. Prepare the LEWAF 18–24 hours in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP.

2. Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. The test medium is filtered, UV-sterilized seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass graduated cylinders or Hamilton syringes to add LEWAF. Dilute LEWAF to desired treatment concentrations in bulk, with enough volume for all treatment replicates and for ALS Environmental sample analysis. Prepare LEWAF dilutions in a 5-L glass bottle and spin the solution for 5 minutes on a stir plate with a 50% vortex. Aliquot the solution into replicate/sample vessels by decanting. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental, as described in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling should be conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C, record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and the RSMAS GLPP. Ship samples overnight on ice to ALS Environmental as soon as possible. Discard unused LEWAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

M.2 Initial Water Quality Measurement

Take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature Measurement SOP, pH Measurement SOP, Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling the test chamber and before adding test organisms. All SOPs can be found in the RSMAS GLPP. Use 10 mL for initial PAH quantification by fluorescence spectroscopy, as described in the RSMAS GLPP.

M.3 Embryo Collection and Experimental Setup

1. Collect the embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain the water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water; place eggs in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a common paracide used to treat marine fish eggs collected from captive marine fish; it improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface; remove them using a soft mesh net and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryos' quality. At 45x magnification, the differentiating cells should be discernible. Discard any embryos that look unfertilized or malformed. Because the need for extra embryos is accounted for and excess embryos are on hand, it is best to remove any embryos that are at all questionable.

3. Transfer 20 embryos into each test replicate in random order using a wide-bore Pasteur pipette. Count the embryos, both as they come out of the pipette and after all have been transferred. Depending on the test, replicate beakers may have WAF treatment solutions or clean water at the start of the test. Exposure times to LEWAF will vary depending on the test and will involve one to two transfers to fresh treatment water (i.e., LEWAF dilution or clean seawater). The transfer of embryos (or larvae if hatched) to LEWAF or clean seawater should also be performed using a wide-bore Pasteur pipette. See test-specific TCTs for details on the exposure interval length and the exposure starting point for each individual test.

4. After appropriate duration, transfer the embryos from the WAF treatment solution to clean source water (or vice versa for tests started with clean water). See test-specific TCT for exposure duration and interval within the test.

5. If the exposure interval is in the middle of the test duration (e.g., during a 96-hour test embryos start out in clean water, get transferred to WAF at 24 hours, then exposed for 24 hours, and then transferred back to clean water from the final 48 hours), perform one more transfer of embryos to clean source water. See test-specific TCT for exposure duration and interval within the test.

6. Following set up or transfer of embryos, cover exposure chambers with glass lids to limit evaporation. Replicates are maintained in the environmental control chamber at 27°C with a 16:8 light/dark photoperiod.

M.4 Measurements

1. Observe and record survival, mortality, missing individuals, and non-treatment mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette.

2. Remove and archive dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP.

Note: Because attempts to separate water and dead animals most likely resulted in destruction of the tissue sample, tissue samples were sometimes stored with small amounts of test water. The volume of test water was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that all of the dead animals were not counted or collected. An animal was considered dead if it was absent from the test chamber, unless there was evidence of non-treatment mortality, such as jumping from the

tank. Cobia and mahi-mahi are not large enough for cannibalism at this life stage, and the static test set-up made this a valid conclusion.

Note: Hatching mortality was recorded, and was defined as the number of animals that died before or immediately post-hatch.

3. Collect a 250-mL composite of each LEWAF dilution at the end of the exposure period after embryos have been transferred out of each of the corresponding replicate vessels and ship the composite overnight on ice to ALS Environmental as described in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP. Store samples at 4°C, record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Ship samples overnight on ice to ALS Environmental as soon as possible.

4. Take daily measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the RSMAS GLPP. Initial measurements should also be taken for each transfer solution before transferring ring embryos. Also remove a 1-mL water sample for ammonia analysis at the end of the exposure and store it at -20°C until assayed. Ensure that all meter calibration logs are filled out.

5. At the end of the test, remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anesthetize all surviving animals with sodium bicarbonate buffered with MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers using a dropper until the animals stop moving. Collect, count, and archive the animals as outlined in the QAPP.

6. Discard remaining test solutions as outlined in *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

Note: A test was said to pass the test criteria if the average control survival exceeded 70% across the four replicates, and if there was no unacceptable deviation in water quality among replicates.

N. Testing Protocol 14: Mahi-Mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Different Exposure Intervals to Oil Slicks – Static

Generally, mahi-mahi spawned in the early morning; eggs were collected by 8:00–10:00 a.m. and were ready to use by 12:00 p.m. The approximate developmental stage of these eggs was the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test water was filtered using UV-sterilized seawater.

N.1 Oil Slick Preparation

1. Perform acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. The test medium is filtered, UV-sterilized seawater. Measure seawater volume using a graduated cylinder.

2. Oil slicks are prepared by evenly spreading 2 g of Slick A or Slick B oil around the internal circumference of a 2.5-inch diameter PVC pipe coupler fitting and suspending the pipe for 4 hours below the surface of the water so that the oil is completely submerged but near the water surface. At the end of 4 hours, the pipe is slowly removed and the embryos added carefully beneath the slick, as described in Section N.3.

N.2 Initial Water Quality Measurement

Before slick formation, take initial measurements of water temperature, pH, DO, and salinity within each test chamber, as outlined in the *Temperature Measurement SOP, pH Measurement SOP, Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and before adding test organisms. All SOPs can be found in the RSMAS GLPP.

N.3 Embryo Collection and Experimental Set-up

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Maintain water temperature throughout the entire collection, prophylactic treatment, and

rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This allows non-viable eggs to settle to the bottom, while viable eggs float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water before placing them in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a commonly used paracide used to treat marine fish eggs collected from captive marine fish; it improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, then remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test setup.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryos' quality. At 45x magnification, the differentiating cells should be discernible. Discard any embryos that look unfertilized or malformed. Because excess embryos are on hand, it is best to remove any embryos that are at all questionable.

3. Transfer 20 embryos into each test replicate in random order using a wide-bore Pasteur pipette. Count the embryos, both as they come out of the pipette and after all have been transferred. Transfer embryos by placing the tip of the Pasteur pipette several millimeters beneath the slick and then expelling embryos. Avoid disrupting the slick.

4. Exposure times to slick oil will vary depending on the test and will involve one to two transfers to fresh treatment water (i.e., from slick oil to clean water or from clean seawater to slick oil). Transfer of embryos (or larvae if hatched) to slick oil or clean seawater should also be performed using a wide-bore Pasteur pipette. See test-specific TCTs for details on exposure interval length and exposure starting point for each individual test.

5. Following set-up or transfer of embryos, cover exposure chambers with glass lids to limit evaporation. Replicates are maintained in the environmental control chamber at 27°C with a 16:8 light/dark photoperiod.

N.4 Measurements

1. Observe and record survival, mortality, missing individuals, and non-treatment mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette.

2. Remove and archive dead animals unless they have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP.

Note: Because attempts to separate water and dead animals most likely resulted in destruction of the tissue sample, tissue samples were sometimes stored with small amounts of test water. The test water volume was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that all of the dead animals were not counted or collected. An animal was considered dead if it was absent from the test chamber, unless there was evidence of non-treatment mortality, such as jumping from the tank.

Note: Hatching mortality was recorded, and was defined as the number of animals that died before or immediately post-hatch.

3. Take daily measurements of water temperature, pH, salinity, and DO for each treatment from a "dummy" beaker as outlined in the RSMAS GLPP. Do not take daily water quality measurements from actual exposure chambers, as this may disrupt the oil slick. Initial measurements should also be taken for each transfer solution before slick formation and transfer of embryos. Also remove 1 mL of water sample for ammonia analysis at the end of the exposure and store at -20°C until assayed. Ensure that all meter calibration logs are filled out.

4. At the end of the test, remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anesthetize all surviving animals with sodium bicarbonate buffered with MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers using a dropper until the animals stop moving. Collect, count, and archive the animals as outlined in the QAPP.

5. Discard remaining test solutions as outlined in *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

Note: The test was said to pass the test criteria if the average control survival exceeded 70% across the four replicates, and if there was no unacceptable deviation in water quality among replicates.

O. Testing Protocol 15: Mahi-Mahi (*Coryphaena hippurus*) Embryo Acute Toxicity Test Following Exposure to Oil Slicks – Static Recirculating

Generally, mahi-mahi spawned in the early morning; eggs were collected by 8:00–10:00 a.m. and were ready to use by 12:00 p.m. The approximate developmental stage of these eggs was the late blastula or early gastrula period. Embryos that entered the shield stage were deemed too old for embryonic testing.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. All test water was filtered using UV-sterilized seawater.

O.1 Oil Slick Preparation

1. Perform acute embryo toxicity tests in 0.8-L glass Imhoff cones with a total test solution volume of 1.8 L (includes a 1-L glass overflow beaker). The test medium is filtered, UV-sterilized seawater. Measure seawater volume using a graduated cylinder.

2. Oil slicks are prepared by evenly spreading 2 g of Slick A or Slick B oil around the internal circumference of a 2.5-inch diameter PVC pipe segment and suspending the pipe for 4 hours below the surface of the water so that the oil is completely submerged but near the water surface. Note that flow through the cones is paused during the 4-hour incubation period. At the end of 4 hours, the pipe is slowly removed and the embryos are carefully added beneath the slick, as described in Section O.3.

O.2 Initial Water Quality Measurement

Take initial measurements (following the 4-hour incubation period) of water temperature, pH, DO, and salinity from each overflow beaker, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP.

O.3 Embryo Collection and Experimental Setup

1. Collect embryos the morning of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater.

Maintain the water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature. Briefly supply aeration to the eggs in the collection bucket to saturate the water. Once saturation has been achieved, remove the air stone and allow the eggs to settle. This separation allows for non-viable eggs to settle to the bottom, while viable embryos float at or near the water surface. After approximately 15 minutes of settling, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35-µm-filtered and UV-sterilized water before placing them in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L. Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a common paracide used to treat marine fish eggs collected from captive marine fish; it improves embryo survival and hatch rate in laboratory conditions. Supply supplemental aeration at a low rate in the treatment vessel to maintain DO levels at saturation throughout the treatment period. Following the 1-hour treatment period, briefly remove the air stone from the vessel to allow the viable eggs to float at the surface, then remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the University of Miami environmental chamber (set to 27°C) for toxicity test set-up.

2. Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo quality. At 45x magnification, the developing embryonic morphology should be clearly discernible. Avoid collecting any embryos that look unfertilized or malformed.

3. Gently transfer 40 embryos into each test replicate in random order using a large-bore Pasteur pipette.

5. Transfer embryos by placing the tip of the Pasteur pipette several millimeters beneath the slick and then expelling the embryos. Avoid disrupting the slick.

4. After all replicates are set up, cover exposure chambers with large glass petri dishes to limit evaporation. Maintain replicates in a temperature-controlled room at 27°C with a 16:8 light/dark photoperiod.

5. Perform daily measurements of water chemistry and physical parameters from overflow beakers, as outlined in the RSMAS GLPP.

O.4 Embryo Exposure

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume is 1.8 L and is circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct the pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure seawater volume using a graduated cylinder.

1. Depending on the test-specific conditions, the water underlying the slick may be first replaced by flushing with clean seawater for 30 minutes before introducing the embryos. The flushing procedure is as follows: while the flow is paused, connect the tubing that is drawing from the 1-L beaker, to a valve attached to a pre-filled intake tube that is drawing from a large reservoir of fresh seawater. Place a second, larger tube on the outflow spout to drain into a bucket for disposal. Return the flow to the Imhoff cone and allow fresh seawater to flush the Imhoff cone for 0.5 hour. After the 0.5-hour flush period, return the tubing from the Imhoff to the 1-L beaker and remove the outflow tubing to allow for overflow to the 1-L beaker, as it was set up before.

2. Just before introducing the embryos, collect a 250-mL composite sample for each set of four replicates by sampling from the corresponding overflow spouts. Ship all samples on ice overnight to ALS Environmental for total PAH analysis, as described in the QAPP.

O.5 Larvae Collection and Measurements

1. At the end of the test, take final measurements for water temperature, pH, salinity, DO, and ammonia in each exposure chamber, as outlined in the RSMAS GLPP. Ensure that all meter calibration logs are filled out.

2. Halt the pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect the tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening the stopcock below the surface and allowing the beaker to slowly drain to a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff during draining. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette.

Record survival, mortality, missing individuals, and non-treatment mortality. Remove and archive numbers of dead animals unless the animals have decomposed, and ensure that all COC documentation is filled out as described in the QAPP and RSMAS GLPP. Retain all dead animals to the extent possible and archive according to the QAPP. Euthanize all surviving animals with an overdose of sodium bicarbonate buffered with MS-222 (2:1 mass of sodium bicarbonate to MS-222) and archive according to the QAPP.

Note: Because attempts to separate water and dead animals mostly resulted in destruction of the tissue sample, tissue samples may have been stored with small amounts of test water. The volume of test water was limited where possible.

Note: Because embryonic animals decay rapidly after death, it was likely that not all dead animals were counted or collected. An animal was considered dead if it was absent from the test chamber, unless there was evidence of non-treatment mortality. Mahi-mahi were not large enough for cannibalism at this life stage, and the closed test set-up made this a valid conclusion.

3. Discard remaining test solutions as outlined in the *WAF and the Toxicity Test Water Disposal Procedures SOP* in the RSMAS GLPP.

4. The test will be said to pass the test criteria if the average control survival meets or exceeds 70% of hatch across the replicates, and if there is no unacceptable deviation in water quality among replicates.

P. Testing Protocol 16: Copepod Acute Toxicity Test

Note: Experiments are set up using a synchronous culture, where all animals are the same age (within 24 hours). Adult copepods will be approximately 17 days old at test initiation.

Before beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP. Obtain all test water from the UV-sterilized seawater system at UMEH.

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP. Prepare HEWAF the morning of experimental setup.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental, as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be overnight shipped on ice to ALS Environmental as soon as possible.

Note: Samples collected for the State of Louisiana tasks must be sent with a separate COC form and in a different shipment from samples collected under tasks conducted under the National Oceanic and Atmospheric Administration (NOAA) contract.

3. Use the bulk WAF solution to make the standard curve dilution series described in *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS GLPP. This dilution series should include a seawater blank and span the concentration range of WAF to be used in the test. Ensure that all documentation is kept.

4. Perform acute toxicity tests in 250-mL glass beakers with a total test solution volume of 200 mL. The test medium is UV-sterilized, 0.45-µm filtered seawater. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, only glass graduated cylinders or Hamilton syringes should be used to add WAF. Perform dilution of WAF to treatment concentrations with enough volume for five treatment replicates and ALS Environmental sample analysis (total volume 1.5 L). Add both water and WAF volumes to a 2-L glass beaker. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels by decanting. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental for analysis, as described in the QAPP. Take 5 mL for initial PAH quantification, as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS

GLPP. Discard unused WAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP*.

5. Add 200 mL of WAF treatment or clean source water to replicate beakers.

6. Set up one non-treatment beaker as described above, without oil, and monitor for representative water quality daily, including dissolved oxygen, pH, and salinity.

7. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity within each test chamber, as outlined in the *Temperature Measurement SOP, pH Measurement SOP*, *Measurement of DO SOP*, and *Measurement of Salinity SOP*, respectively, after filling and before adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

8. Concentrate age-synchronized, adult copepods and place in a clean crystallizing dish. To concentrate copepods, place the dish containing the copepods on the counter in a dark room, and use a small light-emitting diode (LED) light placed against the side of the dish.

9. Using a glass Pasteur pipette, remove 20 copepods from the concentrated adults. Confirm under a dissecting microscope that the correct number of copepods have been removed, and gently transfer animals to the test beaker using a Pasteur pipette, taking care to not allow the pipette to come into contact with the treatment water.

10. Continue to transfer 20 copepods into each test replicate in random order.

11. After all replicates are set up, cover exposure chambers with aluminum foil to limit evaporation. Replicates are maintained in an incubator, with low aeration, at 25°C with a 24-hour light photoperiod. Aeration is accomplished using PE60 tubing with a 22G x 1.5-inch hypodermic needle (plastic hub removed), set to ~ 1 bubble/second.

12. For tests where copepods are fed, feed each beaker daily with 1 x 10^4 cells/mL *Isochrysis* (TISO) and 1 x 10^4 cells/mL *Cheatocerous* (CHGRA). See test-specific TCT for feeding.

13. The test is complete after 48 or 96 hours (see test-specific TCT). At the end of the test, record water quality (dissolved oxygen, pH, temperature and salinity) for each beaker. Take 5 mL for the final PAH quantification, as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS GLPP.

14. Gently pour each beaker through a 40-µm nylon filter to concentrate animals and eggs, and then place them into a clean glass crystallizing dish with a small amount of clean source water.

15. Determine the sex for all adult copepods by microscopic examination of uropod segments and antennae morphology using an inverted compound scope.

16. Determine the number of live and dead adults and nauplii under a variable-power dissecting microscope. Confirm dead animals by gently prodding with a dissection probe while visually looking for movement under high magnification. Remove dead animals and archive according to the QAPP.

17. For some tests, hatching success will be measured after adult survival counts have been determined. See test-specific TCT for list of endpoints. If hatching success is measured, a subset of 20 eggs from each replicate beaker will be pipetted out and placed in a 60 x 15 mm plastic Petri dish with clean seawater and returned to the incubator. The remaining animals from each replicate (live adults and nauplii) will be fixed using formalin and stained with the addition of Rose Bengal to make counting nauplii easier.

18. Count all nauplii from each replicate.

19. Hatching success will be determined after 72 hours using the 20 eggs set aside in Step 12. After 72 hours, samples will be fixed with formalin and stained using Rose Bengal, and all hatched nauplii and unhatched eggs will be counted and hatching success determined.

20. Retain all adult animals and nauplii, to the extent possible, by preserving in 10% buffered formalin. Archive according to the QAPP.

Note: Because attempts to separate water and dead animals will mostly result in destruction of the tissue sample, tissue samples may be stored with small amounts of test water. The volume of test water will be limited where possible.

21. Discard remaining test solutions as outlined in *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

22. The test will be said to pass test criteria provided that the average control survival of adult copepods exceeds 85% across the four replicates, and that there are no unacceptable deviation in water quality among replicates.

Q. Testing Protocol 17: Copepod Nauplii Acute Toxicity Test

Note: Experiments are set up using a synchronous culture, where all animals are approximately the same age (within 24 hours). Nauplii will be approximately 12–24 hours old at test initiation.

Before beginning toxicity tests, pre-clean all glassware according to the *Decontamination SOP*, as described in the QAPP and the RSMAS GLPP. All test water is obtained from the UV-sterilized seawater system at UMEH.

1. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the RSMAS GLPP. Prepare HEWAF the morning of experimental set up.

2. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

Note: Samples collected for the State of Louisiana tasks must be sent with a separate COC form and in a different shipment from samples collected under tasks conducted under the NOAA contract.

3. Perform acute toxicity tests in 250-mL glass beakers with a total test solution volume of 200 mL. The test medium is UV-sterilized, 0.45-µm filtered seawater. Measure the seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, only glass-graduated cylinders or Hamilton syringes should be used to add WAF. Perform dilution of WAF to treatment concentrations with enough volume for 5 treatment replicates and ALS Environmental sample analysis (total volume 1.5 L). Add both water and WAF volumes to a 2-L glass beaker. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels by decanting. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental for analysis, as described in the QAPP. Take 5 mL for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS GLPP. Discard unused WAF according to the procedures in the *WAF and Toxicity Test Water Disposal Procedures SOP*.

4. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity within each test chamber, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*, respectively, after filling and before adding test organisms. All SOPs can be found in the RSMAS GLPP. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

5. Concentrate age-synchronized copepod nauplii and place in a clean crystallizing dish. To concentrate copepods, place the dish containing the copepods on the counter in a dark room, and use a small LED light placed against the side of the dish.

6. Using a glass Pasteur pipette, remove 20 copepods from those that are concentrated by the light. Confirm under a dissecting microscope that the correct number of copepods have been removed, and gently transfer the animals to the test beaker using a Pasteur pipette, taking care to not allow the pipette to come into contact with the treatment water.

7. Continue to transfer 20 copepods into each test replicate in random order.

8. After all replicates are set up, cover exposure chambers with aluminum foil to limit evaporation. Replicates are maintained in an incubator, with low aeration, at 24°C with a 24-hour light photoperiod. Aeration is accomplished using PE60 tubing with a 22 G x 1.5-inch hypodermic needle (plastic hub removed), set to ~ 1 bubble/second.

9. If the test includes feeding, transfer 1 x 10^4 cells/mL *Isochrysis* (TISO) and 1 x 10^4 cells/mL *Cheatocerous* (CHGRA) to each beaker daily.

10. The final survival count is made after 48 hours. At the end of the test, record water quality (dissolved oxygen, pH, temperature, and salinity) for each beaker. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out. Remove 5 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* found in the RSMAS GLPP.

11. Gently pour each beaker through a 40-µm nylon filter to concentrate animals, and then place into a clean glass crystallizing dish. Water from the replicate beakers will be pooled and then sampled for PAH analysis by ALS Environmental. See the QAPP for sampling and shipping procedures. Determine the number of dead animals under a variable-power dissecting microscope. Confirm dead animals by gently prodding with a dissection probe while visually looking for movement under high magnification. Once the dead animals are counted, fix using formalin. and then stain with the addition of Rose Bengal.

12. Count all animals in the fixed solution. Repeat for each replicate beaker. The total number of live animals will be determined from the total count of all stained animals minus the number of animals that were deemed dead before fixation and staining.

13. Retain all animals, to the extent possible, by preserving in 10% buffered formalin. Archive according to the QAPP.

Note: An animal will be considered dead if it is absent from the test chamber unless there is evidence of non-treatment mortality.

14. Discard remaining test solutions as outlined in the *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

The test will be said to pass test criteria provided that the average control survival exceeds 80% across the four replicates, and that there are no unacceptable deviations in water quality among replicates.

R. Testing Protocol 18: Toadfish Adrenal Study

This protocol describes four separate but related toadfish adrenal study trials.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontaminating Glassware* SOP, as described in the *QAPP*. All test water is obtained from the UV-sterilized seawater system at UMEH. Tests were performed using the following protocols:

Adrenal stress response study with large cannulated toadfish, exposed to oil only:

1. One week before exposure, prepare the cannulated toadfish. First, ensure that all toadfish to be cannulated are greater than 100 g in size. Next, anesthetize the toadfish using 1 g/L MS-222 buffered with sodium bicarbonate. Insert cannulas (Clay-Adams PE 50 tubing) filled with heparinized saline into the caudal vein. Secure the cannulas to the fish using 3-0 silk sutures and heat-seal the tip. Allow the fish to recover for up to one week in flow-through chambers.

2. After one week, take a post-recovery blood sample. To collect the blood sample, cut off the heat-sealed tip of the cannula, attach a heparin-filled disposable 1-mL syringe with a 23-gauge needle, and draw out the heparizined saline that fills the cannula. Carefully remove a 100-µL "pre-sample" and set aside. Then, using a new glass EDTA-rinsed Hamilton syringe, collect 200 µL of blood (i.e., 0-hour blood sample). Return the 100-µL pre-sample and then refill the catheter with heparizined saline to prevent the cannula from clotting. Keep the heparin- and saline-filled syringe attached to the cannula and carefully place the syringe and cannula in front of the glass chamber to ensure easy access for the next series of blood samples. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen for later analysis of cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

3. Prepare the WAFs in advance according to the *Protocols for Preparing Water* Accommodated *Fractions SOP*, as described in the QAPP. Prepare the HEWAFs less than 24 hours before exposure.

4. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

5. On the day of exposure, fill 1-L glass chambers with 0.75 L of test solution. Shield the sides and top of chambers to avoid evaporation and disturbance. Aerate each chamber using glass Pasteur pipettes.

6. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity from a control and a WAF-exposed test chamber without organisms, as outlined in the *Temperature Measurement SOP*, *pH measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*. All SOPs can be found in the QAPP and RSMAS GLPP. Following the addition of the WAF, remove 10 mL and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. See respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

7. Transfer the cannulated toadfish to their respective exposure chambers, one fish per chamber.

Note: 20 chambers were used for each treatment (control and oil exposed) and 5 sampling time points. Therefore, 10 of the chambers were used for control fish and the other 10 were used for oil exposure treatment, so that one control and one oil treatment were sampled during each sampling timepoint. This same scenario was repeated to obtain replicate observations.

8. After one hour of exposure, take blood and tissue samples from one control and one oil treatment fish.

9. Sample the control fish before the oil treatment fish. To collect a blood sample, first use a heparin-filled, disposable 1-mL syringe with a 23-gauge needle and draw out the heparizined saline that fills the cannula. Carefully remove a 100-µL “pre-sample” and discard. Then, using a new glass EDTA-rinsed Hamilton syringe, collect 200 µL of blood and temporarily store it on ice.

10. Immediately after collecting the blood sample, over-anesthetize (3 g/L MS-222 buffered with sodium bicarbonate) the sampled fish. Remove and weigh the brain, then dissect out the pituitary, pre-optic region, and the hindbrain. Freeze all three dissected sections, plus the section remaining, in liquid nitrogen. This will be analyzed later for corticotropin releasing factor (CRF) mRNA expression by quantitative real-time PCR (qPCR).

11. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

12. Take 10-mL samples from a control and a WAF-exposed test chamber without organisms and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. In addition, collect 250 mL from a control and a WAF-exposed test chamber without organisms for chemical analysis by ALS Environmental (ALS; formerly Columbia Analytical Services) as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the Analytical Sample Shipping and COC SOP, found in the QAPP and RSMAS Work Plan. Store samples at 4°C, record all necessary sample numbers, and prepare necessary COC documentation, as described in the QAPP and RSMAS Work Plan. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

13. Repeat steps 9 through 12, at the 2, 4, 8, and 24-hour post-exposure sampling timepoints.

14. At 24 hours, take the final measurements for water temperature, pH, salinity, and dissolved oxygen in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Remove 10 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS Work Plan. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Take a composite water sample from each treatment for chemical analysis by ALS Environmental as specified in the QAPP. Ensure that all meter calibration logs are filled out.

15. Discard remaining test solutions as outlined in the WAF and *Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

Adrenal stress response study with large cannulated toadfish that were exposed to oil and air:

1. One week before exposure, prepare the cannulated toadfish. First ensure all toadfish to be cannulated are greater than 100 g in size. Next, anesthetize the toadfish using 1 g/L MS-222 buffered with sodium bicarbonate. Insert cannulas (Clay-Adams PE 50 tubing) filled with heparinized saline into the caudal vein. Secure the cannulas to the fish using 3-0 silk sutures and heat-seal the tip. Allow the fish to recover for up to one week in flow-through chambers.

2. After one week, take a post-recovery blood sample. To collect the blood sample, cut off the heat-sealed tip of the cannula, attach a heparin-filled disposable 1-mL syringe with a 23-gauge needle, and draw out the heparizined saline that fills the cannula. Carefully remove a 100-µL “pre-sample” and set it aside. Then, using a new glass EDTA-rinsed

Hamilton syringe, collect 200 µL of blood (i.e., 0-hour blood sample). Return the 100-µL pre-sample and then refill the catheter with heparizined saline to prevent the cannula from clotting. Keep the heparin- and saline-filled syringe attached to the cannula and carefully place the syringe and cannula in front of the glass chamber to ensure easy access for the next series of blood samples. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

3. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP. Make HEWAFs less than 24 hours before exposure.

4. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

5. On the day of exposure, fill 1-L glass chambers with 0.75 L of test solution. Shield the sides and the top of the chambers to avoid evaporation and disturbance. Bubble air into each chamber using glass Pasteur pipettes.

6. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity from a control and a WAF-exposed test chamber without organisms, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*. All SOPs can be found in the QAPP and RSMAS Work Plan. Following the addition of the WAF, remove 10 mL and immediately dilute with 10 mL of ethanol for the initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. See the respective SOPs for individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and the meter calibration logs are filled out.

7. Transfer the cannulated toadfish to their respective exposure chambers; one fish per chamber. Note that 20 chambers were used for each treatment (control and oil exposed) and 5 sampling time points. Therefore, 10 of the chambers were used for control fish and the other 10 were used for oil exposure treatment, so that one control and one oil

treatment are sampled during each sampling timepoint. This same scenario was repeated to obtain replicate observations.

8. After one hour of exposure, conduct air exposures by removing one control and one oil treatment fish from their chambers and place onto a clean damp towel for 5 minutes. After 5 minutes, return each fish to their respective chambers.

9. After 30 minutes post-air exposure, remove fish from the exposure chambers to sample blood and take tissue samples from one control and one oil treatment fish.

10. Sample the control fish before the oil treatment fish. To collect a blood sample, first use a heparin-filled disposable 1-mL syringe with a 23-gauge needle and draw out the heparizined saline that fills the cannula. Carefully remove a 100-µL "pre-sample" and discard. Then, using a new glass EDTA-rinsed Hamilton syringe, collect 200 µL of blood and temporarily store on ice.

11. Immediately after collecting the blood sample, over-anesthetize (3 g/L MS-222 buffered with sodium bicarbonate) the sampled fish. Remove and weigh the brain, then dissect out the pituitary, pre-optic region, and the hindbrain. Freeze all three dissected sections, plus the section remaining, in liquid nitrogen. These will be analyzed later for CRF mRNA expression by quantitative real-time PCR (qPCR).

12. Centrifuge the blood sample at 16,000 g for 5 minutes, retain plasma, and freeze immediately in liquid nitrogen for later analysis of cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

13. Take 10-mL samples from a control and a WAF-exposed test chamber without organisms and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. In addition, collect 250 mL from a control and a WAF-exposed test chamber without organisms for chemical analysis by ALS Environmental, as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the Analytical Sample Shipping and COC SOP, found in the QAPP and RSMAS GLPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

14. Repeat steps 8 through 13, at the 2, 4, 8, and 24-hour post-exposure sampling timepoints.

15. At 24 hours, take the final measurements for water temperature, pH, salinity, and dissolved oxygen in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Take 10 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Take a composite water sample from each treatment for chemical analysis by ALS Environmental as specified in the QAPP. Ensure that all meter calibration logs are filled out.

16. Discard remaining test solutions as outlined in the *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

Adrenal stress response study with small non-cannulated toadfish exposed to oil only:

1. Two days before exposure, place the non-cannulated toadfish (< 40 g) directly into 1-L glass exposure chambers, one per dish, with clean source water and allow to acclimate for 36–48 hours before starting the test.

2. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP. Prepare HEWAFs less than 24 hours before exposure.

3. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

4. On the day of exposure, after a 36 to –48-hour acclimation period, siphon the source water out of glass chambers and immediately replace it with appropriate WAF treatment or control water to start the exposure.

5. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity from a control and a WAF-exposed test chamber without organisms, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*. All SOPs can be found in the QAPP and RSMAS GLPP. Following the addition of the WAF, remove 10 mL and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. See the

respective SOPs for individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

6. Immediately before transferring toadfish to exposure chambers, collect blood and tissue samples from one toadfish.

7. Collect the blood sample via caudal puncture with a 23-gauge needle on a 1-mL EDTA-rinsed syringe and temporarily store on ice.

8. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

9. Immediately after collecting the blood sample, over-anesthetize the fish using a 1-g/L solution of MS-222 and remove the kidney for in-vitro analysis of spontaneous and ACTH-stimulated cortisol secretion. To prepare the kidney for analysis, finely chop into 1-mm^3 cubes and place in a 24-well cell culture dish with 1 mL of L-15 media. Place the culture dish on an orbital shaker and incubate for 2 hours with periodic L-15 media changes until spontaneous cortisol release is minimal. After the 2-hour pre-incubation period, replace the media with L-15 media + 3.3 x 10-6 M ACTH. Take an initial 35-µL sample of the media and subsequent samples at 30, 60, and 120 minutes after ACTH treatment for analysis of ACTH-stimulated cortisol secretion.

10. To start the test, transfer the toadfish to their respective exposure chambers; one fish per chamber.

Note: 20 chambers were used for each treatment (control and oil exposed) and 5 sampling time points. Therefore, 10 of the chambers were used for control fish and the other 10 were used for oil exposure treatment, so that one control and one oil treatment are sampled during each sampling timepoint. This same scenario was repeated to obtain replicate observations.

11. After one hour of exposure, sample blood and tissue samples from one control and one oil treatment fish.

12. Sample the control fish before the oil treatment fish. Collect the blood sample via caudal puncture with a 23-gauge needle on a 1-mL EDTA-rinsed syringe and temporarily store on ice.

13. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to

MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

14. Immediately after collecting the blood sample, over-anesthetize the fish using a 1-g/L solution of MS-222 and remove the kidney for in vitro analysis of spontaneous and ACTH-stimulated cortisol secretion. To prepare the kidney for analysis, finely chop into 1-mm^3 cubes and place in a 24-well cell culture dish with 1 mL of L-15 media. Place the culture dish on an orbital shaker and incubate for 2 hours with periodic L-15 media changes until spontaneous cortisol release is minimal. After the 2-hour pre-incubation period, replace the media with L-15 media + 3.3 x 10-6 M ACTH. Take an initial 35-µL sample of the media and subsequent samples at 30, 60, and 120 minutes post-ACTH treatment for analysis of ACTH-stimulated cortisol secretion.

15. Take 10-mL samples from a control and a WAF-exposed test chamber without organisms and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. In addition, collect 250 mL from a control and a WAF-exposed test chamber without organisms for chemical analysis by ALS Environmental, as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP and RSMAS GLPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP and RSMAS GLPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

16. Repeat steps 12 through 16, at the 2, 4, 8, and 24-hour post-exposure sampling timepoints.

17. At 24 hours, take the final measurements for water temperature, pH, salinity, and dissolved oxygen in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Remove 10 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* in the RSMAS GLPP. Also remove a my water sample for ammonia analysis and store at -20°C until assayed. Take a composite water sample from each treatment for chemical analysis by ALS Environmental as specified in the QAPP. Ensure that all meter calibration logs are filled out.

18. Discard remaining test solutions of WAF and toxicity test water as outlined in the *Disposal Procedures SOP* found in the RSMAS GLPP.

Adrenal stress response study with small non-cannulated toadfish that were exposed to oil and air:

1. Two days before exposure, place non-cannulated toadfish (< 40 g) directly into 1-L glass exposure chambers with clean source water and allow to acclimate for 36–48 hours before the start of the test.

2. Prepare the WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP. Make the HEWAFs less than 24 hours before exposure.

3. Remove a subsample of each WAF treatment for chemical analysis by ALS Environmental as specified in the QAPP. Each sample jar (provided by ALS Environmental) should be filled to capacity. Sample collection, labeling, and handling will be conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Store samples at 4°C; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP. Samples should be shipped overnight on ice to ALS Environmental as soon as possible.

4. Take initial measurements of water temperature, pH, dissolved oxygen, and salinity from a control and a WAF-exposed test chamber without organisms, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*. All SOPs can be found in the QAPP and RSMAS GLPP. Following the addition of WAF, remove 10 mL and immediately dilute with 10 mL of ethanol for initial PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. See the respective SOPs for individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

5. Before transferring the toadfish to exposure chambers, collect blood and tissue samples from one pre-exposure toadfish.

6. Collect the blood sample via caudal puncture with a 23-gauge needle on a 1-mL EDTA-rinsed syringe and temporarily store on ice.

7. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

8. Immediately after collecting the blood sample, over-anesthetize (3 g/L MS-222 buffered with sodium bicarbonate) the sampled fish. Remove and weigh the brain, then dissect out the pituitary, pre-optic region, and the hindbrain. Freeze all three dissected sections plus the section remaining in liquid nitrogen for later analysis of CRF mRNA expression by quantitative real-time PCR (qPCR).

9. After collecting the pre-exposure samples and prior to transferring the toadfish to the exposure chambers, remove another pre-exposure toadfish and place onto a clean damp towel for 5 minutes. After 5 minutes, place the fish into a 1-L beaker with clean seawater.

10. After 30 minutes post-air exposure, remove the fish and take blood and tissue samples by repeating steps 6 through 8.

11. To start the test, transfer the toadfish to their respective exposure chambers; one fish per chamber.

Note: 20 chambers were used for each treatment (control and oil exposed) and 5 sampling time points. Therefore, 10 of the chambers were used for the control fish and the other 10 were used for the oil exposure treatment, so that one control and one oil treatment were sampled during each sampling timepoint. This same scenario was repeated to obtain replicate observations.

12. After one hour of exposure, conduct air exposures by removing one control and one oil treatment fish from their chambers and place onto a clean damp towel for 5 minutes. After 5 minutes, return each fish to their respective chambers.

13. After 30 minutes post-air exposure, remove the fish from exposure chambers to sample the blood and tissue samples from one control and one oil treatment fish.

14. Collect the blood sample via caudal puncture with a 23-gauge needle on a 1-mL EDTA-rinsed syringe and temporarily store on ice.

15. Centrifuge the blood sample at 16,000 g for 5 minutes, retain the plasma, and freeze immediately in liquid nitrogen. This will be analyzed later for cortisol according to MP Biomedicals catalogue # 07-221102 and ACTH according to MP Biomedicals catalogue # 07-106102 using a radio-immuno assay.

16. Immediately after collecting the blood sample, over-anesthetize (3-g/L MS-222 buffered with sodium bicarbonate) the sampled fish. Remove and weigh the brain, then dissect out the pituitary, pre-optic region, and the hindbrain. Freeze all three dissected sections plus the section remaining in liquid nitrogen. They will be analyzed later for CRF mRNA expression by quantitative real-time PCR (qPCR).

17. Repeat steps 12 through 16, at the 2, 4, 8, and 24-hour post-exposure sampling timepoints.

18. At 24 hours, take the final measurements for water temperature, pH, salinity, and dissolved oxygen in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Take 10 mL for the final PAH quantification as described in the *Measuring Oil in Water Using Fluorescence Spectroscopy SOP* and RSMAS GLPP. Also remove a 1-mL water sample for ammonia analysis and store at -20°C until assayed. Take a composite water sample from each treatment for chemical analysis by ALS Environmental as specified in the QAPP. Ensure that all meter calibration logs are filled out.

19. Discard remaining test solutions as outlined in the *Disposal Procedures SOP* found in the RSMAS GLPP.

S. Testing Protocol 19: Comparison of Static, Static-Agitated, and Static Recirculating Systems for Mahi-Mahi (*Coryphaena hippurus*) and Yellowfin Tuna (*Thunnus albacares*) Embryo Toxicity Testing

This protocol describes tests using mahi-mahi and yellowfin tuna embryos. These tests occurred at different times of day. In general, mahi-mahi spawn in the early morning, and eggs are collected by 8:00–10:00 a.m. and are ready to use by 12:00 p.m. Yellowfin tuna spawn in the early evening and eggs are collected within 2 hours of the spawning event. The approximate developmental stage of the eggs at the initiation of testing is the late blastula or early gastrula period.

Prior to beginning toxicity tests, all glassware was pre-cleaned according to the *Decontamination SOP*, as described in the QAPP. For the mahi-mahi tests, all test water was obtained from the UV-sterilized seawater system at UMEH. For the yellowfin tuna tests, all test seawater collected at the Achotines Laboratory was filtered down to 0.35 µm, UV sterilized, and temperature-matched to the water in which the eggs were collected prior to use.

S.1 Protocol

S.1.1 Initial water quality measurement

Take initial measurements of water temperature, pH, DO, and salinity, within each test chamber in each testing system, as outlined in the *Temperature Measurement SOP*, the *pH Measurement SOP*, the *Measurement of DO SOP*, and the *Measurement of Salinity SOP*, respectively, after filling and prior to adding test organisms. All SOPs can be found in the RSMAS GLPP. Also remove a 5-mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

S.1.2 Embryo collection

Collect embryos in the evening (for yellowfin tuna) or morning (for mahi-mahi) of a spawn by removing the eggs from the egg collector attached to the broodstock tank and placing them in a 20-L bucket filled with seawater. Prior to adding eggs to the bucket, briefly aerate the water in the collection bucket to saturate the water. Once saturation has been achieved, remove the air

stone. Maintain water temperature throughout the entire collection, prophylactic treatment, and rinsing procedure within 1°C of the spawning tank temperature.

Once eggs are in the collection bucket, allow approximately 15 minutes of static conditions during which time non-viable eggs will settle to the bottom of the bucket, and viable eggs will float at or near the water surface. After this settling period, scoop the floating eggs off the surface of the water using a soft mesh net and briefly rinse using 0.35 µm-filtered and UV-sterilized water prior to placement in a treatment vessel (20-L bucket) filled with filtered/sterilized seawater. Stock eggs in the treatment vessel at a density of approximately 300–500 eggs/L.

Apply a prophylactic treatment of Formalin (37% formaldehyde solution) to the eggs at a dosage of 100 ppm for 1 hour. Formalin is a commonly used paracide to treat marine fish eggs collected from captive marine fish and improves embryo survival and hatch rate in laboratory conditions. Reinsert the air stone. Supply supplemental aeration at a very low rate in the treatment vessel to maintain DO levels at saturation levels throughout the treatment period. To evaluate the effects of this prophylactic treatment on fish survival, some tests included paired Formalin and no Formalin treatments as well as longer and shorter Formalin exposures. For the no Formalin treatments, eggs were maintained in the collection bucket with aeration.

Following the prophylactic treatment period, briefly remove the air stone from the vessel to allow viable eggs to float at the surface, then remove them using a soft mesh net, and rinse them using filtered/sterilized seawater. Use at least 20 L of filtered/UV-sterilized water (> 3x rinsing vessel exchange) during rinsing to ensure complete removal of the Formalin. Place embryos in a beaker of filtered/UV-sterilized seawater and transfer to the exposure room (set to 27°C) at the Achotines Laboratory for the yellowfin tuna toxicity test setup or to the University of Miami environmental chamber for the mahi-mahi toxicity test setup.

Use a Leica Zoom2000 stereoscope (or equivalent low-magnification scope) to assess the embryo's quality. At a 45x magnification, the developing embryonic morphology should be clearly discernible. Discard any embryos that appear unfertilized or malformed.

S.1.3 Static exposure experimental setup

Perform static acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. Measure seawater treatment volume using a graduated cylinder. Decant seawater into all replicate beakers for each treatment (see test-specific TCTs for number of replicates).

Transfer 20 embryos into each test replicate in random order. Because embryos are too large for Pasteur pipettes, use a glass eye dropper to count the embryos. Count the embryos both as they

exit the eye dropper and after they have all been transferred. Between test replicates, rinse the eye dropper briefly with Simple Green and distilled water.

After all replicates are set up, cover exposure chambers with glass panes or inverted glass Petri dishes to limit evaporation.

Place vessels in a temperature-controlled room set at 27°C with a 16:8 light/dark photoperiod.

S.1.4 Static-agitated experimental setup

Perform static-agitated acute embryo toxicity tests in 1-L glass beakers with a total test solution volume of 1 L. Measure the seawater treatment volume using a graduated cylinder. Decant seawater into all replicate beakers for each treatment (see test-specific TCTs for the number of replicates).

Transfer 20 embryos into each test replicate in random order. Because embryos are too large for Pasteur pipettes, use a glass eye dropper to count the embryos. Count the embryos both as they exit the eye dropper and after they have all been transferred. Between test replicates, rinse the eye dropper briefly with Simple Green and distilled water.

After all replicates are set up, cover exposure chambers with glass panes or inverted glass Petri dishes to limit evaporation.

Place vessels on a reciprocating shaker in a temperature-controlled room set at 27°C with a 16:8 light/dark photoperiod.

S.1.5 Static recirculating experimental setup

Perform acute embryo exposures in customized 0.8-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. The total test solution volume is 1.8 L and should be circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct the pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Embryos/larvae are retained in the cone using a glass excluder extending from the overflow drain, with a nylon mesh fastened on both sides with silicone O-rings. The test medium is UV-sterilized seawater. Measure the seawater volume using a graduated cylinder. Decant seawater into all replicate beakers for each treatment (see test-specific TCTs for number of replicates). For replicates that incorporate

antibiotic treatment (10 ppm oxytetracycline) in the test solution, add oxytetracycline powder to the bulk volume of test solution and decant from the bulk solution into replicates.

1. Gently transfer 40 embryos into each test replicate using a large-bore Pasteur pipette.

2. After all replicates are set up, cover exposure chambers with large glass Petri dishes to limit evaporation. Replicates are maintained in a temperature-controlled room set at 27°C with a 16:8 light/dark photoperiod.

3. At the end of the test, halt the pump flow to the Imhoff cones by first closing each of the four stopcocks receiving flow from a given pump, and then quickly turning the pump off. Disconnect the tubing from each of the stopcocks, as well as from each beaker, to prevent siphoning. Collect larvae in the corresponding overflow beaker containing ~ 200 mL of exposure media by opening the stopcock below the surface and allowing the beaker to slowly drain into a final volume of ~ 1 L. Use seawater to rinse the sides of the Imhoff cones during draining.

S.1.6 Test termination procedures

1. Observe and record survival, mortality, missing individuals, and non-treatment mortality daily. Observe mortality visually and verify by prodding the animal with a glass Pasteur pipette. Remove and archive dead animals unless they have decomposed. Ensure that all COC documentation is filled out as described in the QAPP. Retain all dead animals to the extent possible and archive according to the QAPP. Perform daily measurements of water chemistry and physical parameters, as outlined in the QAPP. Take the final survival count after 96 hours (mahi-mahi) or 72 hours (yellowfin tuna). Retain all remaining live and dead animals, to the extent possible, and archive according to the QAPP.

   Note: Because attempts to separate water and dead animals mostly resulted in destruction of tissue samples, tissue samples were sometimes stored with small amounts of test water. The volume was limited where possible.

   Note: Because embryonic animals decay rapidly after death, it was likely that some dead animals were not counted or collected. An animal was considered dead if it was absent from the test chamber unless there was evidence of non-treatment mortality, such as jumping from the tank.

   Note: Hatching mortality was recorded, and was defined as the number of animals that died before hatch or immediately post-hatch.

2. Take final measurements for water temperature, pH, salinity, and DO in each exposure chamber, as outlined in the QAPP and RSMAS GLPP. Also remove a 5-mL water sample for ammonia analysis and store at -20°C until assayed. Ensure that all meter calibration logs are filled out.

3. Remove any dead animals and archive as outlined in the QAPP and RSMAS GLPP. Anaesthetize all surviving animals with sodium bicarbonate buffered MS-222 (2:1 mass of sodium bicarbonate to MS-222). Add a concentrated solution to the exposure beakers drop-wise until the animals stop moving. Collect the animals and archive as outlined in the QAPP.

4. Discard remaining test solutions.

8. University of North Texas General Laboratory Procedures and Practices

8.1 Introduction

The goal of these studies was to produce data to parameterize a model to assess the effects from photo-enhanced toxicity based on polycyclic aromatic hydrocarbon (PAH) concentration and site-specific variation in light intensity.

8.2 Methods

Both indoor and outdoor experiments were conducted to establish the full range of toxicity responses of fish and invertebrates to PAH under a wide range of ultraviolet (UV) radiation conditions likely to occur in natural habitats.

Laboratory tests conducted at the Miami University of Ohio (MUO) were used to simulate the diminished levels of UV radiation present at water depths of up to 10 m. These studies were conducted indoors with low light levels; see the MUO General Laboratory Procedures and Practices (GLPP) for details.

Outdoor tests were conducted by University of North Texas (UNT) personnel at the following facilities: the University of Miami, Rosenstiel School of Marine and Atmospheric Science (RSMAS); Florida Gulf Coast University (FGCU); Louisiana University Marine Consortium (LUMCON); and Auburn University (Auburn). Some tests were conducted at Sea Center Texas in collaboration with Stratus Consulting. Additionally, some tests were conducted onsite at UNT. Sunlight was used as a source of UV, and plastic shielding was used to alter exposure wavelengths and intensity.

Outdoor testing apparatus consisted of a water table covered in either UV transparent (Aclar recommended) or UV opaque (Courtgard recommended) plastic sheeting. Courtgard (CP Films, Inc., Martinsville, VA; http://www.plasticstoday.com/sourcebook/cpfilms-inc) is a long-wave-pass plastic that in water transmits photosynthetically active radiation (PAR; 95% 400–800 nm) but blocks most UV radiation (transmits no UV-B 295–319 nm, and only 9% of UV-A 320–400 nm, with a sharp wavelength cutoff and 50% transmittance at 400 nm). Aclar (Honeywell International, Morristown, NJ; http://www.honeywell.com/) is a long-wave-pass plastic that in water transmits both PAR (100% 400–800 nm) and most UV radiation (98% of UV-B 295–319 nm, and 99% UV-A 320–399 nm, with a sharp wavelength cutoff and 50% transmittance at 212 nm). The water table acted simply as a temperature control, and

organisms were never in contact with water from the table. Water was fed into the water table and the rate of flow varied to maintain a certain temperature during the test; see test-specific test conditions tables (TCTs) for details. Plastic sheeting was attached to the water table to allow some airflow over the table for cooling. Test dishes were placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in the water table. The water table was placed in an unshaded location between 8:00 a.m. and 6:00 p.m.

UNT also conducted studies in an indoor UV testing apparatus using high-intensity lights as a source of UV light and fluorescent lights as a source of light without UV. The lights were suspended over laboratory tables, and the height of the high-intensity UV lights was adjusted so that the intensity at the table's surface mimicked the UV intensity of a sunny summer day in the Gulf of Mexico.

8.2.1 Test organism sources and husbandry

This section describes the sources and husbandry for test organisms used for toxicity tests, which were conducted with sheepshead minnow, mahi-mahi, blue crab, oyster, fiddler crab, grass shrimp, red drum, and speckled seatrout.

Sheepshead minnow

Sheepshead minnow were shipped to UNT from MUO; see the MUO GLPP for information regarding sources and husbandry. Sheepshead minnow tests were conducted with artificial seawater (ASW) prepared with a mix of Instant Ocean and Milli-Q water.

Mahi-mahi

Mahi-mahi tests were conducted onsite at RSMAS; see the RSMAS GLPP for information regarding mahi-mahi sources and husbandry.

Blue crab

Blue crab tests were conducted onsite at UNT. For tests conducted prior to 2013, blue crab zoeae were shipped to UNT by personnel at the University of Southern Mississippi Gulf Coast Research Laboratory (GCRL); see the GCRL GLPP for blue crab source and husbandry information. For tests conducted in 2013, blue crab zoeae were purchased from the University of Maryland's Institute of Marine and Environmental Technology blue crab aquaculture facility and shipped to UNT. Blue crab tests were conducted with ASW prepared with a mix of BioSea Marine Mix salts and Milli-Q water.

Oyster

Oyster tests were conducted onsite at FGCU; see the FGCU GLPP for information regarding oyster sources and husbandry.

Fiddler crab

Fiddler crab tests were conducted onsite at Auburn; see the Auburn GLPP for information regarding fiddler crab sources and husbandry.

Grass shrimp

Grass shrimp tests were conducted onsite at UNT. All adult grass shrimp were obtained from the NOAA-Charleston laboratory or GCRL. Animals were shipped overnight to UNT and held in glass aquaria containing ASW (15 ppt; aeration) before testing (generally 48 hours; see individual testing protocols in the UNT GLPP for differences in holding times). Animals were fed fine-ground commercial fish food daily and monitored for mortality/disease before testing. Any animals not used in tests were euthanized and disposed of.

Red drum and speckled seatrout

Red drum and speckled seatrout tests were conducted onsite at Sea Center Texas; see the Stratus Consulting GLPP for information regarding sources and husbandry of red drum and speckled seatrout.

Bay anchovy and red snapper

Bay anchovy and red snapper tests were conducted onsite at LUMCON; see the LUMCON GLPP for information regarding sources and husbandry of bay anchovy and red snapper.

Mysid shrimp

Mysids were shipped to UNT from Aquatic BioSystems (Ft. Collins, CO) and held overnight before testing began.

White shrimp

Pacific white shrimp (*Litopenaeus vannamei*) were shipped overnight to UNT from Shrimp Improvement Systems (SIS) LLC Nucleus Breeding Center in Islamorada, FL, USA. Upon arrival, animals were split among aquariums and housed in mass at 25 ppt with aeration. Cultured shrimp were fed daily with prepared feed (Raceway Plus from Zeigler, MN).

8.2.2 Exposure media preparations

For water exposures, test media were prepared according to established protocols for preparing high-energy and chemically enhanced water accommodated fractions (HEWAF and CEWAF, respectively). See the *Protocols for Preparing Water Accommodated Fractions* in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Each test included at least two UV radiation treatments (full-spectrum solar and solar with UV screened out). For some tests, a 50% full-spectrum solar UV light treatment was also included. Each test included a control [prepared similarly to water accommodated fractions (WAFs) without the addition of oil or dispersant] and different concentrations of WAF.

8.2.3 Phototoxicity model development

Over the past 25 years, a global model of PAH phototoxicity has been developed with major contributions to the area of study coming from Principal Investigators at MUO and UNT (Jeffries et al., 2013). Photo-enhanced toxicity is a function of (1) a particular PAH's ability to generate reactive oxygen species upon absorption of actinic UV radiation, (2) the extent to which a PAH is taken up by an organism (i.e., combination of bioavailability and bioaccumulation potential – also known as fugacity), and (3) the intensity of actinic UV exposure. Model input is thus a combination of structure-activity data on compound-specific levels of phototoxicity, bioaccumulation data, and quantitative information on intensity and duration of UV exposure. Model output is the predicted time-to-death for an organism, exposure to combinations of PAH levels that are taken up by an organism, and UV intensity and duration. This output can be used to estimate site-, time-, season-, or depth-specific phototoxic injury for specific mixtures of PAH. The model takes the form of:

$$TTD = f\left\{\sum_{i=1\text{-}n}([PAH]_i * RPA_i) * (UV)\right\}, \qquad \text{Equation 1}$$

where TTD = predicted time-to-death; $[PAH]_i$ = molar body burden of PAH "i" over PAH "i" to "n;" RPA_i = relative photodynamic action of PAH_i (ratio of phototoxicity intensity of PAH_i and anthracene – a PAH with a median level of phototoxicity); and UV = dose of actinic UV radiation exposure (typically expressed as broad-band UV-A radiation in the range of 320–400 nm).

This model can be used to predict times-to-death under specific conditions or can be used to determine predicted no-effect levels of PAH and UV exposure on a site-specific basis.

Data from tests conducted under the UNT GLPP were modeled using these concepts to predict levels of toxicity and areas of injury within the *Deepwater Horizon* oil spill zone. In addition, the data were incorporated into the global model of phototoxicity, and model comparisons served as one form of validation of both the model and its predictions.

8.2.4 Water quality monitoring

Water quality was monitored as described in the *Water quality measurement standard operating procedure* (SOP) in Section 8.4.1. Dissolved oxygen (DO), pH, conductivity, salinity, ammonia, and temperature were measured in each test dilution stock just prior to renewal. Temperature and DO were measured hourly during the test procedure. Solar radiation was measured using a calibrated, BioSpherical profiling ultraviolet (PUV) radiometer, which took continuous measurements of UV during the entire test period. Calibration was performed and certified by BioSpherical Instruments (San Diego, California).

8.2.5 Analytical chemistry

Water samples were collected as described in the QAPP.

8.3 Reporting and Testing Documentation

All documentation of test procedures, results, etc., was carried out as described in the QAPP. Documentation was provided to Stratus Consulting and electronic and hard copies were kept on file at UNT.

8.4 General Protocols

8.4.1 Water quality measurement standard operating procedure

A. Temperature (YSI ProODO)

1. Using traceable thermometer associated with the optical DO probe, place probe in one replicate of an exposure concentration.

2. Hold probe in replicate test solution until reading stabilizes for 5 seconds.

3. Record reading.

4. Clean probe using analytical detergent and soft sponge with deionized (DI) water.

5. Repeat steps 1 through 4 on one replicate per exposure, per UV exposure (UV exposed and UV shielded).

B. pH (YSI 63)

1. Using manufacturer's standards, test range and calibration of probe. Recalibrate as per manufacturer's instructions if needed.

2. Place probe in test stock or control solution and continue to swirl until reading stabilizes.

3. Record reading.

4. Clean probe using analytical detergent and soft sponge with DI water, and store as per manufacturer's instructions.

C. DO (YSI ProODO)

1. Using saturated solution, verify calibration of probe. Recalibrate as per manufacturer's instructions if needed.

2. Place probe in test stock or control solution and continue to swirl until reading stabilizes.

3. Record reading.

4. Clean probe using analytical detergent and soft sponge with DI water, and store as per manufacturer's instructions.

D. Conductivity (YSI 63)

1. Using manufacturer's standards, test range and calibration of probe. Recalibrate as per manufacturer's instructions if needed.

2. Place probe in test stock or control solution and continue to swirl until reading stabilizes.

3. Record reading.

4. Clean probe using analytical detergent and soft sponge with DI water, and store as per manufacturer's instructions.

E. Salinity (YSI 63)

1. Using manufacturer's standards, test range and calibration of probe. Recalibrate as per manufacturer's instructions if needed.

2. Place probe in test stock or control solution and continue to swirl until reading stabilizes.

3. Record reading.

4. Clean probe using detergent and soft sponge with DI water, and store as per manufacturer's instructions.

F. Total ammonia (Hach colorimetric test)

1. Obtain 5 mL of exposure media for titration.

2. Add test strip according to kit instructions, and compare to colorimetric reference chart to obtain ammonia concentration.

3. Record concentration and dispose of test strip.

4. Repeat for all exposure stocks.

Reference

Jeffries, M.K.S., C. Claytor, W. Stubblefield, W.H. Pearson, and J.T. Oris. 2013. Quantitative risk model for polycyclic aromatic hydrocarbon photo-induced toxicity in Pacific herring following the *Exxon Valdez* oil spill. *Environmental Science and Technology* 47(10):5450–5458.

A. Testing Protocol 1: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Sheepshead Minnow (*Cyprinodon variegatus*)

The survival and hatch success of sheepshead minnow embryos and larvae life stages exposed to WAFs were assessed. For each treatment concentration, there were five replicates exposed to full intensity (100%) ultraviolet (UV) light and five replicates exposed to approximately 10% of the full UV light. Each replicate contained 200 mL of WAF and 10 organisms.

A.1 Testing Apparatus

Testing apparatus for outdoor UV tests with sheepshead minnow was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatment media as static renewal in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Embryo and larvae tests were started in the evening with an overnight soak in their respective treatment WAFs before being exposed to UV the next day. Mortality and hatch success were assessed daily, and tests were carried out until the last organism died or for a maximum of 168 hours for embryos and a maximum of 96 hours for larvae.

A.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number (replicate number).

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10 organisms in each test dish and feed as appropriate (specified on test-specific TCTs).

4. Complete bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight until the next morning.

6. In the morning, turn on the water to water tables to cool to desired temperature.

7. Obtain new, clean 250-mL crystallizing dishes and label with appropriate treatment and replicate numbers.

8. Fill clean dishes with 200 mL of appropriate test medium using glass graduated cylinder.

9. Transfer organisms from old crystallizing dish to new dish containing renewed test medium. Note any mortality (do not replace organisms). All dead test organisms will be sampled and retained according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Collect old dishes, wash, and prepare for afternoon water change. Cover unused test media and place in a refrigerator at 4°C.

11. Label blueboard floats as “UV+” or “UV-” with a marker. Place test dishes in appropriate floats by treatment.

12. By 9:00 a.m., place floats in the water table under appropriate plastic (either UV transparent for UV+ or UV opaque for UV-). Dishes should be floated to maximize direct contact with the cooling water.

13. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

14. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

15. Check temperature and DO hourly, and adjust flow of cooling water as needed.

16. In the afternoon, remove WAF preparations from the refrigerator and place in water bath to equilibrate them to test temperature.

17. After approximately 8 hours of exposure to sunlight, collect dishes and return indoors.

18. Count the number of dead and living organisms and transfer living organisms to new, clean crystallizing dishes containing appropriate test medium (as described above). Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

19. Leave dishes in secure location overnight.

20. Next morning, repeat procedure from step 6.

21. Carry out tests until the last organism in the lowest WAF concentration has died, or for a maximum of 168 hours for embryos and a maximum of 96 hours for larvae.

B. Testing Protocol 2: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Mahi-Mahi (*Coryphaena hippurus*)

The survival and hatching success of mahi-mahi embryo life stages exposed to WAFs were assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light; for details regarding the number of replicates, see test-specific TCTs. In addition, some tests included a 50% UV treatment. Each replicate contained 200 mL of WAF and 10–20 organisms.

B.1 Testing Apparatus

Testing apparatus for outdoor UV tests with mahi-mahi was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatment media without renewal (static) in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table that was placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Due to the typical timing of a mahi-mahi spawn and the rapid development of their embryonic life stage, tests began mid-day with the organisms going out into the sun as soon as the test dishes were prepared. Mortality and hatch success were assessed at the end of test, and tests were carried out for 48 hours.

B.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10–20 organisms in each test dish (as specified in test-specific TCTs).

4. Complete test forms as described in the QAPP.

5. Leave dishes in a secure laboratory area until outside water tables are ready.

6. Turn on water to water tables to cool to desired temperature.

7. Place test dishes in appropriate floats by treatment. Dishes should be floated to maximize direct contact with the cooling water. Ensure blueboard floats are labeled with appropriate UV exposure (e.g., 100%, 50%, 10%).

8. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

9. Start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

10. Check the temperature and DO hourly, and adjust flow of cooling water as needed.

11. After approximately 6–8 hours of exposure to sunlight, count the dead and living organisms. Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

12. After counts, return dishes to blueboard floats in water table and cover test dishes with a tarp to shelter from overnight weather and prevent further sunlight exposure.

13. The next morning, remove tarp and repeat procedure from step 8.

14. Carry out tests for 48 hours.

C. Testing Protocol 3: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Blue Crab (*Callinectes sapidus*) and Fiddler Crab (*Uca longisignalis*) Zoeae

The survival of blue crab and fiddler crab zoeae exposed to WAFs was assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light. In addition, some tests included a 50% UV exposure, with three to five replicates, for each WAF treatment (see test-specific TCTs for details). Each replicate contained 200 mL of WAF and 10–20 organisms.

C.1 Testing Apparatus

Testing apparatus for outdoor UV tests with blue crab and fiddler crab zoeae was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatment as static renewal in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Tests began in the evening with an overnight soak in their respective treatment WAFs before being exposed to UV the next day. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hours (see test-specific TCTs).

C.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10–20 organisms in each test dish and feed as appropriate (see test-specific TCTs for details).

4. Complete test-specific bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight until the next morning.

6. In the morning, turn on water to water tables to cool to desired temperature.

7. If renewal, use a pipette to remove ~ 80% of the test solution out of the chamber and dispense the water through a nylon mesh to avoid inadvertently removing organisms during WAF renewal. If no renewal, skip to step 8.

8. Before replacing WAF, note any mortality (do not replace organisms). Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

9. Refill dish with appropriate test medium so that there is approximately 200 mL of WAF in each dish. Unused test media should be covered and placed in fridge at 4°C.

10. Label blueboard floats with appropriate UV treatment. Place test dishes in appropriate floats by treatment.

11. By 9:00 a.m., place floats in water table under appropriate plastic (e.g., UV transparent for 100% UV). Transfer dishes to blueboard floats making sure to maximize direct contact with the cooling water.

12. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

13. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer, or when unavailable, make manual measurements every 5 to 15 minutes using an Ocean Optics JAZ-EL200 spectrometer. Both the PUV and the JAZ-EL200 measure UV-A, UV-B, and visible light.

14. Check the temperature and DO hourly, adjust the flow of cooling water as needed.

15. After approximately 8 hours of exposure to sunlight, count dead and living organisms in each dish per step 8 above. WAF in dishes should not be renewed at this time.

16. Leave dishes in a secure location overnight.

17. The next morning, repeat procedure from step 6.

18. Carry out tests until the last organism in the lowest WAF concentration has died, or for a maximum of 48–96 hours.

D. Testing Protocol 4: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Oyster (*Crassostrea virginica*) Fertilization, Embryos, Veligers

The survival of eastern oyster embryos and veligers as well as fertilization success (gametes) exposed to WAFs was assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light. In addition, some tests included a 50% UV exposure, with three to five replicates, for each WAF treatment. Each replicate contained 20–200 mL of WAF and 1,000–4,000 organisms (see test-specific TCTs).

D.1 Testing Apparatus

The outdoor testing apparatus consisted of a water table covered in either UV transparent or UV opaque clear plastic sheeting (Aclar and Cortgard recommended). The water table acted as a cooling bath only and organisms were never in contact with cooling water. Water was fed into the water table and the rate of flow varied to maintain a constant temperature during the test (see test-specific TCTs). Plastic sheeting was attached to the system to allow some airflow over the table for cooling. Test dishes were loaded according to test-specific TCTs. Test dishes were then placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in the water table. The water table was placed in an unshaded location between 8:00 a.m. and 6:00 p.m.

D.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 20–200 mL of the corresponding treatment medium using a glass cylinder (see test-specific TCTs). Unused test media should be disposed of appropriately.

3. Place organisms in each test dish and feed as appropriate; the total number of organisms included per dish varied between tests and is specified in test-specific TCTs.

4. Complete bench sheets as described in the QAPP.

5. For all gamete tests, and for embryo and veliger tests prepared in the morning, move prepared dishes immediately to water table floats for 1 hour exposure to UV. For embryo and veliger tests prepared in the late afternoon/evening, leave dishes in a secure area in the dark until next morning.

6. Turn on water to water tables to cool to desired temperature.

7. Label blueboard floats according to the UV exposure treatments included in test. Place test dishes in appropriate floats by treatment.

8. Place floats in water table under appropriate plastic (either UV transparent for UV+ or UV opaque for UV-). Dishes should be floated to maximize direct contact with the cooling water.

9. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

10. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

11. Check temperature and DO hourly, and adjust the flow of cooling water as needed.

12. After ~ 8 hours of exposure to sunlight (or 1 hour in the case of gamete tests), collect dishes and return indoors.

13. For gametes, following the one-hour UV exposure, assess fertilization rates by counting the number of fertilized and unfertilized embryos in a sample of 100 organisms. For embryos and veligers tested before 2013, assess mortality by counting the number of live and dead organisms in a sample of 100 organisms. For embryos and veligers tested in 2013, count the number of live and dead organisms in a 250 µL subsample. Retain all dead test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

14. Leave dishes in a secure location overnight.

15. The next morning, repeat procedure from step 6.

16. Carry out tests for a total of 1 hour sunlight exposure (gametes) or a maximum of 96 hours (embryos, veligers); see test-specific TCTs.

E. Testing Protocol 5: Assessing Photo-Enhanced Toxicity of Maternal Exposure to *Deepwater Horizon* Oil Spiked Sediment or WAF to Fiddler Crab (*Uca longisignalis*) Zoeae

Survival of zoeae hatched from gravid females from the test described in Auburn GLPP Testing Protocol 1 or Testing Protocol 4, depending on the matrix of the maternal exposure, was followed to assess the percentage of zoeal survival in relation to their exposure to oil during egg development. The zoeal survival studies were done outdoors with and without UV exposure. For each female crab, there were three replicates exposed to full UV light and three replicates exposed to approximately 10% of the full UV light. Each replicate contained 200 mL clean ASW at 20-ppt salinity with 20 organisms.

E.1 Testing Apparatus Design

Testing apparatus for outdoor UV tests with fiddler crab zoeae was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatment as static renewal in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hours (see test-specific TCTs).

E.2 Outdoor (UV exposure) Experimental Methods

Aquatic Habitats (AHAB) tanks were checked at least twice a day, first thing in the morning and again in the evening to determine if gravid females had hatched their eggs. If zoeae were found in the morning, they were collected from the filter cups and a subsample was immediately transferred to test dishes to be place outside for the day (see steps 1–4 below). If zoeae were found in the evening, test dishes were prepped and kept in the dark until the following morning. Detailed steps are provided below.

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL clean ASW using a glass cylinder.

3. Place 20 organisms in each test dish.

4. Complete bench sheets as described in QAPP.

5. If morning, go directly to step 6. If later in the day or evening leave dishes in a secure laboratory area in the dark until following morning.

6. First thing in the morning, turn on water to water tables to cool to desired temperature.

7. If test dishes were kept overnight, continue with steps 8–10, otherwise skip to step 11.

8. Pipette out ~ 80% of ASW from each dish.

9. Before replacing water, note any mortality (do not replace organisms). Remove and retain all dead test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Refill each dish to ~ 200 mL with fresh ASW.

11. Place test dishes outside in blueboard floats in water bath under appropriate UV plastic covering. Label blueboard floats as “UV+” or “UV-” with a marker.

12. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

13. After approximately 8–10 hours of exposure to sunlight, bring in all dishes, renew water and count mortalities (see steps 8–10 above).

14. Turn off water to water tables and turn off and bring in UV monitor.

15. Leave dishes in a secure location overnight.

16. The next morning, repeat procedure from step 6.

17. Continue test until the last organism in the lowest WAF concentration has died or for the maximum number of hours specified in test-specific TCTs.

F. Testing Protocol 6: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil Following Different Exposure Periods Using Fiddler Crab (*Uca longisignalis*) Zoeae

The survival of fiddler crab zoeae exposed to WAFs was assessed following different WAF exposure periods. For these tests, the testing apparatus and testing procedures were the same as described in Testing Protocol 3 in the UNT GLPP, except that the WAF exposure period was an additional variable across WAF treatment concentrations (see test-specific TCTs). After a predetermined duration of WAF exposure, zoeae from the dishes were transferred to clean seawater. Survival in the clean seawater, following different exposure durations in WAF was then followed until the end of the test. For each treatment concentration, there were three to five replicates for each WAF treatment, UV dose (10%, 50%, and 100% full spectrum solar), and exposure duration (e.g., 2, 4, and 8 hour) combination (see test-specific TCTs). Each replicate contained 200 mL of WAF and 10–20 organisms. Specific test design details are found in the TCTs for each test. The total test duration was 24–48 hours.

G. Testing Protocol 7: Assessing Photo-Enhanced Toxicity of *Deepwater Horizon* Oil to Mahi-Mahi (*Coryphaena hippurus*) Using Imhoff Cones

The survival and hatch success of mahi-mahi embryos exposed to WAFs in customized Imhoff cones were assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light (see test-specific TCTs).

G.1 Testing Apparatus

Acute embryo exposures were performed in customized 1-L glass Imhoff cones containing an overflow spout for draining into a 1-L glass beaker and a Teflon stopcock on the bottom. Total test solution volume in this testing apparatus was 1.8 L and was circulated between the Imhoff cone and beaker using a peristaltic pump and silicone tubing. Each peristaltic pump can supply flow to four Imhoff cones simultaneously. Direct pump flow such that water is drawn from the glass beaker and delivered to the Imhoff cone via the bottom stopcock at a low flow rate (~ 100 mL/minute) to keep embryos gently suspended and circulating in the cone. Retain embryos/larvae in the cone using a glass excluder extending from the overflow drain with nylon mesh fastened on both sides with silicone o-rings. The test medium was UV sterilized seawater. Seawater volume was measured using a graduated cylinder.

The Imhoff cones were placed in an unshaded location between 8:00 a.m. and 6:00 p.m. and covered with different clear UV plastic sheeting that either let 100% or 10% of the full spectrum solar UV light through (see test-specific TCTs). To control water temperature, the 1-L beaker reservoirs for each Imhoff cone were placed in a water bath where water was fed into the water bath and the rate of flow varied to maintain temperature. Mortality and hatch success were assessed daily, and tests were carried out until the last organism died or for a maximum of 96 hours.

G.2 Test Procedure

1. Label each Imhoff cone/beaker reservoir with test treatment and tank number.

2. Fill with 1.8 L of the corresponding treatment medium using a glass cylinder. Dispose unused test media appropriately.

3. Place organisms in each test dish (see test-specific TCTs).

4. Complete bench sheets as described in the QAPP.

5. Leave Imhoff cones in a secure laboratory area until water table is ready.

6. Turn on water to water bath to cool to desired temperature.

7. Label UV plastic sheet as “UV 100” or “UV 10” with a marker. Place Imhoff cones under appropriate UV plastic (see test-specific TCTs). Place beakers in a water bath.

8. Measure temperature, pH, salinity, and DO content of one randomly selected beaker from each PAH/UV treatment. Place plastic sheets over appropriate UV treatments.

9. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

10. Check temperature and DO hourly, and adjust flow of cooling water as needed.

11. After approximately 6–8 hours of exposure to sunlight, cover Imhoff cones by a steel frame outdoor canopy to shelter from overnight weather and prevent further sunlight exposure. During intense rain events, cover cones using the same outdoor canopy.

12. The next morning, remove canopy and repeat procedure from step 6.

13. Carry out tests until the last organism in the lowest WAF concentration has hatched/died, or for a maximum of 96 hours.

14. At the end of the test, count all dead and living organisms. Retain all test organisms removed according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required. Because of the difficulty in scoring animals in the Imhoff cones, hatch success, survival, mortality, missing, and non-test mortality were recorded at 96 hours only.

H. Testing Protocol 8: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Exposure to Juvenile Mahi-Mahi (*Coryphaena hippurus*) Swim Performance

The swim performance of juvenile mahi-mahi exposed to WAFs was assessed.

H.1 Testing Apparatus

Testing apparatus for outdoor UV tests with mahi-mahi was the same as described in Section 8.2 (Methods) of the UNT GLPP except the exposure tanks were 10-gal glass aquaria. Two to three fish were added to each aquarium. Organisms were exposed to WAF treatment media without renewal (static) for 1 day (~ 4–6 hours of UV). Loading of organisms into chambers never exceeded 0.5 g/L. Test chambers were placed in the water bath in an unshaded location between 8:00 a.m. and 6:00 p.m. Exposures were carried out for 1 day and the animals returned to the RSMAS laboratory where they are kept overnight. Swim performance was assessed the following day.

H.2 Test Procedure

1. Obtain clean 10-gal glass aquaria needed for test. Label each aquarium test treatment.

2. Fill each dish with ~ 8 gal of the corresponding treatment medium. Unused test media should be disposed of appropriately.

3. Place 2–3 organisms in each aquarium.

4. Complete bench sheets as described in the QAPP.

5. Leave aquaria in a secure laboratory area until water tables are ready.

6. In the morning, turn on water to water tables to cool to desired temperature.

7. Place aquaria in water tables. Care should be taken to prevent them from floating.

8. Measure temperature and DO content from each UV treatment. Place plastic sheets over appropriate UV treatments.

9. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

10. Check temperature and DO hourly, and adjust flow of cooling as needed.

11. After approximately 4–6 hours of exposure to sunlight, remove organisms from the test chamber and place in new WAF. Return aquaria to the RSMAS laboratory for an overnight period. Assess swim performance the following day per the RSMAS GLPP Testing Protocol 9.

I. Testing Protocol 9: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil HEWAF Exposure to Larval Grass Shrimp (*Palaemonetes pugio*)

Survival of larvae hatched from gravid females was determined following exposure to WAF/UV light; gravid females were obtained from collections made by NOAA (Charleston) or GCRL. The larval survival studies were done outdoors with and without UV exposure. Larvae were collected that hatched within a 24-hour period.

I.1 Testing Apparatus Design

Testing apparatus for outdoor UV tests with grass shrimp larvae was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to WAF treatments as static renewal in 250-mL glass crystallizing dishes. Loading of organisms into chambers never exceeded 0.5 g/L. Test dishes were placed in blueboard insulation floats and floated in a water table placed in an unshaded location between 8:00 a.m. and 6:00 p.m. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hours (see test-specific TCTs).

I.2 Test Procedure

1. Obtain clean 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Dispose of unused test media appropriately.

3. Place 10 organisms in each test dish and feed as appropriate (see test-specific TCTs for details).

4. Complete test-specific bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight.

6. In the morning, turn on water to water tables to cool to desired temperature.

7. Note any mortality (do not replace organisms). Remove all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

8. Label blueboard floats with appropriate UV treatment. Place test dishes in appropriate floats by treatment.

9. By 9:00 a.m., place floats in the water table under appropriate plastic (e.g., UV transparent for 100% UV). Transfer dishes to blueboard floats, making sure to maximize direct contact with the cooling water.

10. Measure temperature and DO content of five randomly selected dishes from each UV treatment. Place plastic sheets over appropriate UV treatments.

11. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

12. Check the temperature and DO hourly, and adjust the flow of cooling water as needed.

13. After approximately 8 hours of exposure to sunlight, count dead and living organisms in each dish and remove dead organisms per step 7 above. WAF in dishes should be renewed at this time.

14. Leave dishes in a secure location overnight.

15. The next morning, repeat procedure from step 6.

16. Carry out tests until the last organism in the lowest WAF concentration has died, or for a maximum of 48–96 hours (see test-specific TCTs).

J. Testing Protocol 10: Assessing Photo-Enhanced Effects of Spiked Sediment Maternal Exposure to Larval Grass Shrimp (*Palaemonetes pugio*)

Survival of larvae that were exposed to UV, and that hatched from gravid females that were exposed to spiked sediments, was determined. The larval survival studies were done indoors (with and without UV) using a high-intensity lighting system. For each sediment concentration, there were three replicate adult exposure tanks. For each adult exposure tank, there were three replicate dishes of larvae under each UV intensity. Larvae were exposed to 100%, 50%, or 0% of the full UV light. Each replicate contained 200 mL clean ASW at 15-ppt salinity with 10 organisms.

J.1 Testing Apparatus Design

J.1.1 Sediment exposure

Sediments were spiked at UNT following the protocol listed in Section 10.6 of the Pacific EcoRisk GLPP (Protocol for Preparation of Spiked Sediment). Sediment tanks consisted of 20-gal glass aquaria with 1 kg of sediment per aquarium and 7 L of water on a 16:8 light/dark photoperiod. Water was renewed (80%) every other day and the water quality was checked daily. Five adult “early gravid” female shrimp obtained from NOAA (Charleston Laboratory) were placed in each sediment tank. Females were left on sediments for ~13 days and then moved to 2-L glass chambers containing ASW until the eggs hatched. Chambers were checked several times per day and hatched larvae were collected and held in separate glass containers for no more than 24 hours for use in UV survival tests. Larvae from several females within an adult replicate tank were pooled, but larvae between replicate adult tanks were not pooled.

J.1.2 Indoor UV exposure

Testing apparatus for indoor UV tests with larval grass shrimp consisted of overhead high-intensity UV lights (100% UV) or fluorescent (0% UV) lights suspended over a laboratory table. Height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a “sunny” summer day. Because of the height of the lights and room temperature control, no additional water bath cooling was needed. Larvae were exposed in clean water as static renewal in 250-mL glass crystallizing dishes. The total density of organisms in a chamber never exceeded 0.5 g/L. Test dishes were placed on the tabletop under either 100% or 0% UV for

8 hours followed by a 16-hour dark cycle. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hours (see test-specific TCTs).

J.2 Spiked Sediment Exposure Experimental Methods

1. Obtain clean, 20-L glass aquaria needed for test. Label each aquarium with test treatment and tank number.

2. Fill each aquarium with 1 kg of corresponding spiked or control sediment and 7-L ASW.

3. Place five adult "early gravid" female shrimp in each test aquarium.

4. Complete bench sheets as described in the QAPP.

5. Every 24 hours, monitor DO, temperature, ammonia, and salinity according to water quality SOPs described in UNT GLPP. Check adult shrimp for mortality. Record any mortalities and remove any dead shrimp and archive according to the QAPP.

6. After 13 days, remove shrimp from aquaria with sediments and place in 2-L tanks filled with 15-ppt ASW.

7. Check 2-L chambers several times per day for hatched larvae. If larvae are present, collect and hold larvae in separate glass containers with 2 L of clean 15-ppt ASW for no more than 24 hours until transferring them to crystallizing dishes for the start of the UV survival test (see Section J.3). Larvae from several females within an adult replicate tank can be pooled. Do not pool larvae from different replicate tanks.

8. Once an adult female has hatched her eggs, collect and euthanize by placing in a freezer. Archive according to the QAPP.

J.3 Indoor (UV exposure) Experimental Methods

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200-mL clean ASW using a glass cylinder.

3. Place 10 organisms in each test dish.

4. Complete bench sheets as described in the QAPP.

5. Place test dishes under UV lights and turn lights on.

6. Start UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

7. After approximately 8 hours of exposure to UV, turn off the lights, renew water, and count mortalities (do not replace organisms). Remove all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required. Leave test dishes in the dark overnight.

8. The next morning, assess mortality according to step 7 and then repeat UV exposure according to steps 5–7.

9. Continue test until the last organism in the lowest sediment concentration has died or for the maximum number of hours specified in test-specific TCTs.

K. Testing Protocol 11: Assessing Photo-Enhanced Effects of Spiked Sediment Exposure to Adult Grass Shrimp (*Palaemonetes pugio*)

Survival of adults exposed to UV following exposure to spiked sediments was determined. Survival studies were done indoors (with and without UV) using a high-intensity lighting system. For each sediment concentration, there were three replicate tanks. Adults were exposed to full UV light or 0% of the full UV light. Each replicate contained 19 L of clean ASW at 15-ppt salinity with five organisms per tank.

K.1 Testing Apparatus Design

Sediments were spiked at UNT following the protocol listed in Section 10.6 of the Pacific EcoRisk GLPP (Protocol for Preparation of Spiked Sediment). Sediment tanks consisted of 20-gal glass aquaria with 1 kg of sediment per aquarium. Ten female adult shrimp were placed in each sediment tank. Females were held on sediments for 6 days and then moved to 20-L test chambers containing ~ 19 L of clean water for UV exposure. The 10 females from each sediment replicate were split into two groups of 5, one group under 100% UV light and one group under 0% UV light.

The testing apparatus for indoor UV tests with grass shrimp consisted of overhead high-intensity UV lights or fluorescent lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a “sunny” summer day. Because of the height of the lights and room-temperature control, no additional waterbath cooling was needed. Adults were exposed in clean water. The density of organisms loaded into chambers never exceeded 0.5 g/L. Test tanks were placed on the tabletop under either 100% or 0% UV for 8 hours followed by a 16-hour dark cycle. Mortality was assessed daily, and tests were carried out until the last organism died or for a maximum of 48–96 hours (see test-specific TCTs).

K.2 Spiked Sediment Exposure Experimental Methods

1. Obtain clean, 20-L glass aquaria needed for test. Label each aquarium with test treatment and tank number.

2. Fill each aquarium with 1 kg of corresponding spiked or control sediment and 7 L of water.

3. Place 10 adult female shrimp in each test aquarium.

4. Complete bench sheets as described in the QAPP.

5. Every 24 hours, monitor DO, temperature, ammonia, and salinity according to water quality SOPs described in UNT GLPP. Check adult shrimp for mortality. Record any mortalities and remove any dead shrimp and archive according to the QAPP.

6. After 6 days, remove shrimp from the aquaria with sediments and place in a new 20-L glass aquaria filled with ~ 19 L of clean 15-ppt ASW for the start of UV exposure (see Section K.3).

K.3 Indoor (UV exposure) Experimental Methods

1. Obtain clean, 20-L glass tanks needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 19 L of clean ASW using a glass cylinder.

3. Place five organisms from the appropriate sediment tank into each aquaria.

4. Complete bench sheets as described in the QAPP.

5. Place aquaria under UV lights and turn the lights on.

6. Start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

7. After approximately 8 hours of exposure to UV, turn off the lights, renew the water, and count mortalities (do not replace organisms). Remove all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required. Leave aquaria in the dark overnight.

8. The next morning, assess mortality according to step 7 and then repeat UV exposure according to steps 5–7.

9. Continue the test until the last organism in the lowest sediment concentration has died or for the maximum number of hours specified in test-specific TCTs.

L. Testing Protocol 12: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil WAF Exposure to Red Drum (*Sciaenops ocellatus*) and Speckled Seatrout (*Cynoscion nebulosus*)

The survival of red drum and speckled seatrout embryo and larval life stages exposed to WAFs were assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light; for details regarding the number of replicates, see test-specific TCTs. In addition, some tests included a 50% UV treatment. Each replicate contained 200 mL of WAF and 10 organisms.

L.1 Testing Apparatus

The testing apparatus for outdoor UV tests with red drum and speckled seatrout was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to treatment media as static renewal in 250-mL glass crystallizing dishes (10 individuals per dish, 5 replicate dishes per treatment). The density of organisms in chambers never exceeded 0.5 g/L. Test dishes were then placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in a water table. The water table was placed in an unshaded location between 10:00 a.m. and 4:00 p.m.

L.2 Test Procedure

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder (see test-specific TCTs). Prepare treatment medium according to the QAPP. Collect chemistry samples as described in the QAPP. Dispose of unused test media appropriately.

3. Place 10 organisms in each test dish (as specified in test-specific TCTs).

4. Complete test forms as described in the QAPP.

5. Leave dishes in a secure laboratory area until outside water tables are ready.

6. Turn on water to water tables to cool to desired temperature.

7. Place test dishes in appropriate floats by treatment. Dishes should be floated to maximize direct contact with the cooling water. Ensure blueboard floats are labeled with appropriate UV exposure (e.g., 100%, 50%, 10%).

8. Measure the temperature and DO content of five randomly selected dishes from each UV treatment.

9. Place plastic sheets over appropriate UV treatments.

10. Start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

11. After approximately 6 to 8 hours of exposure to sunlight, collect dishes and bring indoors. Hold organisms until ~ 24 hours have passed since larvae were initially loaded into test dishes. For example, if larvae were loaded at 10 p.m. the previous evening and dishes brought inside at 4 p.m., do not proceed to step 12 until 10 p.m. Total test length is 24 hours from the time of initial loading.

12. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

M. Testing Protocol 13: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Slick Exposure on Speckled Seatrout (*Cynoscion nebulosus*)

The survival of speckled seatrout embryos exposed to oil slicks was assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light; for details regarding the number of replicates, see test-specific TCTs. In addition, some tests included a 50% UV treatment. Each replicate contained 200 mL of WAF and 10 to 20 organisms (see test-specific TCTs).

M.1 Testing Apparatus

The testing apparatus for outdoor UV tests with red drum and speckled sea trout was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to treatment media as static renewal in 250-mL glass crystallizing dishes (10 individuals per dish, 5 replicate dishes per treatment). The density of organisms in chambers never exceeded 0.5 g/L. Test dishes were then placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in a water table. The water table was placed in an unshaded location between 10:00 a.m. and 4:00 p.m.

M.2 Slick Preparation

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with test treatment and tank number.

2. Fill each dish with 200 mL of source water. Use the “Tank ID, Dilution, or Stock Code Definitions” datasheet to track tank IDs, respective dilutions, and start/stop dates and times.

3. Weigh 1.5 g of oil in a clean aluminum weigh boat.

4. Tare the polyvinyl chloride (PVC) coupling on the top-loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 1.5 g of oil as possible around the inside of the PVC coupling about 2 cm from the edge.

6. Weigh the PVC coupling with the oil to confirm that the total amount of oil added to the PVC coupling was between 1.4 and 1.6 g. Place oiled PVC on a clean tray to store until start of test.

7. Repeat steps 1–6 for each replicate.

8. Place PVC coupling (oiled side down) into labeled test dishes with source water so that the PVC coupling rests on the bottom of the dish. The water's surface should intersect the oil from the PVC coupling (Figure M.1). Repeat for each replicate.

9. Allow the PVC coupling to soak in the dishes for approximately 4 hours.

Figure M.1. Top view of the crystallizing dish slick exposure set-up.

M.3 Test Procedure

1. Before starting the test, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) in a stock source water sample, as described in the QAPP and Stratus Consulting – Red Drum and Speckled Seatrout GLPP document. Record water quality results on standard "Water Quality Monitoring" datasheets. Note that during testing, the light cycle may vary because of routine hatchery operations.

2. Collect embryos from the egg collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the egg collector. Transfer a sub-sample of embryos from the beaker to Petri dishes for sorting.

3. Use a pipette to collect 10 embryos from the Petri dish. Note that all 10 embryos should be in the pipette simultaneously for transfer to the test dish. If needed, use a separate dish to transfer and count out embryos, and then collect them simultaneously.

4. To avoid disturbing the oil slick, gently place the pipette into the exposure water between the PVC coupling and the edge of the beaker.

5. Simultaneously, expel the embryos while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer of embryos into the test beakers. Record start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" datasheet and replace the exposure vessel back into the water bath. Mark the lip of loaded beakers with tape and repeat the above procedure until all beakers contain embryos.

6. Leave dishes in a secure laboratory area overnight until the outside water tables are ready the next morning.

7. The next morning, turn on water to water tables to cool to desired temperature.

8. Place test dishes in appropriate floats by treatment. Dishes should be floated to maximize direct contact with the cooling water. Ensure blueboard floats are labeled with appropriate UV exposure (e.g., 100%, 50%, 10%).

9. Measure the temperature and DO content of five randomly selected dishes from each UV treatment.

10. Place plastic sheets over appropriate UV treatments.

11. Start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

12. After approximately 6 to 8 hours of exposure to sunlight, collect dishes and return them indoors. Leave the organisms until approximately 24 hours has passed since larvae were initially loaded into test dishes. For example, if larvae were loaded at 10 p.m. the previous evening and dishes brought inside at 4 p.m., do not proceed to step 12 until 10 p.m. Total test length is 24 hours from the time of initial loading.

13. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

N. Testing Protocol 14: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Slick Exposure on Mahi-Mahi, Red Snapper, and Bay Anchovy

The survival of fish embryos exposed to oil slicks with UV light was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV light. Each replicate contained 200 mL of test solution and 10–20 organisms. For details, see test-specific TCTs.

N.1 Testing Apparatus

The testing apparatus consisted of overhead high-intensity UV lights or fluorescent (no-UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter the intensity at the table surface to mimic a sunny summer day. For mahi-mahi testing, a temperature-controlled room was used to maintain temperature of exposure dishes; for bay anchovy and red snapper testing, a recirculating water bath was used to maintain temperature. Exposure dishes were placed under either 100% or 0% UV light for several hours followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 48 hours (see test-specific TCTs).

N.2 Slick Preparation

1. Obtain clean, 250-mL glass dishes needed for test. Label each dish with the test treatment and tank number.

2. Fill each dish with 200 mL of source water. Use the “Tank ID, Dilution, or Stock Code Definitions” datasheet to track tank IDs, respective dilutions, and start/stop dates and times.

3. Weigh 1.5 g of oil in a clean aluminum weigh boat.

4. Tare the PVC coupling on the top-loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 1.5 g of oil as possible around the inside of the PVC coupling, about 2 cm from the edge.

6. Weigh the PVC coupling with the oil to confirm that the total amount of oil added to the PVC coupling was between 1.4 and 1.6 g. Place oiled PVC on a clean tray to store until start of the test.

7. Repeat steps 1–6 for each replicate.

8. Place PVC coupling (oiled side down) into labeled test dishes with source water so that the PVC coupling rests on the bottom of the dish. The water's surface should intersect the oil from the PVC coupling (Figure N.1). Repeat for each replicate.

9. Allow the PVC coupling to soak in the dishes for approximately 4 hours.

Figure N.1. Top view of the crystallizing dish slick exposure setup.

N.3 Test Procedure

1. Before starting the test, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) in a stock source water sample, as described in the QAPP. Record water quality results on standard "Water Quality Monitoring" datasheets. Note that during testing, the light cycle may vary (see test-specific TCTs).

2. Collect embryos from hatchery personnel (see Chapters 7 and 16). Transfer a sub-sample of embryos from the beaker to Petri dishes for sorting.

3. Use a pipette to collect 10–20 embryos from the Petri dish (see test-specific TCTs). Note that all 10–20 embryos should be in the pipette simultaneously for transfer to the test

dish. If needed, use a separate dish to transfer and count out embryos, and then collect them simultaneously.

4. To avoid disturbing the oil slick, gently place the pipette into the exposure water between the PVC coupling and the edge of the beaker.

5. Simultaneously expel the embryos while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer of embryos into the test beakers. Record start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" datasheet and place the exposure vessel back into the water bath. Mark the lip of loaded beakers with tape and repeat the above procedure until all beakers contain embryos.

6. Either leave dishes in a secure laboratory area overnight or immediately place under UV lighting (see test-specific TCTs).

7. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout the UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

8. After UV exposure is complete (see test-specific TCTs for UV exposure duration), collect dishes and turn off lights.

9. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless an analysis of individually identified organisms is required.

O. Testing Protocol 15: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil WAF on Red Snapper and Bay Anchovy

The survival of fish embryos exposed to WAFs was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV light. Each replicate contained 200 mL of test solution and 10–20 organisms. For details, see test-specific TCTs.

O.1 Testing Apparatus

The testing apparatus consisted of overhead high-intensity UV lights or fluorescent (no UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a sunny summer day. A recirculating water bath was used to maintain temperature for snapper and anchovy testing. Test tanks were placed under either 100% or 0% UV for several hours (see test-specific TCTs for UV exposure duration) followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 48 hours (see test-specific TCTs).

O.2 Test Procedure

1. Obtain the clean 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number (replicate number).

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10–20 organisms in each test dish (see test-specific TCTs).

4. Complete bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight until the next morning, or place immediately under UV lights (see test-specific TCTs).

6. Measure temperature and DO content of randomly selected dishes from each UV treatment.

7. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout the UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

8. After UV exposure is complete (see test-specific TCTs for UV exposure duration), collect dishes and turn off UV lights.

9. Count the number of dead and living organisms. Sample all dead test organisms and retain them according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of the individually identified organisms is required.

10. Leave dishes in the dark and in a secure location overnight.

11. The next morning, repeat the procedure.

12. Carry out the tests until the last organism in the lowest WAF concentration has died, or for a maximum of 48 hours.

P. Testing Protocol 16: Acartia Copepod Acute Toxicity Test

The survival of adult copepods exposed to WAF was assessed. For each treatment concentration, there were three to five replicates exposed to full UV light and three to five replicates exposed to approximately 10% of the full UV light; for details regarding the number of replicates, see test-specific TCTs. In addition, some tests included a 50% UV treatment. Each replicate contained 200 mL of WAF and 10–20 organisms (see test-specific TCTs).

P.1 Testing Apparatus

The testing apparatus for outdoor UV tests with adult copepods was the same as described in Section 8.2 (Methods) of the UNT GLPP. Organisms were exposed to treatment media in 250-mL glass crystallizing dishes (10 individuals per dish, 5 replicate dishes per treatment). The density of organisms in chambers never exceeded 0.5 g/L. Test dishes were then placed in blueboard insulation floats (blueboard available at Home Depot, Lowes, etc.) and floated in a water table. The water table was placed in an unshaded location between 10:00 a.m. and 4:00 p.m.

Before beginning toxicity tests, all glassware was pre-cleaned according to the *Decontaminating Glassware Standard Operating Protocol*, as described in the QAPP. All test water was obtained from the UV-sterilized seawater system at the University of Miami Experimental Hatchery (see RSMAS GLPP; Chapter 7).

Note: Experiments were set up using a synchronous culture, where all animals were the same age (within 24 hours). Adult copepods were approximately 17 days old at test initiation.

P.2 Test Procedure

1. Prepare desired WAFs in advance according to the *Protocols for Preparing Water Accommodated Fractions SOP*, as described in the QAPP and RSMAS GLPP. HEWAF is made the morning of experimental setup.

2. Remove a subsample of each WAF for chemical analysis by ALS Environmental as specified in the QAPP. Fill each sample jar (provided by ALS Environmental) to capacity. Conduct all sample collection, labeling, and handling as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP**.** Store samples at 4°C; record all necessary sample numbers and prepare necessary chain-of-custody (COC)

documentation, as described in the QAPP. Ship samples overnight on ice to ALS Environmental as soon as possible

3. Obtain the clean 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number (replicate number).

4. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

5. Before adding test organisms to the aforementioned dishes, take initial measurements of water temperature, pH, dissolved oxygen, and salinity within each representative chamber, as outlined in the *Temperature Measurement SOP*, *pH Measurement SOP*, *Measurement of Dissolved Oxygen SOP*, and *Measurement of Salinity SOP*, respectively. All SOPs can be found in the QAPP and RSMAS GLPP. See the respective SOPs for the individual measurements and collection protocols. Ensure that all necessary COC documentation is prepared and meter calibration logs are filled out.

6. Place 10–20 organisms in each test dish (see test-specific TCTs). To transfer copepods place clean crystallizing dish with adult, age-synchronized copepods on a counter in a dark room, and use a small light-emitting diode (LED) light, placed against the side of the dish to concentrate the animals. Using a glass Pasteur pipette, remove 20 copepods from those that are gathered by the light. Confirm under a dissecting microscope that the correct number of copepods has been removed, and gently transfer the animals to the test dish, taking care to prevent contamination by not allowing the pipette to come into contact with the treatment water.

7. Complete bench sheets as described in the QAPP.

8. After all the replicates are set up, hold them indoors in a secure location overnight.

9. The next morning, move the test dishes to a water bath tank outside. The water bath consists of an 80-gallon, 183 x 51 x 25 cm fiberglass trough, through which 25°C water is continuously run at approximately 50 gallons per minute. The water bath is located in an area where it will receive maximum sunlight throughout the day. The 250-mL beakers are floated in the water bath using a 0.5-inch Styrofoam sheet, through which holes have been punched that exactly fit the beaker diameter. Aeration for each beaker is accomplished using PE60 tubing with a 22G x 1.5-inch hypodermic needle (plastic hub removed), set to approximately 5 bubbles/second. Half of the water bath is covered with a UV-transparent plastic sheet (the UV-exposed treatment), while the other half of the water bath is covered with a UV-opaque plastic sheet (the UV-blocked treatment).

10. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout the UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

11. After UV exposure is complete (see test-specific TCTs for UV exposure duration), collect dishes and turn off UV lights.

12. Perform daily measurements of water chemistry and physical parameters on representative chambers, as outlined in the QAPP and RSMAS GLPP. Feed the animals in each beaker daily with 5×10^3 cells/mL of *Isochrysis* (TISO) and 5×10^3 cells/mL of *Cheatocerous* (CHGRA).

13. Make the final survival count and reproductive output measurement after 96 hours. Gently pour each test dish through a 40-µm filter to concentrate the animals and eggs, and then place the organisms into a clean, glass crystallizing dish. Retain the treatment water for final water quality analysis (see Step 15). Determine the number of live and dead adults under a variable-power dissecting microscope. Confirm dead animals by gently prodding with a dissection probe while visually looking for movement under high magnification. After adult survival counts have been determined, pipette eggs out and place them in clean seawater to determine hatching success. Fix the remaining animals in each beaker (live adults and nauplii) using formalin and stain with the addition of Rose Bengal to make counting nauplii easier.

14. Determine the sex of all adult copepods by microscopic examination of uropod segments and antennae morphology using an inverted compound scope.

15. Retain all adult animals and nauplii to the extent possible, preserved in 10% buffered formalin and archived according the QAPP.

16. Discard remaining test solutions as outlined in *WAF and Toxicity Test Water Disposal Procedures SOP* found in the RSMAS GLPP.

17. The test will be said to pass test criteria if the average control survival exceeds 85% across the four replicates, and if there are no unacceptable deviations in water quality among replicates, as described in the QAPP.

Q. Testing Protocol 17: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil WAF or Dispersant on Mysid Shrimp

The survival of mysid shrimp exposed to WAF or dispersant was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV light. Each replicate contained 200 mL of test solution and 10 organisms. For details, see test-specific TCTs.

Q.1 Testing Apparatus

The testing apparatus for mysid shrimp indoor UV tests consisted of overhead high-intensity UV lights or fluorescent (no-UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter the intensity at the table surface to mimic a sunny summer day. Test tanks were placed on the tabletop under either 100% or 0% UV for several hours (see test-specific TCTs), followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 96 hours (see test-specific TCTs).

Q.2 Test Procedure

1. Obtain the clean 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number (replicate number).

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Unused test media should be disposed of appropriately.

3. Place 10 organisms in each test dish.

4. Complete bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight until the next morning, or place them immediately under UV lights (see test-specific TCTs).

6. Measure temperature and DO content of randomly selected dishes from each UV treatment.

7. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout the UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

8. After several hours of exposure to UV (see test-specific TCTs for times), collect the dishes and return them indoors.

9. Count the number of dead and living organisms. Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Leave dishes in a secure location overnight.

11. The next morning, repeat the procedure.

12. Carry out the tests until the last organism in the lowest WAF concentration has died, or for a maximum of 48 hours.

R. Testing Protocol 18: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Slick Exposure on Mysid Shrimp

The survival of mysid shrimp exposed to oil slicks was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV light. Each replicate contained 200 mL of test solution and 10 organisms. For details, see test-specific TCTs.

R.1 Testing Apparatus

The testing apparatus for indoor UV tests with mysids consisted of overhead high-intensity UV lights or fluorescent (no-UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a sunny summer day. Test tanks were placed on the tabletop under either 100% or 0% UV for several hours (see test-specific TCTs) followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 96 hours (see test-specific TCTs).

R.2 Slick Preparation

1. Obtain the clean, 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number.

2. Fill each dish with 200 mL of source water. Use the “Tank ID, Dilution, or Stock Code Definitions” datasheet to track tank IDs, respective dilutions, and start/stop dates and times.

3. Weigh 1.5 g of oil in a clean aluminum weigh boat.

4. Tare the PVC coupling on the top-loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 1.5 g of oil as possible around the inside of the PVC coupling, about 2 cm from the edge.

6. Weigh the PVC coupling with the oil to confirm that the total amount of oil added to the PVC coupling was between 1.4 and 1.6 g. Place the oiled PVC on a clean tray to store until the start of the test.

7. Repeat steps 1–6 for each replicate.

8. Place the PVC coupling (oiled side down) into labeled test dishes with source water so that the PVC coupling rests on the bottom of the dish. The water's surface should intersect the oil from the PVC coupling (Figure R.1). Repeat for each replicate.

9. Allow the PVC coupling to soak in the dishes for approximately 4 hours.

Figure R.1. Top view of the crystallizing dish slick exposure set-up.

R.3 Test Procedure

1. Before starting the test, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) in a stock source water sample, as described in the UNT GLPP. Record water quality results on standard "Water Quality Monitoring" datasheets. Note that during testing, the light cycle may vary (see test - specific TCTs).

2. Use a pipette to transfer 10 mysids. Note that all 10 individuals should be in the pipette simultaneously for transfer to the test dish. If needed, use a separate dish to transfer and count out mysids, and then collect them simultaneously.

3. To avoid disturbing the oil slick, gently place the pipette into the exposure water between

the PVC coupling and the edge of the beaker.

4. Simultaneously expel the mysids while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer into the test beakers. Record the start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" datasheet and replace the exposure vessel back into the water bath. Mark the lip of loaded beakers with tape and repeat the above procedure until all beakers contain mysids.

5. Either leave the dishes in a secure laboratory area overnight, or immediately place under UV lighting (see test-specific TCTs).

6. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout the UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

7. After the UV exposure is complete (see test-specific TCTs), collect the dishes and turn off the lights.

8. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

S. Testing Protocol 19: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil WAF on White Shrimp

The survival of white shrimp exposed to WAF was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV light. Each replicate contained 200 mL of test solution and 10 organisms. For details, see test-specific TCTs.

S.1 Testing Apparatus

The testing apparatus for white shrimp indoor UV tests consisted of overhead, high-intensity UV lights or fluorescent (no-UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter the intensity at the table surface to mimic a sunny summer day. Test tanks were placed on the tabletop under 100%, 50%, or 0% UV for several hours (see test-specific TCTs), followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 96 hours (see test-specific TCTs).

S.2 Test Procedure

1. Obtain clean, 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number (replicate number).

2. Fill each dish with 200 mL of the corresponding treatment medium using a glass cylinder. Dispose of unused test media appropriately.

3. Place 10 organisms in each test dish.

4. Complete bench sheets as described in the QAPP.

5. Leave dishes in a secure dark laboratory area overnight until the next morning, or place them immediately under UV lights (see test-specific TCTs).

6. Measure temperature and DO content of randomly selected dishes from each UV treatment.

7. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout the UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

8. After several hours of exposure to UV (see test-specific TCTs for times), collect the dishes and return them indoors.

9. Count the number of dead and living organisms. Sample all dead test organisms and retain according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

10. Leave dishes in a secure location overnight.

11. The next morning, repeat the procedure.

12. Carry out the tests until the last organism in the lowest WAF concentration has died, or for a maximum of 96 hours.

T. Testing Protocol 20: Assessing Photo-Enhanced Effects of *Deepwater Horizon* Oil Slick Exposure on White Shrimp

The survival of white shrimp exposed to oil slicks was assessed. Some replicates included exposure to full UV light from a high-intensity lighting system, and some replicates included a 50% UV treatment. Control replicates were exposed to fluorescent lighting with no UV light. Each replicate contained 200 mL of test solution and 10 organisms. For details, see test-specific TCTs.

T.1 Testing Apparatus

The testing apparatus for indoor UV tests with white shrimp consisted of overhead high-intensity UV lights or fluorescent (no-UV) lights suspended over a laboratory table. The height of the lights above the exposure dishes was adjusted to alter intensity at the table surface to mimic a sunny summer day. Test tanks were placed on the tabletop under either 100%, 50%, or 0% UV for several hours (see test-specific TCTs), followed by a dark cycle. Mortality was assessed daily, and tests were carried out for a maximum of 96 hours (see test-specific TCTs).

T.2 Slick Preparation

1. Obtain the clean, 250-mL glass dishes needed for the test. Label each dish with the test treatment and tank number.

2. Fill each dish with 200 mL of source water. Use the “Tank ID, Dilution, or Stock Code Definitions” datasheet to track tank IDs, respective dilutions, and start/stop dates and times.

3. Weigh 1.5 g of oil in a clean aluminum weigh boat.

4. Tare the PVC coupling on the top-loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 1.5 g of oil as possible around the inside of the PVC coupling, about 2 cm from the edge.

6. Weigh the PVC coupling with the oil to confirm that the total amount of oil added to the PVC coupling is between 1.4 and 1.6 g. Place the oiled PVC on a clean tray to store until the start of the test.

7. Repeat steps 1–6 for each replicate.

8. Place the PVC coupling (oiled side down) into labeled test dishes with source water so that the PVC coupling rests on the bottom of the dish. The water's surface should intersect the oil from the PVC coupling (Figure T.1). Repeat for each replicate.

9. Allow the PVC coupling to soak in the dishes for approximately 4 hours.

Figure T.1. Top view of the crystallizing dish slick exposure setup.

T.3 Test Procedure

1. Before starting the test, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) in a stock source water sample, as described in the UNT GLPP. Record water quality results on standard "Water Quality Monitoring" datasheets. Note that during testing, the light cycle may vary (see test - specific TCTs).

2. Use a pipette to transfer 10 shrimp. Note that all 10 individuals should be in the pipette for transfer to the test dish. If needed, use a separate dish to count the shrimp and then transfer them.

3. To avoid disturbing the oil slick, gently place the pipette into the exposure water between the PVC coupling and the edge of the beaker.

4. Simultaneously expel the shrimp while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer into the test beakers. Record the start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" datasheet and return the exposure vessel to the water bath. Mark the lip of loaded beakers with tape and repeat the above procedure until all beakers contain shrimp.

5. Either leave the dishes in a secure laboratory area overnight, or immediately place under UV lighting (see test-specific TCTs).

6. Before UV exposure begins, start the UV monitor. Monitor UV continuously using a BioSpherical PUV multi-wavelength radiometer throughout the UV exposure. The PUV measures wavelengths in the UV-A, UV-B, and visible light spectra.

7. After the UV exposure is complete (see test-specific TCTs), collect the dishes and turn off the lights.

8. Count the number of dead and living organisms. Retain all dead and living test organisms according to the QAPP. Organisms removed from the same exposure chamber at the same time may be frozen and stored in the same container, unless analysis of individually identified organisms is required.

9. University of Maryland General Laboratory Procedures and Practices

9.1 Introduction

The University of Maryland (UMD) conducted toxicity tests to identify toxicological impacts of the 2010 *Deepwater Horizon* oil spill on blue crab and turtles. Red-eared sliders and snapping turtles were used as surrogates for sea turtles. This chapter describes the general laboratory practices and procedures (GLPP) used at UMD.

9.2 Test Organism Sources and Husbandry

9.2.1 Blue Crab: *Callinectes sapidus*

Juvenile blue crabs were obtained from the UMD Institute of Marine and Environmental Technology (IMET) blue crab aquaculture facility. The husbandry techniques used by IMET to rear blue crabs are proprietary. All blue crabs were used in toxicity testing immediately upon receipt from IMET.

9.2.2 Turtles: Red-eared sliders and snapping turtles

One-month-old hatchling snapping turtles [~ 1" straight carapace length (SCL)] and 4" red-eared sliders were purchased from the Concordia Turtle Farm (Louisiana) in September 2012 and March 2013, respectively. The snapping turtles were originally housed in a temperature-controlled room in individual 5-gallon glass tanks, and then later moved to 10-gallon glass tanks. All tanks contained freshwater that was replaced at a minimum of every other day. The temperature of the room was kept at 72°F (22°C) and monitored continuously using a web-based data logger. The turtles were placed under reptile-specific ultraviolet (UV-A/UV-B) bulbs with a 10:14 light:dark cycle.

Red-eared sliders were housed in groups of 25–28 turtles per tank. The tanks were 10 x 250-gallon flow-through tanks that contained 90 gallons of freshwater. The tanks were maintained at ~ 79°F (26°C). Each tank contained a floating basking platform and an overhead reptile-specific UV-A/UV-B lamp for light and heat.

To ascertain health and feeding rate, a visual inspection and monitoring of all turtles were carried out daily. Turtles were fed with commercial pellet food and/or live mealworms every 2 days. Tanks were cleaned every other day. In addition to state and federal permits required to purchase

and move turtle hatchlings < 4-inch SCL across state lines, Institutional Animal Care and Use Committee (IACUC) approval (IACUC # F-CBL-12-06) was obtained for animal collection and housing at the Chesapeake Biological Laboratory (CBL) at UMD.

9.3 Exposure Media Preparations

9.3.1 Field-collected sediments

For the blue crab toxicity test, the following field-collected sediments were obtained from Stratus Consulting:

1. South East Pass Campground 2011
2. South East Pass 2011
3. South Pass Spit 2011
4. Black Hole 2012
5. Black Hole 2011
6. Loomis II 2011.

Preparation procedures for sediments and blended sediments are described in the individual testing protocols.

9.3.2 *Deepwater Horizon* oils

For the turtle studies, turtles were orally dosed with Slick A oil. Preparation of oil/feed mixture and oral dosing procedures are described in the individual testing protocols.

9.3.3 Source water/dilution water

Seawater used for all blue crab exposure studies was prepared in bulk at UMD's Aquaculture Research Center (ARC) using a proprietary mix of salts and charcoal-filtered tap water. Filtered tap water and seawater obtained from ARC were then blended to achieve desired salinity as specified on test-specific test conditions tables (TCTs).

The water used in the turtle exposure tanks was 0.2-µm filtered tap water from CBL.

9.4 Reporting and Testing Documentation

Data management, documentation, quality assurance/quality control (QA/QC), and reporting were handled as described in the Stratus Consulting *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP).

9.5 Water Quality Standard Operating Procedures

9.5.1 Temperature and dissolved oxygen measurements

Temperature and dissolved oxygen (DO) were measured using a YSI ProODO meter with a ProBOD probe.

At the start of each day, the YSI ProBOD probe was calibrated for DO% in a moist environment with deionized (DI) water having a salinity of 0 ppt. For DO measurements in saline test solutions, the salinity was input into the meter so that an internal correction could be used.

Each tank was tested by placing the probe into water deep enough to fully cover the temperature sensor. The sensor reading was allowed to stabilize before either parameter was recorded. The probe was thoroughly rinsed with DI water between treatments, always moving from least- to most-contaminated treatments, and then cleaned at the end of each day before returning to a moist environment.

The temperature (°C) and DO (mg L^{-1}) were recorded on the *Water Quality Monitoring* datasheet found in the Stratus Consulting QAPP.

9.5.2 pH measurements

The pH of each tank was examined using an Accumet Research ARIS pH meter with pH probe.

At the start of each day, the pH meter was calibrated using standard 4.0, 7.0, and 10.0 pH solutions. The calibration from the previous day was cleared and for each standard, the solution was allowed to fully stabilize before the standard was set. The calibration was repeated until a slope > 95% was reached.

The pH and temperature/DO measurements were simultaneously taken to maximize efficiency and time. Both probes were immersed in the water simultaneously. The pH measurement of a tank was recorded once the reading had fully stabilized. If there was a problem with the pH meter probe, ColorpHast indicator strips (6.5–10.0 pH, 0.3 units) were used to measure pH

levels. All pH measurements were recorded on the *Water Quality Monitoring* datasheet found in the Stratus Consulting QAPP.

9.5.3 Salinity measurements

The salinity for each tank was measured using a portable refractometer and disposable pipettes.

When measuring salinity, a small water sample was pipetted out from each tank and one water drop was added onto the lens of the refractometer, ensuring that the lens was completely covered with water but not overflowing. Pipettes were changed between treatments to prevent cross-contamination. The refractometer was raised into the direct light and the salinity was determined by the location of the junction of light and dark regions on the salinity scale.

The salinity of each tank was recorded in the *Water Quality Monitoring* datasheet in the Stratus Consulting QAPP.

9.5.4 Ammonia measurements

The ammonia concentration was measured using an ammonia test kit (NH_3/NH_4^+) from Aquarium Pharmaceuticals. If ammonia levels were below 0.5 mg/L, additional tanks within a treatment were measured using a Hach DR/2400 portable spectrophotometer (Salicylate Method 8155 for Nitrogen, Ammonia).

Using the ammonia test kit (NH_3/NH_4^+), 2.5 mL of water from one tank was pipetted out with a disposable pipette and placed in a round-bottom test tube. Pipettes were changed for each treatment to prevent cross-contamination. Four drops (from each bottle in the kit) were added to the sample and thoroughly mixed. After 5 minutes, the ammonia levels were read using a color chart for "Saltwater" samples that differentiates among 0, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/L ammonia. Any sample with a color that looked to be in-between two of the colors on the chart was designated to be between those two levels of ammonia.

Using the Hach spectrophotometer, 10 mL of sample was added to a flat-bottomed sample cell and an Ammonia Salicylate Reagent Powder Pillow was added to the sample. The cell was capped, mixed thoroughly, and allowed to react for 3 minutes. Once the 3 minutes ended, an Ammonia Cyanurate Reagent Powder Pillow was added to the cell. The cell was capped, mixed thoroughly, and allowed to react for 15 minutes. A seawater sample blank was prepared by the same method in conjunction with the first test sample. Once the 15 minutes ended, the seawater control was measured using the Hach DR/2400 spectrophotometer and zeroed to 0.00 mg/L NH_3-N. The test sample was then measured using the Hach DR/2400, internally normalized to

the seawater blank, and the ammonia level was recorded. All subsequent samples for that day were measured using the same seawater blank. A new seawater blank sample was used each day.

All ammonia levels measured were recorded in a daily *Water Quality Monitoring* datasheet found in the Stratus Consulting QAPP.

9.6 General Turtle Toxicity Testing Standard Operating Procedures

The following sections describe the general standard operating procedures (SOPs) used during turtle toxicity testing, which are summarized in Table 9.1.

Table 9.1. Summary of general turtle toxicity testing SOPs

SOP #	Procedure	SOP #	Procedure
1	Turtle weight and carapace length	9	Packed cell volume (PCV)
2	Preparation of whole blood smears	10	Bicinchoninic acid assay (BCA) protein assay
3a	Subcarapacial venous sinus blood collection	11	Liver tissue homogenization procedure
3b	Cardiac puncture blood collection	12	Total glutathione (tGSH) and glutathione disulfide (GSSG) quantitation in blood cells and liver tissue
3c	Dorsal coccygeal vein blood collection	13	2-Thiobarbituric acid (TBA) reactive substances (TBARS) lipid peroxidation assay for plasma
4	Preparation of blood plasma samples	14	Lipid peroxidation assay for liver
5	Housing, feeding, and monitoring of turtles	15a	Total antioxidant assay for liver
6	Placement/insertion and monitoring of esophagostomy tubes (E-tubes)	15b	Total antioxidant assay for plasma
7	Euthanasia by cervical dislocation (decapitation followed by pithing of the brain)	16a	COMET assay for blood cells
8	Necropsy for blood and tissue collection	16b	COMET assay for liver tissue

9.6.1 SOP #1: Turtle weight and carapace length

1. Confirm that the top-loading balance is in proper working order and properly calibrated
2. Capture and restrain turtle

3. Carefully carry turtle to weighing station
4. Record turtle identification (ID) on the appropriate datasheet
5. Using calipers, measure the carapace length and record it on the appropriate datasheet
6. Place a large weigh boat on the calibrated scale and tare balance
7. Carefully place the turtle inside the container and ensure the container is secured
8. Record the weight on the appropriate datasheet
9. Remove the turtle from the container
10. Place the turtle back into its appropriate housing or continue to dosing or necropsy steps
11. Repeat steps 1–10 until all of the turtles have been weighed.

9.6.2 SOP #2: Preparation of whole blood smears

1. Ensure glass slides are clean, grease- and scratch-free, and have smooth edges without any cuts.

2. Place the label on frosted end nearest the edge; cross-check the label with the sample.

3. Using a micropipette, place a small drop of whole heparinized blood (2–5 µL) close to the end of the slide, approximately 0.5–1.0 cm from the label.

4. Place the short edge of another slide (the smearing slide) flat against the bottom of the slide, approximately 0.5 cm farther along the slide (away from the label) than the blood spot.

5. Hold the "smearing slide" at an angle of 45° from the surface of the flat slide.

6. Move the edge of the "smear slide" toward the blood spot until it touches.

7. Allow the surface tension of the blood spot to move the blood along the width of the smearing slide.

8. Smoothly draw the blood along the length of the slide to create a feathered edge that almost reaches the other end of the slide.

9. Check that the edge is feathered and there are no streaks. If streaks are present, repeat the procedure with a new labeled slide.

10. Allow the smears to air dry or, if necessary, dry the slides under a low stream of air from a blow-dryer.

11. Once the slides are dry, store them in a slide box at room temperature.

12. If any slides are broken or unusable, dispose of them appropriately.

9.6.3 SOP #3: Whole blood collection

Syringe preparation (applies to all three blood collection methods)

1. Aseptically open a syringe and attach a fresh needle if there is not a pre-attached needle.

2. Break the plunger seal by drawing and depressing the plunger.

3. Draw a small volume (sufficient to fill the needle only) of lithium heparin into the syringe.

4. Move the plunger so the lithium heparin coats the length of the needle and syringe, and expel excess anticoagulant.

5. Ensure that there is no fluid in the syringe and no droplet on the end of the needle.

6. Store the needle and syringe at room temperature until use.

7. Several needles and syringes can be prepared prior to sampling turtles.

9.6.4 SOP #3a: Subcarapacial venous sinus blood collection

Two people are needed to perform this method. Person #1 secures the body of the turtle, records the data, and ensures that samples are placed into the correct containers. Person #2 collects the blood.

1. Ensure that the turtle ID on the container label matches the ID of the turtle.

2. Place the turtle on its plastron on a flat surface on the edge of the bench.

3. Secure the turtle's head and use an ethanol swab to sterilize the area on top of the head. Swab toward the neck/body of the turtle where the subcarapacial venous sinus is located.

4. Locate the position for the subcarapacial venous sinus blood draw and adjust the turtle's position accordingly to ensure that the vein is accessible.

5. Insert a 25-gauge needle into the turtle's body, upward toward the carapace at an approximate 20–50° angle.

6. If the needle is properly inserted into the vein, there will be a small flush of blood into the syringe. Take note if it is pure blood, lymph-contaminated blood, or lymph.

7. If the draw is pure blood, gently pull back on the syringe to fill.

8. Turn the syringe gently if the flow begins to slow.

9. If lymph or lymph-contaminated blood is observed, stop the collection, note the sample attempt, and repeat steps 5–8 until a pure blood sample is obtained. If a pure blood sample cannot be obtained after several attempts, proceed with the collection of a sample, and make a note of the lymph contamination on the appropriate datasheet.

10. If more than one syringe volume is required, gently remove the syringe from the needle, replace with a new syringe, and repeat steps 7 and 8.

11. When a sufficient volume is obtained, place a cotton swab over the injection site and gently apply pressure as the needle is removed.

12. After approximately 90 seconds, check to ensure that the bleeding has clotted.

13. Remove the needle from the syringe and carefully decant the blood into a labeled microtainer or vacutainer, as appropriate.

14. Record the blood volumes obtained on the appropriate datasheet and make a note if lymph contamination occurred and an estimate of the lymph:blood ratio.

15. Prior to separating blood into aliquots for different sample preparations, gently invert the vacutainer tube 10 times.

16. For plasma preparation, centrifuge blood within 1 hour of blood collection. See SOP #4 for the plasma preparation method.

17. Store samples as appropriate (e.g., 4°C, freeze samples on dry ice, store at -80°C; specified in the test-specific Testing Protocols).

18. Ship samples to the appropriate laboratory as specified in the test-specific Testing Protocols.

9.6.5 SOP #3b: Cardiac puncture blood collection

Two people are needed to perform this method. Person #1 secures the body of the turtle, records the data, and ensures that samples are placed into the correct containers. Person #2 collects the blood.

1. Ensure that the turtle ID on the container label matches the ID of the turtle.

2. Immediately after euthanasia, prepare the turtle for the cardiac blood draw. For snapping turtles, place the needle between scutes. For sliders, drill a hole in the plastron to allow the needle to enter the body cavity.

3. Insert a 23-gauge needle into the body of the turtle toward the heart at an approximate 90° angle.

4. Gently pull back on the syringe to fill.

5. If more than one syringe volume is required, gently remove the syringe from the needle and replace.

6. Repeat steps 3 and 4.

7. Remove the needle and carefully decant the blood into a labeled microtainer or vacutainer, as appropriate (specified in the test-specific Testing Protocols).

8. Record the blood volumes obtained on the appropriate datasheet.

19. Prior to subsampling blood for different sample preparations, gently invert the vacutainer tube 10 times.

20. For plasma preparation, centrifuge blood within 1 hour of collection. See SOP #4 for the plasma preparation method.

9. Store samples as appropriate (e.g., 4°C, freeze on dry ice, store at -80°C; specified in the test-specific Testing Protocols).

10. Ship samples to appropriate laboratory as specified in the test-specific Testing Protocols.

9.6.6 SOP #3c: Dorsal coccygeal vein blood collection

Two people are needed to perform this method. Person #1 secures the body of the turtle, records the data, and ensures that samples are placed into the correct containers. Person #2 collects the blood.

1. Ensure that the turtle ID on the container label matches the ID of the turtle.

2. Restrain the turtle on a table, plastron down, with its head covered with a towel to minimize stress to the animal.

3. Extend the tail away from the body and down to expose the cranial end of the coccygeal vein, and use an ethanol swab to sterilize the area.

4. To collect blood, use a 22-, 23-, or 25-gauge 1-inch needle, with either a 1 mL or 3 mL syringe attached (depending upon the blood volume required; see test-specific Testing Protocols).

5. Insert the needle at an approximate 90° angle in the dorsal midline, aiming for the coccygeal vertebrae. Once the vertebrae are touched, apply negative pressure and pull the needle back slowly until the dorsal coccygeal vein is reached.

6. Gently pull back on the syringe to fill.

7. Remove the needle and carefully decant blood into a labeled microtainer or vacutainer, as appropriate (specified in the test-specific Testing Protocols).

8. Record blood volumes obtained on the appropriate datasheet.

9. Prior to subsampling blood for different sample preparations, gently invert the vacutainer tube 10 times (if appropriate).

10. For plasma preparation, centrifuge blood within 1 hour of blood collection. See SOP #4 for plasma preparation method.

11. Store samples as appropriate (e.g., 4°C, freeze on dry ice, store at -80°C; specified in the test-specific Testing Protocols).

12. Ship samples to appropriate laboratory, as specified in the test-specific Testing Protocols.

9.6.7 SOP #4: Preparation of blood plasma samples

1. Place the labeled blood-filled microtainer or vacutainer tubes into an appropriate refrigerated (4°C) centrifuge.

    a. Spin vacutainer tubes at > 2,000 x g for 10 minutes
    b. Spin speed and time may be modified according to manufacturer's specifications; if different than above, record on the appropriate datasheet or laboratory notebook.

2. Check that the blood is sufficiently separated.

3. Note if there is any lysis (by looking for color such as yellow/pink/red in the supernatant), and record on the appropriate datasheet.

4. Using a micropipette, transfer appropriate volumes of heparinized plasma or serum into the appropriate cryovials or microcentrifuge tubes. See the test-specific Testing Protocols.

5. Record the samples that were prepared on the appropriate inventory form.

6. Store as directed in the test-specific Testing Protocols.

7. Repeat until all samples have been processed.

9.6.8 SOP #5: Housing, feeding, and monitoring of turtles

Turtle feeding

1. Feed turtles daily. On Wednesdays and Fridays, feed turtles live, large mealworms. On all other days, or if live mealworms are unavailable, feed turtles commercial pellet feed. Provide each turtle with enough food that amounts to at least 3–4% of its body mass.

2. Each morning, before daily tank cleaning, inspect tanks for remaining food. Record on the appropriate datasheet if any uneaten food remains. Carefully monitor these individuals to determine amount of time they are not eating and any other signs of stress.

Turtle tank cleaning

1. During the study, clean turtle tanks daily.

2. For each experimental tank, remove the water using a pump. Then pump clean water from the reservoir back into the tank. Refill the tanks so that they are approximately

1/3 full. Ensure that clean water from the reservoir has been heated to 26°C before pumping into the experimental tanks.

3. For all experimental tanks with oil-dosed turtles, ensure that the water is filtered through an oil removal system (e.g., sand/activated charcoal) before draining to the sewage system. If the water is heavily contaminated with oil, collect it for hazardous waste disposal.

Environmental and turtle health monitoring

1. Check temperature-controlled water baths daily to ensure that temperatures are maintained.

2. Monitor all individual tanks for temperature and record on the appropriate datasheets.

3. At least once daily, inspect each turtle for any signs of stress or poor health. If a wound is observed, apply Neosporin with a cotton swab. Inspect the exposed skin of each turtle for any signs of bacterial or parasitic infections, redness/swelling, and edema. If any signs of stress occur, note this on the appropriate datasheets.

4. During the experimental procedures, measure the turtle weight and carapace length daily.

5. Note any change in the turtle's normal behavior.

6. Before water changes, note the presence of uneaten food and/or the presence of feces.

9.6.9 SOP #6: Placement/insertion of E-tubes

1. Complete the E-tube procedure at least 48 hours prior to the start of any experiment to allow for an appropriate recovery time.

2. Using proper handling techniques, remove the turtle from the container.

3. Before E-tube insertion, weigh the turtle and note its heart rate on the appropriate datasheets.

4. To start the procedure, inject the turtle with either 2.5 mg/kg propofol (red-eared sliders) or 5 mg/kg propofol (snapping turtles), and note the time of administration.

5. When the turtle is determined to be under sedation (again note the heart rate on the appropriate datasheets), the surgery can be initiated with an injection of meloxicam (0.2 mg/kg intramuscular).

6. Measure the distance from the insertion site to mid-body and use this measurement to determine how much of the E-tube will be inserted into the esophagus/stomach. Make the incision in the caudal cervical region on the left side of the turtle, just cranial to the carapace margin.

7. Always use sterile techniques, including sterile preparation of the surgery site.

8. Insert a hemostat through the mouth and into the esophagus to the incision location, and apply pressure to push the tissue laterally. This will help the hemostat tip to be easily palpable. Use a scalpel blade to incise the skin and esophagus, exposing hemostat tips. Grasp the end of the esophageal tube in hemostats and retract to the mouth, then direct the tube tip toward the stomach. Use the hemostats to guide the tube tip down the esophagus and toward the stomach.

9. Secure the tube to the skin with purse string and the Chinese finger locking suture pattern with non-absorbable, monofilament sutures. Further secure the tube to the shell with non-heating epoxy or similar material. Affix the tube to the dorsal aspect of carapace.

10. Fill the E-tube with sterile saline and close the external end of tube with a removable plug.

11. After surgery is completed, make a mark with a permanent marker on the E-tube next to the sutures. This mark will help assess if the tube moves (i.e., the turtle pulls them out).

12. Inject (intramuscular) lidocaine (2 mg/kg) at the external E-tube site, and note the time of the injection and the heart rate of the turtle. Place the turtle in a recovery tank and observe it until it has regained all normal behavior. Once recovered, place the turtle into its individual tank containing a minimal amount of water (so that it does not cover the E-tube site).

13. Monitor the turtle closely (at least twice daily) for any signs of stress or abnormal behavior for the next 48 hours. Inspect the E-tube site for signs of irritation.

9.6.10 SOP #7: Euthanasia by cervical dislocation (decapitation followed by pithing of the brain)

Two people are needed to perform this method. Person #1 secures the body of the turtle. Person #2 performs the cervical dislocation.

1. Using proper handling techniques, remove the turtle from the container.

2. Stabilize the turtle to reduce its movement (Person #1).

3. Carefully stretch out the neck and locate vertebrae (Person #1).

4. In one movement, use the scalpel to completely separate the head from the neck and immediately take the head and insert a needle to pith the brain (Person #2).

9.6.11 SOP #8: Necropsy for blood and tissue collection

Before beginning necropsy, ensure that all sample containers, vials, tubes, and slides are labeled and all labels have been cross-checked with the sample inventory. Photograph and record all abnormalities. Ensure the turtle ID number and date are clearly visible in each photograph.

Blood collection prior to euthanasia

1. Weigh the turtle and record carapace length (as detailed in SOP #1).

2. Euthanize the turtle by cervical dislocation following SOP #7.

3. Collect blood according to SOP #3b. The carcass is ready for dissection when a sufficient blood volume is collected to meet all of the study requirements or no further blood can be obtained.

4. Record the number of blood tubes collected.

5. Process blood per SOP #4 to get appropriate plasma and serum samples (see test-specific Testing Protocols).

6. Record the sample ID information on the appropriate datasheet.

7. Store and aliquot blood according to the test-specific Testing Protocols.

8. Before necropsy, take digital pictures of the turtle dorsally and ventrally on a sheet labeled with the turtle's ID. Include a ruler for scale in each photograph. Record all photographs on the appropriate datasheet.

Necropsy

1. Using the dremel tool, cut the carapace to separate and allow access to the body cavity.

2. Open the body cavity, being careful not to puncture or move organs.

3. Record any abnormalities on the appropriate datasheet.

4. Collect blood according to SOP #3b. Collect as much blood as possible.

5. Following the blood draw, record any abnormalities on the appropriate datasheet.

6. After the cardiac blood draw, take a digital image of the all organs in situ. Ensure that the study ID, turtle ID, and date are clearly visible in the photograph. Record the photograph on the appropriate datasheet.

7. Locate the gall bladder and insert a 25-gauge needle to remove the bile.

8. Place the bile in a labeled glass vial, record the volume, and immediately place the vial in liquid nitrogen to freeze.

9. After the bile is frozen, place the sample in a -80°C freezer for long-term storage.

10. Remove the liver and weigh the whole organ. Record the weight on the appropriate datasheet.

11. Collect three subsamples from the liver (3 x 0.5 g); place in labeled, individual cryovials; and flash freeze samples in liquid nitrogen. Store the samples in a -80°C freezer until analysis. Record on the appropriate datasheet.

12. Place the remaining liver sample in 10% neutral buffered formalin (NBF) for histological analyses. *Note*: if the remaining liver sample is more than 5-mm thick, cut even slices into the large tissue sample to allow formalin perfusion and adequate fixation.

13. Cut out the lungs and heart and separate the heart from the lungs.

14. Assess the lungs for any abnormalities, weigh, and place the organ with the liver in a specimen jar with 10% NBF.

15. Before weighing the heart, open the heart chambers and wash them with sterile saline to remove blood and clots, and pat dry (repeat if necessary).

16. Once weighed, assess the heart for any abnormalities and place it in a specimen jar with 10% NBF.

17. Next, remove the thyroid, located in the neck, just anterior to blood vessels near the clavicle, and place it in a specimen jar with 10% NBF.

18. Cut and remove the whole gastrointestinal (GI) tract (from the throat to the anus), including the spleen.

19. Slice the GI tract vertically and rinse with sterile saline to remove all of the consumed food.

20. Check the GI tract for parasites, lesions (particularly at the E-tube insertion site), and edema. Then, prepare sections for histopathology:

    a. *Parasites*: to identify parasites, remove a subsample and fix in a separate vial with 10% NBF. If parasites are attached, leave in place.

    b. *Lesions*: remove all lesions from the GI tract, cutting them into pieces that are no more than 0.5-cm thick (length is not important). On each lesion sample, include an edge with some normal tissue. Place into a specimen jar with the rest of the organs and 10% NBF.

    c. *Edema*: If edema is observed, measure the wall thickness using calipers.

21. Once the GI tract is removed, the kidneys, adrenals, and gonads will be visible. Verify the sex of the turtle if possible.

22. Remove the kidneys, adrenals, and gonads as one unit and place in a specimen jar with 10% NBF.

23. Photograph the carcass with the label clearly visible.

24. Place the carcass in a labeled bag and freeze at -20°C.

9.6.12 SOP #9: Packed Cell Volume

1. Place a micro-hematocrit capillary tube in a blood-filled microtainer, vacutainer, or microcentrifuge tube, and fill the capillary tube 2/3 to 3/4 full via capillary action; do not overfill. Use a heparin tube if the blood has not already been exposed to heparin. Alternatively, carefully touch the tip of a heparinized micro-hematocrit capillary tube to a well-rounded drop of blood formed at the blood collection site after a blood sample has been collected via a syringe. A heparinized micro-hematocrit tube can be recognized by a red ring on the tube. Do not allow the capillary tube to puncture the skin. Hold the tube at a downward angle to avoid air bubbles. Fill the tube 2/3 to 3/4 full via capillary action; do not overfill. Invert the tube to ensure the blood comes into contact with the heparin.

2. Fill one end of the tube with a sealant such as plasticine or Critoseal. Hold the clay perpendicular to the laboratory bench, holding the micro-hematocrit tube as close to the

end as possible that will be filled with clay. Gently and carefully push the end of the tube into the clay 2–3 times. Avoid excessive pressure as it will cause the tube to break.

3. Centrifuge the sealed micro-hematocrit tubes in a hematocrit centrifuge for 2 minutes at > 12,000 x g.

4. Measure the PCV with a card reader.

9.6.13 SOP #10: Bicinchoninic acid assay protein assay

In general, BCA assay samples were prepared using the Thermo Scientific Pierce BCA Protein Assay Kit #23227 (Rockford, IL). Samples were analyzed using a Molecular Devices SpectraMax 96-Well Spectrophotometer; if samples were very diluted or limited in volume, the Thermo Scientific Pierce MICRO BCA Assay Kit # 23235 was used.

Preparation of standards and working reagents

1. **Preparation of diluted bovine serum albumin (BSA) standards.** Dilute the contents of one BSA standard ampule into several Eppendorf tubes as detailed in Table 9.2 below.

2. **Preparation of BCA working reagent (WR).** The WR must be made fresh on the day of the assay. For each microplate, 20 mL of WR is needed. Prepare the WR by mixing 50 parts of BCA Reagent A from the kit with 1 part of BCA Reagent B from the kit (i.e., mix 20 mL Reagent A with 400 µL Reagent B). Mix and keep at room temperature until use.

Table 9.2. BSA standard preparation

Vial	Dilutant volume (µL)	BSA volume and source (µL)	Final BSA concentration (µg/mL)
A	0	300 of stock	2,000
B	125	375 of stock	1,500
C	325	325 of stock	1,000
D	175	175 of vial B	750
E	325	325 of vial C	500
F	325	325 of vial E	250
G	325	325 of vial F	125
H	400	100 of vial G	25
I	400	0	0

Assay procedure (microplate procedure; sample to WR ratio = 1:8)

1. Pipette 25 µL of each standard or unknown sample replicate into a microplate well. *Note*: if the sample size is limited, pipette 10 µL of each unknown sample and standard (i.e., the sample to WR ratio is 1:20 and the working range will be limited to 125–2000 µg/mL), and use the alternative assay kit. Samples may be diluted with 1 x phosphate buffered saline (PBS) before use if needed.

2. Add 200 µL of the WR to each well (duplicate or triplicate; refer to plate layout design), and mix plate on a plate shaker for 30 seconds (or in the spectrophotometer).

3. Cover the plate with sealing tape and incubate at 37°C for 30 minutes (time can be increased up to 2 hours for dilute samples).

4. Cool the plate to room temperature.

5. Measure absorbance at 562 nm on a plate reader.

6. Subtract the average 562-nm absorbance measurement of the blank standard replicates from the 562-nm measurements of all other individual standards and unknown samples.

7. Prepare a standard curve by plotting the average blank-corrected, 562-nm measurement for each BSA standard versus its concentration in µg/mL.

8. Use the standard curve to determine the protein concentration of each unknown sample. *Note*: a quadratic or best-fit curve will provide more accurate results than a linear fit.

Data management and records management

1. Check that the standard curves generated fall within the normal ranges and that r^2 values are > 95%.

2. Verify that the samples fall within the standard curve (i.e., the dynamic range of the assay – (20–2,000 µg/mL); rerun any samples that do not fall within this range by diluting in 1 x PBS.

3. Check that the % coefficient of variation (CV) of replicate values (only do this when triplicates are used) is no greater than 10%. Rerun any samples with a CV that is > 10%.

9.6.14 SOP #11: Liver tissue homogenization procedure

1. Remove the liver samples (stored in cryovials) from the -80°C freezer and place on ice.

2. Weigh the samples and record weights.

3. Depending upon the specific assay (SOP #12, #14, or #15a), use the volume of buffer suggested and add the liver and buffer to a homogenization tube. Keep on ice.

4. Use a pestle attached to a drill set to a low speed to homogenize the sample on ice for 1–2 minutes until an even homogenate is formed.

5. Add the homogenate to one or two (number will depend on the assay being used) labeled Eppendorf tubes and spin for 5 minutes at 4°C at 3,000 x g.

6. Remove the supernatant and place it in new labeled Eppendorf tubes.

7. Keep on ice if assayed immediately; otherwise, place in N_2, and store at -80°C if not assayed immediately.

8. Discard the pellet.

9.6.15 SOP #12: Total glutathione and glutathione disulfide quantitation in blood cells and liver tissue

The tGSH/GSSG assay samples were prepared using the GT40 Assay Kit from Oxford Biomedical Research (Oxford, MI). Samples were analyzed using a Molecular Devices SpectraMax 96-Well Spectrophotometer.

General information

1. Never leave the reagent bottles open; replace the caps as soon as the necessary volume is removed. Use aseptic techniques when opening and dispensing reagents.

2. Store the components of the kit at the temperatures specified on the labels.

Reagent preparation

1. ***NADPH (nicotinamide adenine dinucleotide phosphate) reagent***: Reconstitute contents of the vial with a 500-µL assay buffer. Add this to a 5.5-mL assay buffer and vortex (i.e., mix in a vortex mixer). Keep on ice.

2. ***Reductase reagent***: Add 30-µL Reductase reagent to a 6-mL assay buffer. Keep on ice.

3. ***DTNB (Dithionitrobenzoic acid) reagent***: Reconstitute contents of the DTNB vial with a 500-µL assay buffer. Add this to a 5.5-mL assay buffer and vortex. Keep at room temperature.

4. ***Standard curve preparation***: Add the indicated volume of assay buffer and 10 µmol/L GSSG standard stock according to Table 9.3. Keep tubes on ice.

Table 9.3. Preparation of tGSH/GSSG assay standards

Standard	tGSH conc. (µmol/L)	GSSG conc. (µmol/L)	Assay buffer volume (µL)	10 µmol/L GSSG stock volume (µL)	Final volume (µL)
S7	3.0	1.5	850	150	1,000
S6	2.0	1.0	900	100	1,000
S5	1.5	0.75	925	75	1,000
S4	1.0	0.50	950	50	1,000
S3	0.5	0.25	975	25	1,000
S2	0.25	0.125	987.5	12.5	1,000
S1	0.10	0.05	995	5	1,000
B0	0	0	1,000	0	1,000

Sample preparation

Whole blood

1. Remove whole blood samples from the -80°C freezer just before use and place on ice

2. Use whole blood directly without further preparation.

To prepare GSSG whole blood sample

1. Add 30 µL of DTNB reagent to the microfuge tube.

2. Add 100 µL of whole blood immediately after thawing on ice to the tube and gently mix. Keep at room temperature for 5–10 minutes.

3. Add 270 µL of ice-cold 5% metaphosphoric acid (MPA) and vortex.

4. Centrifuge at 1,000 x g for 10 minutes at 4°C.

5. Add 50 µL of the supernatant to a 700-µL assay buffer in a new tube and mix. Keep on ice until use.

6. Discard the pellet.

To prepare tGSH whole blood sample

1. Add 50 µL of whole blood to 350 µL of ice-cold 5% MPA and vortex.

2. Centrifuge at 1,000 x g for 10 minutes at 4°C.

3. Add 25 µL of the supernatant to a 1,500-µL assay buffer in a new tube and mix. Keep on ice until use.

4. Discard the pellet.

Liver tissue

1. Remove liver samples from the -80°C freezer just before use and place on ice

2. Once thawed, split the sample into two pieces (no more than 0.2–0.3 g each) and weigh each piece; one will be for the tGSH assay (Tube A) and one for the GSSG assay (Tube B).

To prepare GSSG liver sample

1. Prepare 1 x PBS tissue buffer with scavenger (i.e., add 3 mL of DTNB reagent into 25 mL of 1 x PBS tissue buffer).

2. Add a volume of this buffer to the tissue sample so that the tissue mass to buffer volume ratio is 1:5 (i.e., for 0.2-g tissue, add 1,000 µL of tissue buffer with scavenger (1 x PBS and DTNB reagent). Homogenize on ice. Keep on ice until centrifugation.

3. Once several samples are prepared, spin at 4°C for 10 minute at 10,000 x g.

4. Decant supernatant into an Eppendorf tube, mix well, and then split supernatant into two equal volumes. Keep one on ice for the assay, and store the second replicate sample at -80°C.

5. Add 130 µL of liver preparation supernatant to a new tube and gently mix. Keep at room temperature for 5–10 minutes (note time for each sample on the appropriate datasheet).

6. Add 270 µL of ice-cold 5% MPA and vortex.

7. Centrifuge at 1,000 x g for 10 minutes at 4°C.

8. Carefully transfer the supernatant to a new tube and mix (pellet can be discarded).

9. Add 50 µL of the supernatant to 700 µL of assay buffer in a new tube and mix. Keep on ice until use. Freeze the rest of the supernatant on N_2 and store at -80°C. It may be used for up to 6 months.

To prepare tGSH whole blood sample

1. Add a volume of buffer to the tissue sample so that the tissue mass to buffer volume ratio is 1:5 (i.e., for .2-g tissue, add 1,000 µL of 1 x PBS tissue buffer). Homogenize on ice. Keep on ice until centrifuged.

2. Once several samples are prepared, spin at 4°C for 10 minutes at 10,000 x g.

3. Decant the supernatant into an Eppendorf tube, mix well, and then split the supernatant into two equal volumes. Keep one on ice for the assay. Store the second replicate sample at -80°C.

4. Add 50 µL of the liver preparation supernatant to 350 µL of ice-cold 5% MPA and vortex.

5. Centrifuge at 1,000 x g for 10 minutes at 4°C.

6. Carefully transfer the supernatant to a new tube and mix (pellet can be discarded).

7. Add 25 µL of the supernatant to 1,500 µL of the assay buffer in a new tube and mix. Keep on ice until use. Freeze the rest of the supernatant on N_2 and store at -80°C. It may be used for up to 6 months.

Sample assay procedure (for both prepared whole blood and liver tissue supernatants)

1. Add 50 µL of standards, samples, or blank to the corresponding wells on the microplate
2. Add 50 µL of DTNB reagent to each well
3. Add 50 µL of Reductase reagent to each well
4. Mix the wells by tapping the plate and incubate at room temperature for 5 minutes
5. Add 50 µL of NADPH Reagent to each well
6. Place the plate in the SpectraMax microplate reader and, using the kinetic function, record the change of absorbance at 412 nm by taking readings every minute for 10 minutes.

Calculations

Rate determination (mean V): The change in absorbance, or optical density (OD), is a linear function of the tGSH concentration in the reaction mixture,

$$OD = slope * minutes + intercept$$

Calibration curves: Prepare an 8-point calibration curve for both tGSH and GSSG (see Table 9.3). For each concentration of tGSH/GSSG, calculate the net rate. The net rate is the difference between the sample or standard and the blank rate. For tGSH, use the tGSH standards 0, 1.0, 2.0, and 3 µmol/L. For GSSG, use the 0, 0.05, 0.125, and 0.25 µmol/L GSSG data points.

tGSH and GSSG concentration in samples: The general form of the regression equation describing the calibration curve is:

$$Net\ rate = slope * tGSH + intercept$$

Therefore, to calculate the tGSH or GSSG concentration from the tGSH or GSSG calibration curves, use:

$$tGSH\ (or\ GSSG) = \frac{net\ rate - intercept}{slope} * dilution\ factor$$

***tGSH/GSSG ratio*:** the tGSH/GSSG ratio is calculated by dividing the difference between the tGSH and the GSSG concentration by the concentration of GSSG using:

$$Ratio = \frac{tGSH - 2(GSSG)}{GSSG}$$

QA/QC

1. If sample volumes allow, run all standards and samples in triplicate; at a minimum, run all samples in duplicate.

2. Check that the standard curves generated fall within the normal ranges and that r^2 values are > 95%.

3. Check that the samples fall within the standard curve (i.e., the dynamic range of the assay 1.0–3.0 µmol/L tGSH or 0.05–0.25 µmol/L GSSG); rerun any samples that do not fall within this range by diluting in 1 x PBS.

4. Check that the CV of replicate values (do this when triplicates are used) is no greater than 10%. Rerun any samples with a CV of > 10%.

9.6.16 SOP #13: 2-Thiobarbituric acid reactive substances lipid peroxidation assay for plasma

The TBARs lipid peroxidation assay samples were prepared using the Lipid Peroxidation TBARS Colorimetric FR40 Assay Kit from Oxford Biomedical Research (Oxford, MI). This kit was modified for the FR45 fluorescent plate method. Samples were analyzed using a Molecular Devices SpectraMax 96-Well Spectrophotometer.

General information

1. Do not leave the reagent bottles open. Replace the caps as soon as the required volume is removed.

2. Sample blanks for each sample are recommended for this assay, which contain the sample plus the acid reagent alone (i.e., no Indicator Solution).

3. The acid reagent freezes at temperatures of 15°C or colder. Before use, remove it from the kit, which is stored at 4°C, and allow to thaw at room temperature for 1 hour.

Sample preparation

1. Remove plasma samples from the -80°C freezer just before assay. Place on ice.

Reagent preparation

1. ***Acid reagent:*** Remove from kit that is stored at 4°C, and allow to thaw at room temperature for 1 hour.

2. ***Indicator solution***: Add 10 mL of the Acid Reagent to the powdered contents of one vial of TBA and shake until completely dissolved.

3. ***20 µmol/L malondialdehyde (MDA) standard stock***: Dilute the 10 mmol/L MDA standard 1:500 in DI water (i.e., 20 µL of 10 mmol/L MDA standard and 9.98 mL of water).

4. ***Prepare the set of seven standards as described in Table 9.4***. *Note*: MDA is provided as a MDA-TBA salt in a slightly basic buffer, as MDA itself is not stable. When mixed with Indicator Solution, the MDA-TBA molecule is acidified and generates MDA quantitatively.

Table 9.4. Preparation of lipid peroxide assay standards

Standard	MDA conc. (µmol/L)	Water volume (µL)	20 µmol/L stock volume (µL)
S0	0	400	0
S1	0.5	390	10
S2	1.0	380	20
S3	2.5	350	50
S4	5.0	300	100
S5	10	200	200
S6	15	100	300
S7	20	–	400

5. ***Plasma preparation***: To deproteinate plasma before use, add 2.5 µL of butylated hydroxytoluene (BHT) stock solution and 157.5 µL of 1 x PBS (pH 7.4) to 100 µL of plasma. Then add 20 µL of saturated Indicator Solution (in DI water) and 20 µL of saturated ammonium sulfate solution (in DI water). Vortex to mix and centrifuge samples at 15,000 x g for 10 minutes. Transfer the supernatant to a new tube and use for the assay. Discard the pellet.

Sample assay procedure

Preparation of standards and samples

1. Add 200 µL of each of the standards or samples to a microcentrifuge tube
2. Add 200 µL of Indicator Solution to each tube and mix.

Preparation of sample blanks

1. Add 100 µL of each of the samples to a microcentrifuge tube.

2. Add 100 µL of Acid Reagent to each tube and vortex.

3. Incubate all standard, sample, and blank tubes in the water bath at 65°C for 45 minutes.

4. Transfer two 150-µL aliquots of each standard/sample (in duplicate) or blanks (single) to the designated wells on the microplate.

5. Read the plate at 532 nm.

Calculations

Plot the absorbance at 532 nm (A_{532}) of each standard versus its MDA concentration and perform a linear regression analysis:

$$A_{532} = m[MDA] + b$$

where A_{532} is the absorbance at 532 nm, m is the slope of the standard curve, [MDA] is the µmol/L concentration of MDA in the sample, and b is the y-intercept. Calculate the concentration of analyte in each unknown using:

$$[MDA] = \frac{A_{532} - b}{m} * dilution\ factor$$

QA/QC

1. If sample volumes allow, run all standards and samples in triplicate; at a minimum, run all samples in duplicate.

2. Check that the standard curves generated fall within the normal ranges and that r^2 values are > 95%.

3. Check that the samples fall within the standard curve (i.e., the dynamic range of the assay, 1.0–20 µM); rerun any samples that do not fall within this range by diluting in 1 x PBS.

4. Check that the CV of replicate values (do this if triplicates are used) is no greater than 10%. Rerun any samples with a CV > 10%.

9.6.17 SOP #14: Lipid peroxidation assay for liver

The liver lipid peroxidation assay samples were prepared using the FR22 Assay Kit from Oxford Biomedical Research (Oxford, MI). Samples were analyzed using a Molecular Devices SpectraMax 96-Well Spectrophotometer.

General information

1. Do not leave the reagent bottles open. Replace caps as soon as the required volume is removed.

2. Do not allow the capped reagent bottles to sit at room temperature for any extended period of time.

Sample preparation

1. Homogenize the liver tissue in 5–10 mL of ice-cold PBS buffer (pH 7.4) containing 5 mmol/L BHT (which prevents sample oxidation) per gram of liver tissue (e.g., homogenize a 1-g liver tissue sample in 10 mL 1 x PBS with 100 µL of stock BHT; add 1 mL of 1 x PBS and 10 µL of stock BHT to a 0.1-g liver sample).

2. Centrifuge the homogenate at 3,000 x g for 10 minutes at 4°C. Split the supernatant into two Eppendorf tubes: one (~ 500 µL) is used for this assay and kept on ice until use; and the second is any remaining sample, which should be frozen in N_2 and stored at -80°C immediately. Dilute the homogenate if needed with the homogenization buffer. Discard the pellet.

Reagent preparation

1. ***Reagent R1***: Dilute Reagent R1 3:1 with dilutant (i.e., 12 mL R1 and 4 mL dilutant). Do this immediately before use (keep on ice until use).

2. ***20 mmol/L MDA standard***: Dilute 10 mmol/L MDA standard 1:500 in DI water (i.e., 20 µL of the 10 mmol/L MDA and 9.98 mL of water) to obtain a 20 µmol/L MDA standard stock solution.

3. Prepare the set of seven standards as described in Table 5 just before use. Keep on ice until use.

Table 5. Preparation of liver lipid peroxidation standards

Standard	Volume of DI water (µL)	Volume of 20 µmol/L MDA stock (µL)	MDA concentration (µmol/L)
BLK	600	0	0
S1	570	30	1.0
S2	525	75	2.5
S3	450	150	5.0
S4	300	300	10.0
S5	150	450	15.0
S6	0	600	20.0

Sample assay procedure

1. Add 140 µL of each of the standards, blanks, or samples to a microcentrifuge tube. Keep at room temperature.

2. Add 455 µL of diluted Reagent R1 to each tube and vortex.

3. Add 105 µL of 37% HCl (12 N HCl) to each tube and vortex.

4. Incubate all tubes in the water bath at 45°C for 60 minutes.

5. Centrifuge the samples at 15,000 x g for 10 minutes to obtain a clear supernatant.

6. Place the supernatant into a new tube to mix and immediately transfer three 150 µL aliquots of each standard/sample to the designated wells on the microplate.

7. If needed, centrifuge briefly for a few seconds to remove bubbles (up to ~ 300 x g).

8. Read the plate at 586 nm.

Calculations

Plot the absorbance at 586 nm (A_{586}) of each standard versus its MDA concentration and perform a linear regression analysis:

$$A_{586} = m[MDA] + b$$

where A_{586} is the absorbance at 586 nm, m is the slope of the standard curve, [MDA] is the µmol/L concentration of MDA in the sample, and b is the y-intercept. Calculate the concentration of analyte in each unknown using:

$$[MDA] = \frac{A_{586} - b}{m} * dilution\ factor$$

QA/QC

1. If sample volumes allow, run all standards and samples in triplicate; at a minimum, run all samples in duplicate.

2. Check that the standard curves generated fall within the normal ranges and that r^2 values are > 95%.

3. Check that the samples fall within the standard curve (i.e., the dynamic range of the assay, 1.0–20 µM); rerun any samples that do not fall within this range by diluting in 1 x PBS.

4. Check that the CV of replicate values (do this if triplicates are used) is no greater than 10%. Rerun any samples with a CV of > 10%.

9.6.18 SOP #15: Total antioxidant assay for liver (SOP #15a) and plasma (SOP #15b)

The total antioxidant assay samples were prepared using the Total Antioxidant Power TA02 Assay Kit from Oxford Biomedical Research (Oxford, MI). Samples were analyzed using a Molecular Devices SpectraMax 96-Well Spectrophotometer.

Sample preparation procedure

Liver tissue

1. Remove samples from the -80°C freezer and record weight of tissue in total antioxidant assay sample inventory.

2. Calculate the 7 x volume of 1 x PBS buffer to add, and record the volume added on the appropriate datasheet.

3. Add tissue to the homogenization tube (keep on ice) with the 7 x volume of ice-cold PBS (pH 7.4) per gram of tissue. Homogenize the tissue on ice.

4. Pour homogenate into one or more pre-labeled 1.5 or 2 mL Eppendorf tubes and centrifuge at 3,000 x g for 10 minutes at 4°C.

5. Remove the supernatant for the assay and store samples on ice. A minimum of 700 µL is required for the TA02 assay.

6. Place any remaining sample in N_2 and store frozen at -80°C.

7. Dilute samples as needed with the 1 x PBS solution.

8. Discard the pellet.

Plasma samples

1. Remove plasma samples from the -80°C freezer and place on ice.

2. Use plasma samples directly without further preparation.

Preparation of reagents and standards

1. Equilibrate the dilution buffer, copper solution, and stop solution to room temperature 30 minutes prior to beginning assay.

2. Using a sterile syringe and needle, add 1.0 mL of absolute (200 proof) ethanol directly to the Trolox Standard vial through the unopened rubber stopper. Then remove 1 mL of air from the tube and add another 1.0 mL of ethanol to the vial.

3. Vortex the vial for 30–60 seconds, or until the standard is completely dissolved. The standard stock is now 2.0 mmol/L.

4. Prepare a set of standards (see Table 6) using 2-mL tubes (*Note*: for triplicate standards, a volume of > 600 µL is needed).

Table 6. Preparation of total antioxidant assay standards

Standard	Trolox final conc. (mmol/L)	Water (µL)	Transfer volume (µL)	Transfer source	Final volume (µL)
S5	2.0	None	2,000	Stock	1,200
S4	1.0	800	800	S5	800
S3	0.5	800	800	S4	800
S2	0.25	800	800	S3	800
S1	0.125	800	800	S2	1,600
Blank	0	800	None		800

Sample assay procedure

1. Dilute both the samples (plasma or liver supernatants) and the standards 1:40 in the provided dilution buffer (e.g., to pre-labeled tubes, add 780 µL of dilution buffer and 20 µL plasma/liver homogenate or standard). Mix by vortex.

2. Place 200 µL of the diluted standards or samples in triplicate into each well in the 96-well plate. Refer to the plate assay template sheet.

3. Place the 96-well plate into the centrifuge and briefly spin to remove bubbles.

4. Read the plate at 450 nm for a reference plate measurement, and save the file with the file name “pre-read.”

5. Add 50 µL of the copper solution to each well and incubate for 3 minutes at room temperature.

6. Add 50 µL stop solution to each well and respin the plate briefly as outlined in step 3 to remove bubbles.

7. Reread the plate at 450 nm and save the file with the file name "post-read."

8. Save files on the SpectraMax microplate reader computer, and to all other storage devices.

Calculations

Calculate the net absorbance by subtracting the absorbance readings from the first plate read (pre-read) from those of the second plate read (post-read). Plot the net absorbance of the standards versus their Trolox concentration and perform a linear regression analysis:

$$A_{450} = m[Trolox] + b$$

where A_{450} is the absorbance at 450 nm, m is the slope of the standard curve, [Trolox] is the mmol/L concentration of Trolox equivalents in the sample, and b is the y-intercept. Calculate the concentration of analyte in each unknown using:

$$[Trolox] = \frac{A_{450} - b}{m} * dilution\ factor$$

Values can also be expressed as µmol/L copper reducing equivalents (CRE) by multiplying the mmol/L equivalents of Trolox by 2,189.

QA/QC

1. If sample volumes allow, run all standards and samples in triplicate; at a minimum, run all samples in duplicate.

2. Check that the standard curves generated fall within the normal ranges and that r^2 values are > 95%.

3. Check that the samples fall within the standard curve (i.e., the dynamic range of the assay, 0.125–2.0 mmol/L); rerun any samples that do not fall within this range by diluting in 1 x PBS.

4. Check that the CV of replicate values (do this when triplicates are used) is no greater than 10%. Rerun any samples with a CV of > 10%.

9.6.19 SOP #16: COMET assay for blood cells (SOP #16a) and liver tissue (SOP #16b)

Carry out this method under minimal light.

Preparation of samples

Whole blood cells

1. Add 5 µL of whole blood to a microcentrifuge tube containing Hank's balance salt solution (HBSS) + 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.6).

2. Mix and immediately assay 10 µL of prepared whole blood cells using the COMET assay.

Liver cells

1. To a glass microscope slide, add two to three droplets (around 100–150 µL) of cold HBSS and HEPES solution.

2. Place slide on ice, or metal block chilled by ice, to keep cold.

3. Then place freshly collected liver (about 2–3 mm in radius) onto the slide with HBSS and HEPES solution and mince into small pieces.

4. Pipette the solution through a 70 µm filter into an Eppendorf tube and repeat.

5. Centrifuge the solution at 1,500 x g.

6. Remove the supernatant and discard.

7. Collect the pellet from the bottom for the assay.

Preparation of stock solutions, glass slides, and fresh working solutions

Stock lysing solution [10 mmol/L Tris, 200 mmol/L NaCl, 100 mmol/L disodium ethylenediaminetetraacetic acid (Na_2EDTA), N-Laurylsarcosine]: Add 37.2 g of Na_2EDTA, 146.1 g of NaCl, and 1.2 g of Tris base to 600 mL of DI water. Then add ~ 3–4 g of NaOH to get these chemicals into solution. When in solution, adjust to a pH of 10 using NaOH pellets (will need about 8 g of pellets). Add 10 g of N-Laurylsarcosine and bring up to 890 mL with DI water. Filter to sterilize using 0.2 µm filters. Keep solution at room temperature.

Stock 200 mmol/L Na_2EDTA: Add 37.22 g of Na_2EDTA to 500 mL of DI water. Store solution at room temperature.

Stock 10 mol/L NaOH: In fume hood, using goggles, laboratory coat, and gloves (reaction is highly exothermic), add 40 g of NaOH to 100 mL DI water. Store solution at room temperature.

Electrophoresis buffer (2 L, pH > 12; store at 4°C): Add 40 mL of NaOH stock to 10 mL Na_2EDTA stock and bring up to 2 L with DI water. Store solution at 4°C until use.

Lysing solution [110 mL; store at 4°C; stock lysing solution with 10% dimethyl sulfoxide (DMSO) and 1% Triton X-100 surfactant]: Add 11 mL of DMSO to 1.1 mL of Triton X-100, bring up to 110 mL with stock lysing solution, and mix well. Add to slide staining tray and store at 4°C.

Neutralization buffer (store at 4°C): Add 48.5 g of Tris base to 800 mL DI water, adjust pH to 7.5 with HCl, and then bring up to 1,000 mL with DI water. Store at 4°C.

HBSS + HEPES (pH 7.6): Add 0.6507 g of HEPES to 250 mL HBSS. Aerate and adjust pH to 7.6. Filter through a 0.2 µm filter to sterilize. Store at room temperature.

Ethidium bromide (EtBr; store in the dark at 4°C): Use nitrile gloves when handling these reagents. Dilute EtBr 1:5000 to make a 1:100 solution by adding 100 µL of EtBr to 9.9 mL DI water. Then dilute 1:100 EtBr solution to a 1:5000 solution by adding 1 mL of the 1:100 solution to 49 mL of HEPES + HBSS.

1% normal melting point agarose (NMPA): Add 1 g NMPA to 100 mL PBS, and boil to melt. Use immediately to coat glass slides.

0.75% low melting point agarose (LMPA): Add 0.075 g LMPA to 10 mL HBSS + HEPES buffer, and boil to melt. Store at 37°C before use.

Coating of glass microscope slides

Using unfrosted, uncleaned microscope slides, etch numbers into the upper right-hand of the slide using a diamond pen. Then etch "**X's**" across the lower 3/4 of the slide surface. One at a time, dip slides about 3/4 of the total slide length in the hot 1% NMPA. Wipe the back of the slides and place on aluminum foil at 37°C in a drying oven. Once the slides dry, store at room temperature in a Ziploc bag containing a slide tray and desiccant.

Assay procedure

1. After slides are prepared according to the procedure described above, cover the slides with foil.

2. Remove coated slides from the drying oven and place on a paper towel until ready for use.

3. Make a single cell suspension from the liver and blood as detailed above.

4. Pipette 10 µL of mixed cell suspension onto Parafilm and quickly mix with 100 µL of 37°C LMPA from the water bath.

5. Pipette 100 µL of mixture quickly down the length of the left side of the slide. Place the coverslip over the pipetted mixture, dropping from the left side of the slide down to spread in an even, thin layer.

6. Allow mixture to solidify in the dark, on an ice-chilled metal block for 5–10 minutes. Note all times on appropriate datasheet.

7. Once the agarose has solidified, carefully remove the coverslip.

8. Place the slide into the freshly made lysing solution in a slide box stored at 4°C. Make a note of which side of each slide has the agarose when putting them in the slide storage box.

9. Allow cells to lyse for at least 1 hour but not more than 24 hours (there will be some precipitate in the bottom of the lysing chamber). Note all times on the appropriate datasheet.

10. While cells are lysing, remove the freshly made electrophoresis buffer from the refrigerator and put into the electrophoresis chamber. Connect the power supply and set a constant voltage to 25 V. Adjust the electrophoresis buffer height to achieve ~ 100 milliamps (mA). *Note*: adding buffer increases amps, removing buffer reduces amps. Turn off the chamber so that the slides can be placed into it.

11. After cells have lysed, carefully remove and rinse the fronts and backs of the slides with DI water using a squeeze bottle. Place all slides on the rack.

12. Once all slides have been placed on the rack, quickly place them into the electrophoresis chamber. Insert slides into chamber at an angle (maximum of 16 slides per run). Once all the slides are in the chamber, start timing (*Note*: the exact positions can be moved during

this time so they are in straight lines). Keep slides in the electrophoresis solution for 10 minutes.

13. After 10 minutes, turn on the power supply (*Note*: the buffer volume can be quickly adjusted if needed to maintain the 100 mA start). Electrophorese the slides for 10 minutes, after which disconnect the power supply.

14. Remove the slides, let most of the excess buffer drip off, and then rinse the slides in neutralization buffer by placing the slides in a glass slide tray with the solution for 10 minutes. Drain the buffer from the slides and place the slides on paper towels with the agarose side up.

15. Place the slides in a slide tray with ice-cold ethanol or methanol for 5 minutes.

16. Remove the slides and lay them out on aluminum foil to dry in the dark at room temperature for 30 minutes–1 hour.

17. When dry, store the slides in a microscope slide box with desiccant in the dark at room temperature.

18. To rehydrate to analyze the slides, pipette 50 μL of EtBr diluted with HEPES-buffered HBSS and place a coverslip on the slide. Using the epifluorescent microscope under green light at 20 x objective (total x 400 magnification), use the Komet software (Kinetic Imaging, UK) to score 50 cells per slide in random order. Measure deoxyribonucleic acid (DNA) tail %, tail length, and tail moment.

A. Testing Protocol 1: Chronic Exposure of Juvenile Blue Crabs to Oil Contaminated Sediments

A.1 Experimental Setup

A.1.1 Experimental design overview

Each sediment toxicity test consisted of six test sediment preparations, including five test sediments (contaminated) and a control (reference). Eight replicate aquarium tanks (8,000 mL total volume with standpipes for water changes set to 6,000 mL water volume) were used for each of the six sediment types (48 tanks in total). Contaminated and reference field-collected sediments were provided by Stratus Consulting. Each tank was divided into three sections and one organism was placed into each section. There were 24 total organisms per treatment (i.e., 8 × 3).

All exposures lasted 31 days. Water quality parameters, including temperature, salinity, pH, ammonia, and DO, were recorded daily. A partial flow-through system was used to maintain water quality conditions. Daily water changes were conducted for 40 minutes at a flow rate of 200 mL/minute using a pressurized pumping system (resulting in a change of 1.3 x the volume of the tank). This system maintained all water quality parameters (including ammonia) at appropriate levels. During exposures, the light cycle was held at 14L:10D. Crabs were fed portions of squid tissue in the evening, and in the morning all excess food was removed and food consumption was recorded. To adequately maintain DO levels, oxygen delivered through air stones provided gentle aeration to the tanks.

During exposure, organisms were monitored daily for mortality and sublethal endpoints as described below. All endpoint data were recorded on the appropriate bench sheets provided by Stratus Consulting.

A.1.2 Tank design

The glass tanks (8,000 mL total volume) used for the experiment had a circular hole cut into the bottom of each tank to secure a standpipe that allowed for water outflow that did not disturb the sediment (volume of water maintained in each tank was set at 6,000 mL). The tanks were placed in two columns of 24 tanks (three treatments on each side), and propped up on rows of wood to allow the standpipe to drain into a long trough.

The stand pipe included a polyvinyl chloride (PVC) male fitting adapter sealed to the bottom of the tank around the hole with professional, aquarium grade 100% silicone. A PVC female

adapter was fitted to the male adapter and a PVC pipe, cut to give each tank a standpipe level of 6,000 mL water volume, was tightly fitted into the female adapter. When additional sealing was needed to prevent leaks, a washer was added between the male and female adapters and polytetrafluoroethylene (PFTE) tape was used to seal the connections. To prevent loss of any large particles of sediment, screens covered the top of the standpipe. All plastic pieces making up the standpipe were properly cleaned according to the Stratus Consulting QAPP and conditioned with seawater (25 ppt salinity) for at least 24 hours prior to use.

The tanks were divided by perforated plastic pieces cut to the height and width of the tanks with holes large enough to allow water and small particle transfer between sections within a tank, but small enough to prevent organisms from moving between sections. Dividers were pre-conditioned with seawater at 25 ppt salinity for at least 24 hours before use. Professional, aquarium grade 100% silicone was used to seal the dividers to the tank bottoms, and the silicone was allowed to dry for 24 hours.

Once the silicone dried, the entire tank, including dividers and standpipe, was conditioned with 6,000 mL of seawater (25 ppt salinity) for at least 24 hours prior to the addition of sediment. This water was siphoned out and fresh seawater added before the sediment was added.

A suspended airline system was implemented in which a long pipe with separate air valves for each tank was suspended less than a foot above the tanks. Aeration tubing was attached to each valve and an air stone to each tube. The aeration took place in the middle section for each tank.

Tanks were covered by thick, translucent plastic coverings from UMD's ARC algal culture facility. Plastic coverings were conditioned in seawater for at least 24 hours before use. Each row of eight treatments for both columns was covered by one plastic covering (three total coverings) that was creased in the middle to prevent movement from one column to another. Small holes were cut into the plastic covers for the airlines to be placed in the middle section of each tank.

To further prevent crabs from moving between tanks, permanently sealed, translucent plastic tubes (same plastic as coverings above) filled with water were placed over the top of the plastic cover to create a tight seal between the tanks and sections, while still allowing light penetration. These tubes did not come into direct contact with the tanks or the water in the tanks, but only the tops of the plastic covers.

A.1.3 Sediment preparation

Each sediment type was prepared separately using a stainless steel mixing bowl. Mixing bowls were cleaned and rinsed with solvent prior to use and between sediment types (the names and lettering of the different treatment blends are listed below).

A. South East Pass Campground 2011
B. South East Pass 2011
C. South Pass Spit 2011
D. Blend 1:1.7 (Loomis II 2011: Black Hole 2012)
E. Black Hole 2012
F. Black Hole 2011.

For all treatments except blend D, 1 kg of sediment was weighed in the mixing bowl. Seawater (1 kg at 25 ppt salinity) was added to the bowl (1:1 sediment to seawater ratio), and the sediment was allowed to thaw. The sediment and seawater were mixed thoroughly by hand using a stainless steel spoon until well-homogenized. These sediment mixtures were used to fill the tanks and 8-oz sediment sampling jars. Sediment sampling jars were sent to ALS Environmental for chemical analysis. Sediment preparation methods were documented in a laboratory notebook and proper datasheets.

For blend treatment D, 370.4 mg of Loomis II 2011 and 629.7 mg of Black Hole 2012 were weighed in the mixing bowl to get a 1:1.7 ratio. Seawater (1 kg at 25 ppt salinity) was added to the bowl with the sediments, and the sediments were allowed to thaw. The sediments and seawater were mixed thoroughly until well-homogenized.

A.1.4 Filling tanks with sediment

Each tank was prefilled with 4,800 mL of seawater (25 ppt salinity) prior to adding the sediment. The remaining seawater was added after a 24-hour settling period in order to minimize the risk of sediment exiting through the drain pipe.

Each tank and sampling jar was filled with sediment in a series of four 50-g increments, for a total of 200 g of sediment. Tanks were filled with sediment using pre-cleaned stainless steel measuring cups that were cleaned and rinsed with solvent between each sediment addition.

Sediment was scooped from the mixture using a four-tiered layering method (see Figure A.1).

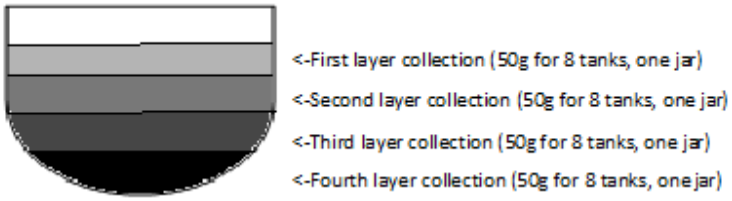

Figure A.1. Four-tiered layering method

The eight tanks and one jar were filled first by collecting from the top, "first" layer. After all the tanks and the jar had been filled once, they were filled again from the subsequent layer. This was repeated for a third and fourth time until all the tanks and jars had 200 g of total sediment/seawater mixture, always scooping from the top most-volume. Each scoop contained 50 g of mixture.

Weights of the sediment scoops were documented before and after pouring sediments into the tanks. Measuring cups were rinsed with seawater between scoops to remove any excess sediment and the cups were tared to 0.00 g before weighing each new scoop of sediment.

The 50 g of mixture in each scoop was distributed within the three sections of each tank (~ 1/3 of the scoop into each divided section). The scoop of sediment was added gently to reduce loss of sediment out of the drain pipe.

Any remaining sediment was placed into sample jars to be archived and stored at -20°C. Sample jars of sediment/seawater mixture were properly labeled as described in the Stratus Consulting QAPP, documented, and stored at -20°C.

The sediment was allowed to settle in the tanks for at least 24 hours while covered with gentle aeration to maintain DO levels for the next day's addition of blue crabs.

A.1.5 Addition of organisms to treatments

After the sediment had settled for at least 24 hours, composite water samples for chemical analysis were collected as described in Section A.5 and stored in a locked cooler at 4°C. After the composite water samples were collected, water in each tank was added gently to reach the 6,000 mL level (i.e., to the tip of the standpipe), with attempts to minimize sediment disruption.

After all the tanks had been filled, juvenile blue crabs were added. The organisms were taken out of the holding tank in the ARC facility and separated by size. As much as possible, crabs of similar size were selected for use in the study to minimize the overall size range of the crabs. The size range of the study depended upon the crabs available for use. Only healthy crabs with at least one cheliped and one swimming leg were chosen for the study, with preference given to crabs with two of each.

Before being added to the tanks, each crab was weighed, a photograph was taken on a grid for length/width measurements, and the sex was determined. The measurements and times that each crab was added to the tank were documented on an appropriate bench sheet.

Crabs were randomly selected from the holding buckets. Crabs were added to the "a" section (innermost) of each tank for every treatment first, moving sequentially through treatment and

tank number. After all the "a" sections were filled, the "b" sections (middle) for each tank for every treatment were filled. Lastly, all of the "c" sections (outermost) were filled.

Once all the crabs for a treatment were in their respective tank sections, seawater-conditioned plastic covers were placed on top of the tanks to protect the tanks from debris as well as to prevent crabs from moving between tanks. The air stones and tubing were placed into the water in the "b" (middle) section of each tank and kept at sufficient air pressure to maintain DO levels while not disturbing the sediment. To further seal the tanks, permanently sealed translucent plastic tubes filled with water were placed on top of the plastic cover. Airlines were checked constantly to ensure that the air stones remained in the water.

A.1.6 Water change

A large reservoir of water was filled at the start of each day for the water change using a mixture of filtered tap water and seawater prepared at the ARC facility. The seawater and filtered tap water were mixed until the desired test salinity of 25 ppt was reached.

Daily water changes were conducted on eight tanks (one treatment) at a time until all treatments had received a water change. The flow rate of the water change was fast enough to provide an efficient tank exchange (approximately 1.3 x the volume of the tank, i.e., 8,000 mL) of water without disturbing the sediment layer. The water change of each tank lasted 40 minutes at a flow rate of 200 mL/minute, using a pressurized pumping system with individual valves for each tank.

The water being exchanged flowed out of the standpipe into a long drainage well and collected in an activated carbon filter apparatus. The treatment effluent percolated through the activated carbon, removing any contaminants before flowing into a sewer drain. The activated carbon filter was replaced halfway through the 31-day experiment with a new activated carbon filter. Used activator carbon filters were packaged and given to Environmental Health and Safety employees at IMET for hazardous waste disposal.

During the water change, the plastic sheets covering the tanks were moved so that only the front sections of each tank were exposed to minimize any potential movement of organisms from one tank to another. The front sections were also closely monitored during the water change. Individual water valves were monitored to ensure that all valves were releasing the standard flow rate and any valve with a flow rate deviation was properly adjusted.

Times for each treatment's water change were recorded in the notes section of the *Water Quality Monitoring* bench sheet.

A.2 Daily Monitoring Endpoint

Every crab was checked daily for general-monitoring endpoints, including mortality, molting, feeding, and growth (weight and carapace length/width). Crabs were checked for general-monitoring endpoints one treatment at a time to minimize any potential crab movement between tanks. Water tube barriers were removed from the top of the plastic cover and placed in designated storage areas. The covers were lifted and clamped into the airline system pipe to allow access to all of the tanks for one treatment. The clamps were cleaned and rinsed with solvent between treatments to prevent cross-contamination.

General monitoring checks began before the start of the water changes, unless the water quality prohibited visualization, in which case crabs were viewed during and/or after the water changes. For example, treatments containing finer sediments that reduced the water clarity were examined after the daily water change for that treatment to increase visibility.

The visibility of crabs could be difficult for the middle and inner tanks, particularly for treatments with finer sediments. Before checking the crabs, air stones were removed temporarily from the water, and were replaced immediately after general monitoring endpoints had been examined for a treatment.

If the visibility was still reduced, a pre-cleaned glass beaker was used to reduce the path length and a head lamp or flashlight was used to aid visualization. The glass beaker was cleaned and rinsed with solvent between treatments and after use.

All daily general monitoring endpoints were recorded on the *Test Performance Monitoring Bench Sheet*, provided in the Stratus Consulting QAPP, for mortality and sublethal endpoints.

A.2.1 Mortality

A *Test Performance Monitoring Bench Sheet* was filled out daily for all tanks, scoring crabs as alive or dead and indicating in the notes which individual crab number died in a tank. The mortality was recorded as a treatment mortality if the crab died from the treatment and as a non-treatment mortality if the crab died from other sources (e.g., cannibalism). In tanks where the crabs were not visually moving or if they had buried themselves in the sediment, mortality was assessed by gently probing either the sediment (without disturbing and resuspending the sediment) or the actual crab. Crabs not responsive after gentle prodding were removed from tanks (see below) and scored as dead.

All dead crabs were removed from the tanks as soon as possible. For each dead crab (when possible), a photograph was taken for length and width measurements and recorded in the sublethal *Test Performance Monitoring Bench Sheet*. All dead crabs were individually wrapped

in foil that was labeled on the inside and outside of the foil packet. The foil packet with the crab was then placed into a labeled 8-oz glass jar or plastic bag. Labels included the date removed, individual crab ID (treatment-tank-crab letter), and observer initials. Dead organisms from each treatment were kept in separate jars and all jars were stored in a locked freezer at -20°C.

A.2.2 Molting

All crabs were examined for molting daily and scored as molted (Yes or No) on the sublethal *Test Performance Monitoring Bench Sheet*. If the crabs buried themselves in the sediment, making them difficult to visualize, the sediment was gently probed to improve visibility of the crabs. Likewise, all thicker sediments were gently probed to help find any potential molted shells.

If a crab had molted, the molt shell was removed and photographed to validate the carapace size. If enough of the shell molt remained to determine the sex of the crab, it was recorded in the sublethal *Test Performance Monitoring Bench Sheet*.

Every crab that molted was examined for growth endpoints (weighed and photographed for carapace length and width) the following day.

A.2.3 Food consumption

The food consumption of the each crab was scored daily from 0 to 2 on the sublethal *Test Performance Monitoring Bench Sheet*. A score of 0 indicated No Feeding (all of the food remained). A score of 1 indicated Intermediate Feeding (some of the food had been consumed). A score of 2 indicated Complete Feeding (food was completely gone). As with the molt scoring, all thicker sediments were also probed to ensure all excess food was found.

Any remaining food in the tanks was removed using pre-cleaned stainless steel tongs and placed into plastic bags for disposal. Tongs were cleaned and rinsed with solvent between treatments.

A.2.4 Growth endpoints

Weight of crab

1. Crabs that had molted the previous day were gently removed from the tank using a pre-cleaned, large stainless steel serving spoon. The crab was picked up by the posterior region and wrapped in a paper towel for transport.

2. The crabs were removed as quickly and gently as possible, attempting to minimize disturbance of the sediment and stress to the crab. Excess water brought up with the crab was drained back into the tank and the spoon was dipped multiple times back into the treatment water to remove any attached sediment. The spoon was cleaned and rinsed with solvent between treatments.

3. A pre-cleaned, 150-mL beaker containing approximately 50 mL fresh seawater (25 ppt salinity) was tared to 0.00 g on an analytical balance.

4. The crab was gently blotted with the paper towel to remove excess water and placed into the beaker on the analytical balance.

5. The weight and sex of the crab were recorded in the sublethal *Test Performance Monitoring Bench Sheet*.

Length and width of carapace

1. A camera was set up on a tripod prior to starting these measurements. The camera position and zoom were standardized for each daily group of crabs to allow for quicker analysis in Image J.

2. A grid of known distances was taped to a plate of glass with clear packaging tape and used to measure the length and width. The grid size was 0.25 in. (6.35 mm). The grid was placed under the camera and the focus was adjusted until the proper distance and clarity were reached.

3. For each treatment, tank number and crab letter ID labeled cards were created and used to identify the crab within each photograph. For each photograph, the appropriate card was placed in the top corner of the grid/photograph.

4. The crab was taken out of the beaker used for measuring weight and gently blotted with a paper towel to remove excess water.

5. The crab was placed on the grid and held in place with a thin, wooden stick, making sure that the stick did not cover either of the lateral spines or the top/bottom of the carapace for the photograph.

6. Immediately after the photograph was taken, the crab was placed back into its respective tank to minimize the time the crab spent out of the tank.

7. The analysis of length and width of the carapace was done using Image J software. The length and width boxes on the sublethal *Test Performance Monitoring Bench Sheets* were

left blank until those measurements were conducted. Once the length and width were measured, those data were then filled in on the original sheets for the day the crab was photographed.

A.3 Daily Water Quality Measurements

Water quality parameters, including temperature, salinity, pH, and DO, were recorded daily in all tanks when possible, or for a minimum of two tanks per treatment. Ammonia level was recorded for at least two tanks per treatment every day (using a random sampling design). Ammonia levels were monitored in all tanks of a treatment at least once weekly. All water quality parameters were measured in the outermost, front section of each tank. Water quality measurements, as well as the time each measurement was taken, were recorded on the *Water Quality Monitoring* bench sheet provided in the Stratus Consulting QAPP. See the UMD GLPP document for details on water quality measurement procedures.

A.4 Feeding

Daily, each crab was fed small pieces of squid tissue one treatment at a time to minimize the potential for movement of crabs between tanks. Squid tissue, pre-shredded and stored in a freezer at -20°C, was thawed before being fed to the crabs.

After the water change, the pre-shredded squid was removed from the freezer and allowed to partially thaw in a thin layer of fresh seawater (25 ppt salinity) to the point where individual pieces could be removed. Feeding was the last task completed each day.

Each crab was fed one large, pre-shredded squid or two smaller, pre-shredded squid pieces depending on how the squid was shredded. Initially the total amount of food fed to the crabs was between 0.10 and 0.15 g, and the amount of food fed to the crabs increased as the crabs molted.

The timing of feeding was documented in the laboratory notebook and in the notes section of the *Water Quality Monitoring* bench sheet. Any remaining food was documented and removed the following morning. See Section A.2.3 for more details.

A.5 Sampling

Both sediment and overlying water samples were collected throughout the experiment. Analytical chemistry samples were handled, labeled, and shipped to ALS Environmental according to the Stratus Consulting QAPP, with the following specifications:

- ***Initial sediment sampling:*** When preparing tanks for sediment exposures, sediment samples were collected from each treatment and control group, and prepared as a "ninth" replicate during the aliquoting of sediment into the replicate tanks plus one analytical chemistry sediment glass jar (8-oz). These initial sediment samples were sent to ALS Environmental for chemical analysis.

- ***Final sediment sampling:*** Composite sediment samples were collected from each treatment group and control tanks at the end of the experiment. First, all or most of the water was siphoned from each tank. Next, all of the sediment from within a treatment group was composited into a stainless steel mixing bowl. Care was taken to avoid adding water when transferring sediments to the mixing bowl. Composited sediments were then mixed using a pre-cleaned, large stainless steel spoon. Once the sediment was thoroughly mixed, a pre-cleaned ¼-cup stainless steel scoop was used to fill the analytical chemistry glass sediment jar (8-oz). This process was repeated for each treatment group and control using clean, decontaminated sample processing equipment. Final sediment samples were sent to ALS Environmental.

- ***Composite water sampling:*** Composite water samples were collected from each treatment group and control just before the crabs were added (time zero), every other day for 6 days, then once a week for the duration of the experiment. Time zero samples were taken just prior to adding the crabs; all other sampling was performed before the daily water changes. At each time point, a subsample from each of the eight replicate tanks within each treatment was collected and composited into a clean decontaminated glass mixing container. Subsamples were collected by dipping a clean, decontaminated ¼-cup stainless steel scoop into each tank. For consistency, the sample was always taken from the front section of each tank. The same transfer beaker was used for all tanks within a single treatment. A clean, decontaminated beaker was used when collecting subsamples from each additional treatment. The scoop was filled to the brim for each subsample to ensure that the same volume was added from each tank to the composite. After thoroughly mixing subsamples, the composite was used to fill the required analytical chemistry containers for requested chemical analyses (see the Stratus Consulting QAPP). This process was repeated for each treatment group and control. Water samples were sent to ALS Environmental for analysis.

The standard chain-of-custody (COC) forms provided in the Stratus Consulting QAPP were used to request chemical analyses and relinquish samples to ALS Environmental.

A.6 Additional Endpoints

A.6.1 Dissection of tissue for molecular endpoints

Hemolymph and hepatopancreas tissues from one crab per treatment were extracted to examine DNA damage (i.e., single-strand breaks) with the Comet Assay. Remaining hepatopancreas tissue from each crab was extracted for genetic molecular biomarker analysis (see below). Before the experiment began, hemolymph and hepatopancreas tissues were also extracted from 10 unexposed crabs (Time 0 hour crabs) collected from the same holding tanks as the test organisms were collected from. These samples were exposed to hydrogen peroxide to be used as a positive control for the Comet Assay.

From each crab, the hemolymph was extracted first to minimize the impacts of stress on the DNA of the hemolymph. Immediately after the crab was removed from the water, approximately 50 µL of hemolymph was extracted directly into a syringe with a 23-gauge needle that had been coated with and contained approximately 50 µL of crustacean anticoagulant (1:1 hemolymph). The hemolymph was extracted from the crab's hypobranchial sinus through the arthrodial membrane between the chelae and the first walking leg. The crustacean anticoagulant contained 0.3 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 25 mM citric acid, and 3 mM ethylenediaminetetraacetic acid (EDTA) at a pH of 6. The hemolymph/anticoagulant solution was transferred into Eppendorf tubes and placed on ice. The tubes were then centrifuged at 800 x g for 10 minutes at 4°C to concentrate the cells. The supernatant of each tube was discarded and the pellet was resuspended in 50-µL anticoagulant, which was kept on ice until processing for the Comet Assay. The exposure of the tubes to light was minimized.

After removal of the hemolymph, the crabs were placed on ice for at least 5 minutes (until torpid) before dissecting. Crabs were dissected one at a time with pre-cleaned dissection equipment rinsed with solvent. First, the hepatopancreas was dissected and a small subsample taken for the Comet Assay. The subsample was immediately processed by mincing it in 300 µL of HBSS-HEPES with a buffer capacity of pH 7.6 using a clean razor blade. To keep the sample cold, it was minced on a strip of Parafilm on top of a cold metal aluminum sheet placed on ice. The rest of the hepatopancreas tissue was placed into pre-labeled cryovials containing RNA stabilizing solution (RNAlater). After allowing the RNAlater solution to permeate the tissues overnight, the cryovials were placed into a sealed, labeled cryovial box and stored in a locked -80°C freezer. If RNAlater solution was not available, the cryovials were flash frozen in liquid nitrogen and stored on dry ice during the remaining processing until they could be permanently stored in a locked -80°C freezer.

For the positive control samples, 10 µL of the resuspended hemolymph or the minced hepatopancreas tissue in HBSS-HEPES was placed into Eppendorf tubes containing a solution of 50 µM hydrogen peroxide in cold HBSS-HEPES and left on ice for 30 minutes before analysis

with the other samples. These tubes were then centrifuged at 800 x g for 10 minutes at 4°C to concentrate the cells. The supernatant of each tube was discarded and the pellet containing the cells was resuspended in 50 µL anticoagulant for hemolymph or 50 µL HBSS-HEPES for hepatopancreas, kept on ice, and covered with foil. The positive controls were processed as quickly as possible to minimize potential impacts from residual hydrogen peroxide in the resuspension.

A.6.2 Alkaline single cell gel electrophoresis (Comet Assay) protocols

Microscope slides were coated with 1% NMPA in PBS and allowed to dry at 37°C in the dark. These slides were stored in slide boxes within plastic bags containing desiccant.

From the hemolymph resuspensions, minced hepatopancreas tissues, or resuspended positive controls above, 10 µL was added to 100 µL of 0.6% LMPA (37°C) in HBSS-HEPES pH 7.6 and layered over the NMPA layer on the slides. Two replicate slides were made for each crab sample. Coverslips were placed onto slides and the agarose was allowed to polymerize for at least 5 minutes on a metal tray over ice. Following solidification, the coverslips were removed and the slides were placed into cold (4°C) lysing solution (10% DMSO, 1% Triton X-100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris Base, 1% sodium sarconsinate; pH 10) for at least 1 hour but not more than 24 hours at 4°C in the dark.

Once removed from the lysing solution, slides were rinsed with distilled water, placed on a horizontal gel electrophoresis tray, and covered with cold (4°C) electrophoresis buffer (0.20 M NaOH, 1 mM EDTA; pH > 12) for 10 minutes to allow the DNA to unwind. Electrophoresis was conducted at 25 V, 300 mA for 10 minutes. Slides were removed and placed in a cold (4°C) neutralization solution (0.4 M Tris, pH 7.5) for 5 minutes (repeated for 3 washes for a total of 15 minutes). Slides were then drained and placed in 100% ethanol (4°C) for 5 minutes, allowed to dry in a dark container overnight, and then placed in a desiccated slide box until processing by the image analysis system.

The slides were reconstituted with 2 µM/mL EtBr in HBSS-HEPES and examined using an epifluorescent microscope (Olympus BX50) with a green filter at 40 x magnification (QImaging Retiga 1300 camera). The Komet 5.5 Software's (Kinetic Imaging, Liverpool, UK) image analysis package was used to score the cells. From each duplicate slide, 50 non-overlapping cells were randomly selected for quantification. The results were expressed as means ± standard error of the means in terms of the percentage of DNA in the tail (% tail DNA), tail length (µm), and Olive tail moment.

A.6.3 Gene expression protocols

The hepatopancreas tissue samples from all of the Time 0 hour and treatment crabs were homogenized using a Fast Prep Bead Breaker method, and the messenger RNA (mRNA) was extracted using an RNAqueous Kit (Life Technologies). The extracted mRNA for each crab was treated with deoxyribonuclease (DNase) using a TURBO DNA-free kit (Life Technologies) to remove any genomic DNA contamination and then reverse transcribed to complementary DNA (cDNA) using a High Capacity ribonucleic acid (RNA)-to-cDNA Kit (Life Technologies) for absolute quantitative polymerase chain reaction (qPCR) analysis. For each sample, a no reverse transcription (No RT) control was also conducted in parallel to the reverse transcription (RT) reaction to examine genomic DNA contamination in the cDNA sample remaining after the DNase treatment.

Primers for each gene of interest were designed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) from either existing blue crab mRNA sequences in GenBank or from Expressed Sequence Tags (EST) database hits observed by using BLAST with known mRNA sequences for other crab species in a Blue Crab EST Library available in Dr. Al Place's laboratory at IMET.

Polymerase chain reaction (PCR) products for each gene of interest were run on a 1.5% agarose gel, extracted using a QIAquick Gel Extraction Kit (QIAGEN), and ligated into a TOPO pCR-II plasmid. These plasmids were transformed into chemically competent TOP10 *E. coli* (Invitrogen). The plasmids were then purified from the *E. coli* using a ZR Plasmid Miniprep-Classic Kit (Zymo Research) and the plasmid DNA were quantified on a NanoDrop 2000 spectrophotometer. The purified products and plasmids were sequenced by the IMET BioAnalytical Services Laboratory and the orientation of the sense strand of product insert in relation to the T7 promoter was verified. The plasmid DNA for each gene was linearized using a restriction digest (Hind *iii*), and complete digestion was verified by gel electrophoresis. The linearized plasmids were then appropriately diluted to a six-point serial dilution series and run on qPCR to examine the qPCR efficiency for all genes.

To conduct absolute quantification, the plasmid inserted products were transcribed into RNA using a TranscriptAid T7 High Yield Transcription Kit (Fermentas). The RNA concentrations for each gene were measured on a NanoDrop 2000 spectrophotometer and six-point RNA serial dilutions were prepared to give a known copy number for the RT reaction. Each RNA dilution was reverse transcribed to cDNA of a known copy number. Absolute qPCR was conducted using the Power SYBR Green Kit on an Applied Biosystems 7500 Fast Real-Time PCR System. For each gene, the crab cDNA samples, the six-point cDNA standards, and the "no template" controls were run in triplicate. The No RT crab cDNA samples were run in duplicate for arginine kinase (AK) with AK cDNA standards in triplicate to examine genomic DNA contamination. A dissociation curve was produced for all samples and standards. A linear regression equation was produced for the cDNA standards of each gene and used to determine the copy number of each

crab sample. Copy numbers were then normalized to the total RNA concentration (ng) and reported as copy numbering total RNA.

A.6.4 Histology

At the beginning of the experiment, five organisms were selected at random from the holding tank and dedicated for histology. At the end of the study, one crab from every tank, provided there were not two mortalities within a tank, was dedicated for histology (i.e., eight crabs per treatment).

Whole crabs were placed on ice until torpid (up to 15 minutes). Chilled crabs were injected with a few drops of fixative (Bouin's solution) using either a 26.5-G or a 23-G needle and a syringe of 1 or 3 mL. Fixative was injected under the carapace by putting the needle into the viscera through the ventral shell if it was thin (< 1 g crab) or through the membrane between the swimming leg and the carapace. Care was taken to avoid moving the needle tip laterally once it was inserted into the viscera as this could tear organs and tissues. When necessary, it was acceptable to make two injections, one on either side, to ensure the complete penetration of fixative. The penetration of fixative was visible as a change in opacity or color (if Bouin's solution was used) under the ventral shell.

Once the crab was injected with fixative, it was placed in > 10 volumes of fixative in a sealable container. For example, a 0.5 g crab would be placed in at least 5 mL of fixative. For crabs under 3 g, 50-mL conical polypropylene tubes were used. For crabs that were too large to fit into a 50-mL tube, medical specimen jars or histology jars were used. Each crab was placed in its own 50-mL conical tube or jar. Suitable labels were applied to tubes or jars before testing to avoid mixing up treatments. Because labels could be rubbed off or erased by later solvents or acids, a label was also put inside each tube or jar. This label was written in soft pencil (not a pen) on filter paper. Crabs were stored at 4°C in fixative for at least 96 hours and up to 14 days.

The fixative was later decanted, the crabs were rinsed in distilled water for 1 hour, and then placed in 70% ethanol (10 volumes) for at least 1 day. Traces of fixative were rinsed from the crab and container with 3–5 mL of 70% ethanol. Then the containers were filled with the > 10 volumes of fresh ethanol for long-term storage.

A.6.5 Polycyclic aromatic hydrocarbons bioaccumulation

At the beginning of the experiment, 10 unexposed organisms were selected at random from the holding tank to serve as controls for the analysis of polycyclic aromatic hydrocarbon (PAH) bioaccumulation. At the end of the study, the remaining crabs from each treatment (i.e., any

crabs not used for the histological or molecular endpoints) were collected for PAH bioaccumulation.

The crabs to be used for PAH bioaccumulation were first weighed and photographed for length/width of carapace as detailed in Section A.2.4. The crabs were then placed on ice for at least 5 minutes and then individually wrapped in foil, labeled, and stored in bags. All crabs from each treatment were stored in the same bag and stored at -20°C for potential bioaccumulation analyses.

B. Testing Protocol 2: Turtle Stress Response Trials

For this study, baseline corticosterone assessments were conducted followed by a standard adrenocorticotropic hormone (ACTH) challenge. The methods used were similar to those used previously with mink (Mohr et al., 2010). All SOPs referred to in this section can be found in the UMD GLPP.

B.1 Experimental Design

Both red-eared sliders and snapping turtles were tested. Each study included three treatment groups: (1) a high ACTH dose group (50 IU/kg), (2) a low ACTH dose group (10 IU/kg), and (3) a restraint and handling group using the dilutant only (0.9% sterile saline) to account for potential stress related to handling during ACTH injections and blood draws. Blood samples were collected at different time points for up to four hours following the ACTH injection to determine when the maximum corticosterone response was achieved in the two turtle species.

Because the handling and the blood draw itself can interfere with stress response, each animal had blood collected at the beginning of the test (i.e., pre-injection) and then at post-injection. There were limitations on the maximum volume of blood that could be drawn from repeated blood draws on the same individual. The goal was to collect 0.6 mL of blood at each time point. For red-eared sliders, blood samples were collected at 60, 120, 180, and 240 minutes post-injection and for snapping turtles, due to the limited number of turtles available, blood samples were collected at 60, 120, and 240 minutes post-injection. For red-eared sliders, each group included 16 animals (total n = 48 turtles), and for the snapping turtles, each group included 12 animals (total n = 36 turtles). This allowed sampling of four replicate individuals at each time point.

B.2 Turtle Housing and Maintenance during Experiment

At least one day prior to the scheduled start of any experiment, turtles were selected from a pool of individuals and placed in individual 10-gallon glass tanks in the second temperature-controlled room. The temperature-controlled room was set at 26°C and tanks were placed on stainless steel metal shelves with attached UV-A/UV-B reptile lamps to provide the required light and heat conditions. Before being placed in the experimental tanks, each turtle was weighed and given a unique test ID number, which was placed on a label on the outside of the tank. Each turtle had a notebook assigned to it to keep track of daily health (feeding, behavior, temperature) monitoring. The turtles remained in their individual tanks for the duration of the experiment. As outlined in the housing and monitoring SOP #05, the turtles were fed, their water was changed, and they were monitored for signs of stress on a daily basis.

After the test was completed, the snapping turtles were returned to their original 5-gallon tanks in the first temperature controlled room and the sliders were returned to two 200-gallon tanks (~ 20 turtles per tank). All red-eared sliders used in this test were housed separately from the remaining turtles. They were also uniquely marked by drilling holes in the scutes so they could be identified in subsequent studies.

B.3 Dosing (injection of ACTH or control solution) and Blood Collection

At the beginning of the test, all turtles had an initial blood draw to establish baseline corticosterone levels in the individual. Depending on which method could more easily be performed, blood was drawn from the subcarapacial venous sinus or from the dorsal coccygeal vein as outlined in SOP #3a and #3c, respectively. Care was taken to draw pure blood; however, if a lymph-contaminated blood draw occurred, it was noted along with an estimate of volume in the sampling datasheets. In order to minimize effects of handling stress on baseline corticosterone results, samples were collected within 2–6 minutes of turtles first being handled. Immediately following the baseline blood draw, each turtle was injected with its respective dose of ACTH or 0.9% sterile saline control. Before injection, the injection site was cleaned with an ethanol swab and air dried. Turtles received an intraperitoneal injection using a 26 gauge needle, and the volume injected reflected the dose group and was based on the weight of the turtle. For the restraint and handling group, a saline injection with volumes similar to the 50 IU/kg group was completed to mimic the handling experienced by the ACTH groups. All blood draws were conducted according to SOP #3a or #3c. Blood samples were processed according to SOP #04, and the plasma samples were sent to Cornell University for analysis. The goal was to collect at least 400 µL of plasma (~ 800 µL of whole blood) so that the plasma samples could be split and stored in two replicate vials. Plasma samples were stored at -20°C until shipped. If enough blood was collected for replicate vials, the second replicate remained at CBL for long-term storage. At least 100 µL of plasma (~ 200 µL of whole blood) was needed for each analysis (i.e., one of the replicate vials).

B.4 Documents and Records

Toxicity testing information documented in notebooks, datasheets, photograph files, and logs; COC forms; and shipping forms followed procedures described in the Stratus Consulting QAPP. Additional datasheets specific to this study were also used. Please refer to the QAPP for all instructions pertaining to recordkeeping, sample label protocols, and document retention requirements.

C. Testing Protocol 3: Oral Dosing of Oil in Turtles

For these tests, red-eared sliders or snapping turtles were dosed orally with oil for 14 days to determine sublethal impacts to turtles following exposure to oil. A variety of biological and chemical endpoints were used to determine exposure to and effects of the oil. Specifically, the endpoints that were examined investigated the extent of PAH metabolites, oxidative stress, histological modifications and impacts to hypothalamic-pituitary-adrenal axis (HPA), in addition to blood chemical and biological endpoints. All SOPs referred to in this section can be found in the UMD GLPP.

C.1 Experimental Design

Both red-eared sliders and snapping turtles were tested in separate experiments. Each study included two treatment groups and one control group. The control group was dosed orally with a food slurry bolus followed by 0.9% sterile saline to account for the potential stress related to dosing and handling. The treatment groups were dosed orally with daily doses of Slick A oil followed by a food slurry bolus and 0.9% sterile saline. Each group had 16 animals for a total of 48 animals. Six of the turtles in each group were necropsied at the end of the 14-day period. The other 10 turtles had a pre-dose blood sample collected (for baseline corticosterone levels, and blood chemical and biological endpoints), and then were dosed with ACTH. After a set period of time (see SOP #10), the turtles underwent necropsy so that a post-dose ACTH blood sample could also be collected.

To dose the turtles orally, feeding E-tubes were surgically inserted by a trained veterinarian according to SOP #6 at least 4 days before experimental dosing. Each individual turtle was weighed (see SOP #1) and dosed on a daily basis for the 14-day period. If a turtle showed any signs of stress or removed their E-tube, they were removed from the study. Turtles were inspected daily for signs of feeding, regurgitation, feces production, and any other signs of stress/discomfort, including issues related to the E-tube surgical site. At the end of the 14-day study, the remaining turtles were necropsied. Blood/tissue samples were collected and stored appropriately or sent directly to the contracting laboratories as outlined in Table C.1. To examine the adrenal response to oil dosing, 10 turtles in each group underwent an ACTH challenge, which included the collection of a pre-ACTH blood sample to establish a corticosterone baseline (and to analyze blood chemistry and biological endpoints), after which the ACTH was injected, and a final blood sample was collected. Necropsies were performed, and tissue samples were processed and collected according to SOP #8. Blood samples were collected according to SOP #3b.

Table C.1. Analysis detail, sample containers, preservation techniques, and holding times for each type of sample/analysis/ endpoint conducted.

Analysis	Sample type (minimum volume)	Preparation details	Storage container	Storage conditions	Analysis lab/ sent details
Red blood cell (RBC)/white blood cell (WBC) counts and morphology (1)	60 µL heparinized whole blood	Blood smear, prepare 3 slides at CBL (SOP #2) and 3 slides at Miami	Glass slides in plastic slide box	Room temperature in slide box	Send to Miami and N. Stacy (for morphology)
RBC/WBC counts and morphology (2); hematocrit (PCV)	100 µL heparinized whole blood	Remove from vacutainer after careful mixing	Labeled plastic 1.5 mL cryovial with internal O-ring	Keep at 4°C, do not freeze or place direct on ice	Send to Miami and N. Stacy (for morphology) overnight on blue ice
Hemoglobin concentration	100 µL heparinized whole blood (500 µL for validation)	Remove from vacutainer after careful mixing	Labeled plastic 1.5 mL cryovial with internal O-ring	Keep at 4°C, do not freeze or place direct on ice	Send to Miami overnight on blue ice
PCV	30–40 µL heparinized whole blood	Remove from vacutainer after careful mixing	Microhematocrit capillary tube; see SOP #9	Keep at 4°C do not freeze or place direct on ice; analyze in < 12 hours	Analysis at CBL
Blood chemistry panel (based on bird work and reptile panel); see Table C.2 for list	320 µL heparinized plasma (for all) except glutamate dehydrogenase (GLDH)	Spin whole blood at 2,000 g for 10 minutes at 10°C, remove supernatant (plasma)	Labeled plastic cryovials with internal O-ring (one for 300 µL and one for 100 µL sample)	Freeze on dry ice and store at -70°C	Send to Miami overnight on dry ice
GLDH	100 µL heparinized plasma	Spin whole blood at 2,000 g for 10 minutes at 10°C, remove supernatant (plasma)	Labeled plastic cryovials with internal O-ring	Freeze on dry ice and store at -70°C	Send to Miami overnight on dry ice
Plasma electrophoresis (albumin and globulin)	20 µL heparinized plasma (volume also included in the 320 µL complete blood count sample)	Spin whole blood at 2,000 g for 10 minutes at 10°C, remove supernatant (plasma)	Labeled plastic cryovials with internal O-ring	Freeze on dry ice and store at -70°C	Send to Miami overnight on dry ice

Table C.1. Analysis detail, sample containers, preservation techniques, and holding times for each type of sample/analysis/ endpoint conducted.

Analysis	Sample type (minimum volume)	Preparation details	Storage container	Storage conditions	Analysis lab/ sent details
Corticosterone	150 µL heparinized plasma	Spin whole blood at 2,000 g for 10 minutes at 10°C, remove supernatant (plasma)	Labeled plastic 1.5 mL cryovials with internal O-ring	Freeze on dry ice and store at -20°C	Send to Cornell overnight on dry ice
Oxidative stress (tGSH/GSSG, blood)	50 µL heparinized RBCs	Spin whole blood at 2,000 g for 10 minutes at 10°C, remove supernatant (plasma)	Labeled plastic 1.5 mL cryovials with internal O-ring	Freeze on dry ice and store at -70°C	Analysis at CBL (Cayman kit)
Oxidative stress (tGSH/GSSG, liver)	One 0.5 g piece of liver	Slice piece from whole organ, and weigh	Labeled plastic 2 mL cryovials with internal O-ring	Flash freeze in liquid nitrogen and store at -70°C	Analysis at CBL (Cayman kit)
Oxidative damage (lipid peroxidation)	One 0.5 g piece of liver	Slice piece from whole organ, and weigh	Labeled plastic 2 mL cryovials with internal O-ring	Flash freeze in liquid nitrogen and store at -70°C	Analysis at CBL (Oxford kit)
CYP1A enzymes (liver)[a]	One 0.5 g piece of liver	Slice piece from whole organ, and weigh	Labeled plastic 2 mL cryovials with internal O-ring	Flash freeze in liquid nitrogen and store at -70°C	Analysis at CBL
PAH and metabolites[a]	0.1 mL or more of bile	Collect bile with needle and syringe before removing liver, transfer to sample container	Solvent rinsed, baked, or trace clean Amber glass vial with polytetrafluoroethylene lid	Freeze on dry ice and store at -70ºC	Samples stored at CBL
Histopathology	GI tract, liver (gallbladder emptied), kidney, spleen (half), adrenal, thyroid, heart, lungs, gonads, muscle tissue (one hind leg)	Collect bile, liver, and spleen samples first (for other analyses), then fix in 10% NBF; slice thick tissue if needed	Single labeled jar (1 L) with at least 10x volume NBF; decant and replace formalin after 24 hours	Room temperature	Analysis by Zoo/Exotic Pathology Service

Table C.1. Analysis detail, sample containers, preservation techniques, and holding times for each type of sample/analysis/ endpoint conducted.

Analysis	Sample type (minimum volume)	Preparation details	Storage container	Storage conditions	Analysis lab/ sent details
DNA damage (COMET assay); blood and liver cells	20 µL heparinized blood, 10 mg liver tissue	Remove 20 µL from vacutainer and place in tube, place liver in tube on ice	Plastic tube	Keep on ice in the dark, and assay in < 2 hours	Analysis at CBL (needs immediate processing)
Transcriptomics (blood)[a]	500 µL heparinized blood	Remove 500 µL from vacutainer and place in PAXgene tube, invert tube 8–10 times	PAXgene tube	Store upright at room temperature for 2–6 hours, then store at 2–8°C for < 5 days; if a delay in shipping occurs, store at -20°C after 5 days	Send to Hollings Marine Laboratory overnight on ice

a. Indicates samples that were collected but not analyzed.

Table C.2. Specific endpoints to test in the blood chemistry panel and associated endpoints (University of Miami). Analytes are listed in order of priority.

Chemistry endpoint to test	Volume of plasma	Cumulative volume of plasma required
Plasma electrophoresis (albumin and globulins)	20 µL	One 320-µL plasma sample for both endpoints
Total protein (TP)	300 µL[a]	
GLDH	100 µL	A separate 100-µL plasma sample for GLDH
Gamma-glutamyl transpeptidase (GGT)	[a]	
Glucose (Glu)	[a]	
Uric acid	[a]	
Creatine phosphokinase (CPK)	[a]	
Aspartate aminotransferase (AST)	[a]	
Lactate dehydrogenase (LDH)	[a]	
Triglycerides	[a]	
Sodium (Na)	[a]	
Calcium (Ca)	[a]	
Chloride (Cl)	[a]	
Phosphorus (Phos)	[a]	
Cholesterol	[a]	
Alkaline phosphatase (ALkPhos)	[a]	

a. Grouped samples in reptile panel.

C.2 Turtle Housing, Maintenance, and Monitoring during Experiment

At least one day prior to dosing, turtles were selected from a pool of individuals and placed into individual 5.5-gallon glass tanks in one of two temperature-controlled water baths (98” long x 48” wide). A unique test ID (turtle ID number) was placed on the outside of each tank so that individual turtles were readily identified. Tanks were also color-coded to aid in quick ID of the dose group (i.e., controls had white tape, low dose had green tape, and high dose had red tape). After E-tube insertion, the turtles were placed into the temperature-controlled water baths in individual 5.5-gallon tanks. Once placed in the external water bath, the heated external recirculating water maintained the water temperature between 77°F and 79°F (25–26°C). To monitor water temperatures, individual thermometers were placed into each water bath as well as in each individual 5.5-gallon tanks. Turtles from each of the three treatment groups were placed

in a random design between the two temperature-controlled water baths. UV-A/UV-B strip lights (on a 10:14 hour light:dark regime) and basking UV-A/UV-B heat ceramic bulbs were placed above the water baths to maintain light quality and air temperature.

Each turtle was weighed (body weight; g) and measured (carapace length; mm) before being placed into their tank. Each turtle had a health monitoring datasheet assigned to it to keep track of daily health (feeding, behavior, other visual observations), as well as an experimental monitoring datasheet to record experimental conditions such as daily feeding (amount, type, and time), daily cleaning, and temperature and light regimes. The turtles remained in their individual tanks for the duration of the experiment. The water was changed and turtles were fed, as outlined in SOP #5, and they were monitored for signs of stress on a daily basis. Turtle weight and length measurements were taken daily before each dosing and at the end of the study before the turtle was euthanized and necropsied.

C.3 Oral Dosing of Oil and/or Control Solutions Using the E-Tubes

The toxicant in this study was the Slick A oil provided by Stratus Consulting. Turtles were randomly assigned to one of the three treatment groups using n = 16 turtles per group. Each turtle was assigned individual test numbers that were placed on the outside of each tank. In addition, each tank had a group (dose) color code. Only one turtle was confirmed to be a male; thus, treatment groups could not be balanced by gender. The three treatment groups were as follows:

1. Control group
2. 100 mg of oil per kilogram body weight group
3. 1,000 mg of oil per kilogram body weight group.

Before the initiation of each test, turtles were allowed to recuperate from the E-tube surgery for a minimum of 48 hours prior to oil dosing. At the beginning of the test and daily for the 14-day period, all turtles were briefly removed from their 5.5-gallon tanks (for approximately 2 minutes), weighed, and re-dosed with the appropriate volume of oil based on the turtle's dosing group and weight. Body weight and carapace length measurements were collected according to SOP #1 and recorded on the appropriate datasheets. Any external sign of injury or issues with the E-tube were noted on the appropriate datasheets.

To prepare the oil dose for the turtles, the oil was mixed with the food slurry to form a homogeneous mixture. This mixture helped minimize the retention of oil onto the E-tube wall and ensured that an accurate low dose could be delivered. The food slurry was prepared by mixing 30 mL of DI water and six small scoops of the powdered feed. For the low dose group, 20 g of the food slurry and 1 g of Slick A oil were weighed into a solvent-rinsed glass beaker. The mixture was then well-homogenized. The low-dose animals were weighed and their weight

(in kg) multiplied by 2.1 to determine the weight (in mg) of feed mixture to dose the turtle (i.e., the target dose was 100 mg oil and 2,000 mg feed per kg turtle).

For the high-dose group, 20 g of the food slurry and 10 g of Slick A oil were weighed into a solvent-rinsed glass beaker. The mixture was then well-homogenized. The high-dose animals were then weighed and their weight (in kg) multiplied by 3 to determine the weight in mg of feed mixture to dose the turtle (i.e., the target dose was 1,000 mg oil and 2,000 mg feed per kg turtle).

To dose the turtles, the necessary amount of oil/food mixture was drawn into a syringe. The end cap from the E-tube was then removed and the end of the E-tube and cap wiped with ethanol. The oil/food mixture was placed down the E-tube by inserting the Luer lock syringe into the end of the E-tube. After the addition of the oil/feed mixture, a volume of 0.9% sterile saline equaling 3 mL per kg turtle body weight was inserted via syringe into the E-tube to flush all of the oil/feed slurry into the stomach and also to prevent any future blockage of the E-tube. The E-tube end cap was replaced and the turtle was placed back into its individual glass aquarium.

To calculate the exact dosing for each animal, the syringe with the oil/food mixture was weighed before and after dosing. Weights and lengths of the turtles; and weights of the oil, feed, and saline doses were recorded on the appropriate datasheets.

Dosing continued for a maximum of 14 days; however, some turtles were removed from the study earlier because their E-tube was no longer functional (i.e., either it was removed by the turtle or it became blocked). Some turtles were also removed from the study early due to deterioration of health. The condition of each turtle was monitored at least once daily and recorded on the appropriate datasheets. We noted any considerable changes in weight and overt signs of stress such as lethargy, persistent recumbency, or decreased intake of food or fecal production.

We recorded the times each turtle was fed and when their water was changed on the appropriate datasheet. In addition, estimated feces production, the amount of feeding, and the approximate volume of regurgitated/excreted oil was recorded daily. The appearance of an oil sheen in the water was noted and attempts were made to score the amount of oil on the water using a visual score system. In addition, a daily health assessment of each turtle was recorded on the appropriate datasheet, including noting the external appearance (skin color, etc.), recording any observations of infection or injury at the E-tube insertion site, and assessments of behavior. The dates and times of oil dosing and body weight/length on days of dosing were recorded on the appropriate datasheet.

C.4 Euthanasia, Blood Sample Collection, and Necropsy

Necropsies were performed at the termination of the dosing, except for turtles whose health deteriorated to the point that the individual could not be retained in the study, or turtles that lost their E-tubes. These turtles were necropsied at the time of their removal from the study. Signs of damage by the E-tube and/or infection at the site of insertion were recorded during necropsy.

Before necropsy, each turtle was weighed and the carapace length measured. This information was recorded on the appropriate datasheet. Then, turtles were photographed ventrally and dorsally (photographs were logged on the appropriate datasheet) and blood was collected via cardiac puncture according to SOP #3b prior to euthanasia. The blood sample was processed according to the endpoints listed in Table C.1. Then they were euthanized according to SOP #7 and a necropsy was performed. The necropsy of each turtle followed the procedures in SOP #8.

During necropsy, the heart, kidneys, liver, lungs, GI tract, spleen, thyroid, thymus, gonads, and adrenal glands were collected. All organs were assessed for gross abnormalities. If present, a photograph of the abnormality was taken. If the abnormality was a discrete lesion, it was removed with a section of undamaged tissue attached and placed in 10% NBF. All abnormalities and/or lesions were recorded on the appropriate datasheet. Once assessed, all organs were placed in an appropriately labeled specimen jar containing 10% NBF for subsequent histopathological analyses. Before placing the liver in a specimen jar, sub-samples were removed, as discussed below, for subsequent analyses. In addition, the GI tract was flushed of any consumed food and cleaned (only if the diameter was greater than 0.5 cm) before adding to the specimen jar. If edema was noted in the GI tract, the wall thickness was also assessed. No tissue sections placed in the NBF were more than 5-mm thick. Any tissues or lesions larger than this were sliced (like a bread loaf) to allow formalin perfusion and adequate fixation.

If present, bile was collected for chemical analysis of PAH metabolites. To collect bile, the contents of the gall bladder, which was located before removal of the liver, was collected via syringe and transferred to a cryovial. The bile was then flash frozen in liquid nitrogen and stored at -80°C. If volumes of bile were less than 0.1 mL, individual samples were pooled so PAH metabolite analyses could be performed.

After the removal of bile, the whole liver was removed and weighed. After weighing, four sub-samples (approximately 2 g each) were collected from the right (non-gall bladder containing) lobe of the liver. These liver sub-samples were placed in individual cryovials, flash frozen in liquid nitrogen (or dry ice), and stored at -80°C for potential future assessment of cytochrome P450 (CYP1A) enzyme activity, oxidative stress endpoints (i.e., tGSH and lipid peroxidation), and PAH analyses. After collection of these samples, all remaining liver tissues were placed in the specimen jar containing 10% NBF. If the thickness of the liver was greater than 5 mm, the liver was scored using a scalpel before placing it in a specimen jar with 10% NBF.

For the GI tract, the whole organ was removed and at regular intervals (at least five times) over the length of the tissue the GI tract was scored with the scalpel blade. Any consumed food in the upper and lower tracts was then removed by rinsing with PBS. After cleansing, the GI track was assessed for potential edema and lesions. Then the GI tract was placed in the histology specimen jar containing the rest of the organs in 10% NBF.

After completion of the necropsy, any remaining pieces of tissue were placed into the body cavity of the respective turtle and the carcass was placed into a labeled plastic bag for archiving in the -20°C freezer.

The appropriate datasheets were used to document necropsy observations and tissue samples (excluding blood) for each turtle. The date and time of necropsy, the weight of each organ/tissue sample removed, a description of any gross abnormalities (in addition to digital image file information), and the disposition of tissue slices (buffered formalin, cryovial in dry ice/liquid nitrogen) were recorded.

C.5 Blood Collection and Processing

As described above, at the end of the 14-day period, turtles were weighed and carapace length recorded according to SOP #1. A sub-set of 10 turtles underwent an ACTH challenge to examine adrenal responses to oil dosing. Prior to ACTH dosing, blood was collected according to SOP #3b for analysis of all routine blood chemistry and biological endpoints, and to establish a baseline corticosterone level for the ACTH challenge. Turtles undergoing an ACTH challenge then received a 50 IU/kg dose after the initial blood draw. A final blood sample was then taken after 60 minutes to assess final corticosterone levels.

These turtles were then euthanized according to SOP #7 and necropsied according to SOP #8. Blood was collected directly from the heart immediately prior to euthanasia via cardiac puncture according to SOP #3b and recorded on the appropriate datasheet.

All blood samples were processed according to SOP #4 and the resultant plasma samples stored at -80°C. Plasma preparations were divided into at least three aliquots for different analyses (i.e., Aliquot 1 of 150 μL was sent on dry ice overnight to Cornell University for corticosterone analyses; Aliquot 2 of 100 μL and Aliquot 3 of 320 μL were sent on dry ice overnight to the University of Miami for plasma proteins and blood chemistry panels, respectively; Aliquot 4, containing all remaining plasma, was archived at CBL at -80°C).

In addition, whole heparinized blood (before plasma preparation) was collected for blood smear preparation and PCV analysis. For blood smears, three slides were prepared per turtle by CBL using well-mixed, heparinized whole blood according to the SOP #2. These three slides were

shipped unstained to the University of Miami, where one of the three CBL blood smears was stained with Diff-Quick. In addition, three more slides were prepared by the University of Miami with whole blood that was shipped overnight. Of the three blood smears prepared by the University of Miami, two of these slides were stained with New Methylene Blue (NMB). Then all six slides were shipped to Dr. Nicole Stacy at the University of Florida, where she stained the remaining three unstained slides with Wright-Giemsa. Whole blood smears were used for WBC estimates, and WBC and RBC morphologic assessments.

For blood smear preparation and PCV analysis, 100 µL of whole blood was collected and sent to the University of Miami. In addition, another 100-µL aliquot of whole heparinized blood was collected and sent to the University of Miami for the hemoglobin assay. At CBL, a small sub-sample (< 50 µL) of whole heparinized blood was used for the PCV assessments according to SOP #9, and the heparinized RBCs produced during the separation of the blood plasma from whole blood was frozen for tGSH analysis according to SOP #12.

If enough whole heparinized blood remained, 0.4 mL of blood was transferred to a Qiagen PAXgene tube containing blood stabilization solution. Immediately after the blood transfer, the PAXgene tube was gently inverted 8–10 times, then stored upright at room temperature for 2-6 hours before moving to a refrigerator (2–8°C).

For a list of all blood/plasma samples that were collected and their respective analyses, see Table C.1.

C.6 Documents and Records

Toxicity testing information was documented in notebooks, datasheets, photograph files and logs, COC forms, and shipping forms and followed procedures described in the Stratus Consulting QAPP. Please refer to the QAPP for all instructions pertaining to recordkeeping, sample label protocols, and document retention requirements.

Appendix Reference

Mohr, F.C., L. Lasley, and S. Bursian. 2010. Fuel oil-induced adrenal hypertrophy in ranch mink (Mustela vison): Effects of sex, fuel oil weathering, and response to adrenocorticotropic hormone. *Journal of Wildlife Diseases* 46(1):103–110.

10. Pacific EcoRisk General Laboratory Procedures and Practices

10.1 Background

Pacific EcoRisk (PER) is an environmental consulting firm conducting research and testing in the field of environmental toxicology. Stratus Consulting contracted with PER to perform standardized testing of whole sediment acute toxicity on *Leptocheirus plumulosus* using methods that were developed by the U.S. Environmental Protection Agency (EPA). PER was also contracted to perform standardized testing of water accommodated fractions (WAFs) on mysid shrimp and the diatom *Skeletonema costatum*. After the conclusion of these tests, PER provided Stratus Consulting with reports describing the procedures used to conduct each test. Considering that these tests followed EPA standardized guidelines, PER did not write a General Laboratory Procedures and Practices (GLPP) document. Stratus Consulting used information from PER reports to prepare a PER GLPP in the *General Laboratory Protocols and Procedures: Deepwater Horizon Laboratory Toxicity Testing* document.

10.2 Methods

10.2.1 Test organism sources

Leptocheirus plumulosus

These tests were performed on the amphipod *L. plumulosus*, which were obtained from a commercial supplier (Chesapeake Cultures, Hayes, Virginia). A sample of the same sediment used to culture the amphipods (termed "Lab Control") was also obtained from the amphipod supplier for use as one of the control sediment treatments.

Mysid shrimp (*Americamysis bahia*)

The *A. bahia* used in this test were obtained from a commercial supplier (Aquatic Indicators, St. Augustine, FL). Upon receipt at the laboratory, the test organisms were held in aerated tanks containing reverse osmosis, deionized (RO/DI) water adjusted to a salinity of 25 ppt via addition of an artificial sea salt (Crystal Sea – bioassay grade). Test organisms were fed brine shrimp nauplii *ad libitum* during this pre-test holding period.

Diatom (*Skeletonema costatum*)

S. costatum were ordered from The Culture Collection of Algae at the University of Texas at Austin. Test cultures were ordered far enough in advance to ensure that the algae culture was in the log growth phase before set-up. Algae were cultured at PER at the desired test salinity for at least two weeks before test initiation.

10.2.2 Source of natural seawater for testing

The natural seawater used in the tests for all species was obtained from the University of California Davis Granite Canyon Marine Laboratory; this water was stored at the PER laboratory in a 3,000-gal insulated high-density polyethylene tank at 4°C. Prior to use in these tests, 150 gal of this water was archived in a large carboy (a sample of this 150-gal "batch" was collected and sent to the analytical laboratory for source water chemical analyses). A sample of every "batch" that was used was sent to ALS Environmental for source water analyses. This water was stored in a temperature-controlled room at 4°C for use in these tests in order to ensure that the water used in all of the Stratus Consulting testing was of the same origin. This seawater was adjusted to the desired test salinity [see test-specific test conditions tables (TCTs)] via the addition of Type I laboratory water (RO/DI water); these diluted natural seawaters are referred to using the adjusted salinity level (e.g., 20 ppt seawater).

10.2.3 Biological testing procedures

The methods used in conducting this testing followed established guidelines:

Leptocheirus

- Standard E1367-99: Standard Guide for conducting 10-day static toxicity tests with marine and estuarine amphipods (ASTM, 1999)
- Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods (U.S. EPA, 1994).

A. bahia

- Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition (EPA/821/R/02/012; U.S. EPA, 2002).

S. costatum

- Standard Guide for Conducting Static 96-hour Toxicity Tests with Microalgae (ASTM E1218-97a; ASTM, 1997).

10.2.4 Solid-phase sediment toxicity testing

Solid-phase sediment tests were conducted to evaluate the potential adverse impacts of the sediments on the benthic community. These tests involved exposing a benthic amphipod (*L. plumulosus*) to test sediments under static conditions for 10 days, after which the survival of the amphipods was determined and evaluated.

10.2.5 Sediment samples

Tests were conducted with both field-collected sediments provided to PER by Stratus Consulting and reference sediment that was spiked at the University of Mississippi Gulf Coast Research Laboratory (GCRL) and shipped to PER. The methods used by GCRL to spike the sediment are in Section 10.6.

10.2.6 Reference toxicant testing of *L. plumulosus*

To assess the sensitivity of the amphipods used in these tests to toxic stress, a reference toxicant test was run concurrent with the solid-phase sediment testing. The amphipod reference toxicant test consisted of a 96-hour water-only exposure to KCl with survival (%) as the test endpoint. The resulting test response data were analyzed by PER to determine key dose-response point estimates [e.g., EC50 (the concentration for which 50% of the test organisms were affected)]; all of the statistical analyses were made by PER using the CETIS software. The test responses were compared to the "typical" response range established by the mean ± 2 standard deviations (SD) of the point estimates generated by the 20 most recent previous reference toxicant tests performed by this laboratory.

10.2.7 Acute toxicity of WAFs on mysid shrimp and *skeletonema*

High-energy WAFs (HEWAFs) and low-energy WAFs (LEWAFs) were prepared at PER according to the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP; located in Attachment 3) for mysid shrimp and *S. costatum* testing. Routine water quality characteristics [pH, dissolved oxygen (DO), and salinity] were determined for each

treatment test solution before the start of the test. A subsample of each test solution was also collected for PAH analysis, and shipped to ALS Environmental for analysis.

10.3 Pore Water Characterization for Sediment Tests and Additional Sediment Sampling

Dummy test replicates for each sample/treatment were sacrificed at the time of test initiation and test termination for sediment pore water characterizations (pH, salinity, total sulfides, and total ammonia). The overlying water in each test replicate selected for the pore water characterization was carefully siphoned off to minimize any disturbance of the sediments, after which the remaining sediment was transferred to a 750-mL centrifuge bottle. The sample sediment was centrifuged at 2,500 G for 30 minutes, after which the overlying supernatant was carefully decanted from the centrifuge bottle and water quality characteristics were determined.

At the time of the pore water characterization (test initiation and test termination), additional test replicates were similarly sacrificed to provide bulk sediment for chemical analyses. The overlying water was siphoned out as before, after which the sediment was transferred into a 250-mL glass sample jar (provided by the analytical laboratory). The sample containers were wrapped in bubble wrap and securely packed inside a cooler with crushed ice. A temperature blank was included in each cooler. The original signed chain-of-custody (COC) forms were placed in a sealed plastic bag and taped to the inside lid of the cooler. Appropriate packaging tape was wrapped completely around the cooler. A “This Side Up” arrow label was attached on each side of the cooler, a “Glass – Handle with Care” label was attached to the top of the cooler, and the cooler was sealed with custody seals on both the front and the back lid seams. These samples were shipped via overnight delivery to ALS Environmental (Kelso, Washington) for the chemistry analysis.

10.4 Quality Assurance/Quality Control Review

The biological testing of these sediments incorporated standard quality assurance/quality control (QA/QC) procedures to ensure that the test results were valid, including the use of negative laboratory controls, positive laboratory controls, test replicates, and measurements of water quality during testing. QA procedures that were used for sediment testing are consistent with methods described in the EPA and the ASTM International (ASTM) guidelines. For tests performed with field-collected sediments, sediments for the bioassay testing were stored appropriately at -20°C at the request of Stratus Consulting and the aliquots used for testing were broken off of the frozen samples prior to thawing. For tests performed with spiked sediments, sediments for the bioassay testing were stored appropriately at 4°C at the request of Stratus

Consulting until they were processed prior to testing. Sediment interstitial water characteristics were within test acceptability limits at the start of the tests. The overlying water in these solid-phase sediment toxicity tests consisted of natural seawater diluted to the test salinity using Type I laboratory water.

All measurements of routine water quality characteristics were performed as described in Section 10.5 *Water Quality Laboratory Standard Operating Procedures*. All biological testing water quality conditions were within the appropriate limits. Laboratory instruments were calibrated daily according to PER standard operating procedures (SOPs), and calibration data were logged and initialed.

10.5 Water Quality Laboratory Standard Operating Procedures

10.5.1 Conductivity/salinity analysis SOP

This is the SOP for the Thermo Scientific Orion 3-Star meter.

Calibration of meter

1. Turn on Conductivity/Salinity meter.

2. Press the “Calibrate” button. There should be a number (i.e., 0.475) displayed below CELL. This is the cell constant. Record this number.

3. Place the probe in 100 µS standard at 25°C. The meter will automatically recognize the standard. When the reading stabilizes at 100.0, press “Calibrate Again.”

4. Repeat with the 1,413 µS and 12.9 mS standards. After the reading for the 12.9 mS standard has stabilized, press MEASURE.

5. A new value for the cell constant will be displayed. Record this number. Verify as described in the next section before use.

Verification of calibration of meter (required minimum of daily)

1. Turn on Conductivity/Salinity meter.

2. Place the probe in a standard National Institute of Standards and Technology (NIST) salinity or conductivity solution, making sure that the standards are at 25°C.

3. Adjust the readout of the meter to display conductivity (µS/cm) and take the reading of the lowest standard (100 µS) and compare to the standard value. If readout is within 5% of the standard value, the meter is properly calibrated. If the reading is outside the 5% range, follow the steps in the previous section to calibrate the meter.

4. Record the reading into the Conductivity/Salinity logbook.

Measurement procedure

1. With the instrument prepared for use and the probe calibrated, select the appropriate measurement parameter (conductivity of salinity).

2. Place the probe into the test solution. There is no need to agitate the probe or the sample to measure.

    a. Hold the probe by the sensor-body at all times. Do not hold the probe by the cable at any point during rinsing or measurement. Holding the probe by the cable could result in damage to the wiring and to the probe reading incorrectly.

3. While the meter is measuring the solution, the units symbol (µS/cm) will blink. Once the reading has stabilized, the symbol will stop blinking and the value displayed can be recorded.

4. Rinse the electrode with Type I water and proceed to measure the next sample.

Storage

1. Rinse electrode with Type I water and place it in the holder.

2. Turn the meter off.

Maintenance

1. All conductivity verification standards must be changed out weekly. Replace conductivity standard bottles every 2 weeks.

2. Inspect all conductivity probes for damage at least once a week, but preferably every day during calibration.

3. Keep an eye on the bottles of conductivity standard solutions. If a bottle is getting low (less than one-fourth full) or the standard is going to expire, it must be replaced prior to calibration.

4. Record all standard changes and maintenances in the "Conductivity Log Book."

10.5.2 Dissolved oxygen analysis SOP

This is the SOP for the Thermo Scientific 3-Star RDO Meter.

Air calibration

1. Turn on the RDO Meter.

2. Place the probe in a chamber with 100% saturated air conditions, making sure that the probe membrane is not touching anything or has any water droplets on it.

3. Press CALIBRATE.

4. Record Pre-Calibration % Saturation and let stabilize. In a few seconds, it should stabilize to 100%, and this should be recorded.

5. Press the UP arrow, and record the air pressure in torr (barometric pressure). Press the DOWN arrow, to return to mg/L, and the meter is ready to use.

Measurement procedure

1. Submerge the end of the probe in the water sample. There is no need to agitate the probe or the sample to measure. Record the value in mg/L once the reading has stabilized.

    a. The probe should be held by the sensor-body at all times. Do not hold the probe by the cable at any point during rinsing or measurement. Holding the probe by the cable could result in damage to the wiring and the probe reading incorrectly.

Salinity adjustment

1. When measuring DO in salty samples (> 15 ppt), enter the sample salinity into the meter prior to measurement.

2. Press SETUP.

3. Press the bottom-right button (with three rectangles and an arrow) once. The screen should read RES.

4. Press the DOWN arrow three times. The screen should read SALF.

5. Press the bottom-right button again. The curser should be on the bottom row.

6. Use the bottom-left button (with the number 8 and arrows) to scroll between digits, and the arrow buttons to change the numbers until the value on the screen reflects the salinity of the sample.

7. Press the bottom-right button again to save. Press MEASURE to return to measurement mode.

8. Repeat the process to return salinity to zero after the measurement of salty samples is complete.

Maintenance

1. Change Type I water in the probe storage chamber weekly and refill to the fill line with new water.

2. Inspect probes for any damage at least once a week, but preferably daily during calibration.

3. Record all water changes and weekly maintenance in the “DO log book.”

4. Replace probe caps annually.

10.5.3 pH analysis SOP

This is the SOP for the Beckman pHi 410/ISE/mV meter.

Three-point calibration of meter

1. Turn pH meter on and check that all electrodes are properly attached. The screen display should show the following:

    a. A “pH” symbol in the upper left hand corner. If not displayed, press the 1 button to place the meter in “pH” mode.

b. "ATC" indicating that automated temperature compensation is functioning properly. If no "ATC" is displayed, the probe is not properly attached, or the built-in thermometer is not functioning.

c. If a battery icon is displayed, the batteries should be changed out within 25 hours of the icon first appearing.

d. If the word "bat" is displayed, the batteries are too low to provide reliable measurements. The meter must not be used until batteries are changed out.

2. Press the pH button to enter pH mode.

3. Press CAL then DELETE. This will clear the previous calibration from the memory of the meter.

4. Place the probe in the first buffer solution (Pink pH 4.01).

5. Press CAL. The Cal icon will begin flashing.

6. If the buffer displayed on the screen is correct, press ENTER. The pH buffer value will flash until calibration is complete.

   a. If the pH buffer is incorrect, press the arrow keys to select another buffer.

7. When calibration is complete for the current buffer, the display will show a flashing Cal 2 and the next pH buffer value will display (e.g., 7.00).

8. Remove the probe from the PINK pH 4.01 buffer and rinse with DI water.

9. Place the probe in the YELLOW pH 7.00 buffer and repeat steps 6 through 8 above, but use the pH 7.00 buffer.

10. Place the probe in the BLUE pH 10.01 buffer and repeat steps 6 through 8 above, but use the pH 10.01 buffer.

11. Calibration is complete when the final calibration point has been entered. The meter will beep three times and display the slope (4.01–10.01), mV offset at pH 7.00 and the number of calibration points.

12. All slopes must be between 95% and 105%. If not, a flashing E04 will be displayed in the center of the screen. In this case, the calibration procedure must be repeated, beginning with step 2. If the slopes are within the acceptable range, proceed to step 13. If the slope

is not acceptable, proceed with recalibration and/or seek the guidance from the equipment manual for remedies.

13. Press the pH or ENTER button to return to the main screen. You are ready to measure pH values. The pH or ENTER button must be pressed in order to measure pHs correctly.

Procedure

1. Turn on the pH meter. Ensure that daily calibration is complete. If, not refer to the *Three-point calibration of meter* section.

2. Remove the pH electrode from the electrode holder, and rinse with DI water.

    a. The probe should be held by the sensor body at all times. Do not hold the probe by the cable at any point during rinsing or measurement. Holding the probe by the cable could result in damage to the wiring and the probe reading incorrectly.

3. Place pH electrode into a sample.

4. The pH value will rise or fall depending on the solution being measured. There is no need to agitate the probe or the sample to measure. When the pH value displayed on the meter is stable and the display reads “Ready,” the meter has stabilized and the value displayed may be recorded onto the appropriate data sheet.

5. Remove the electrode from the sample and rinse with Type I water.

6. Proceed to the next sample.

7. When the pH analyses are completed, place clean electrode back into the electrode holder.

Storage

1. At the end of the day, store the pH probes in a storage solution in order to maintain a properly functioning probe.

2. Place the probe into the Erlenmeyer flask containing the storage solution and submerge the tip of the probe in the solution.

3. Turn the meter off.

Maintenance

1. All pH probe storage solutions must be changed out weekly.
2. All pH buffer solutions must be changed out weekly.
3. All pH probes should be thoroughly cleaned once a week.
    a. Soak probes in pH probe cleaning solution for 30 minutes.
    b. Rinse thoroughly with DI water before proceeding to calibration of meters.
4. Record all buffer changes and weekly maintenance in the "pH log book."

10.5.4 Sulfide analysis SOP

Theory of operation

The following SOP outlines the procedures for testing sulfide concentrations in wastewater. This spectrophotometric method is equivalent to EPA 376.2 for wastewater and is based on Hach Method 8131 for sulfide. This test can detect sulfide concentrations from 0 to 800 µg/L.

Supplies needed

1. Hach spectrophotometer DR/4000
2. Two matching 25 mL cuvettes
3. Type I water for sample blank
4. Sulfide 1 Reagent, sulfuric acid (Hach Cat. No. 1816-32)
5. Sulfide 2 Reagent, potassium dichromate (Hach Cat. No. 1817-32)
6. Small graduated cylinder
7. 50-mL Erlenmeyer flask (turbid samples only)
8. Bromine Water (turbid samples only; Hach Cat. No. 2211-20)
9. Phenol Solution (turbid samples only; Hach Cat. No. 2112-20).

Procedure

1. Switch on the spectrophotometer and allow it to self-calibrate.

2. Select Hach program #3500.

3. Pour 25 mL of sample into a cuvette (for turbid samples, please see next section). Pour 25 mL of Type I water into the matching cuvette.

4. Add 1 mL of Sulfide 1 Reagent to each cuvette and immediately swirl.

5. Add 1 mL of Sulfide 2 Reagent to each cuvette and immediately swirl. A pink color will develop, and the solution will turn blue if sulfide is present.

6. Press the START TIMER button on the spectrophotometer, which begins a 5-minute countdown. Allow the solutions to react during this period.

7. When the timer beeps, place the DI blank in the cell holder and close the lid. Press ZERO.

8. The display should read 0 µg/L S^2.

9. Place the sample cuvette in the cell holder and close the lid and press READ. The results will be displayed.

Turbid samples

1. If testing a turbid sample, prepare a sulfide-free sample blank to use in place of the Type I blank.

2. Measure 25 mL of sample into a 50 mL Erlenmeyer flask.

3. Add bromine water dropwise with constant swirling until a permanent yellow color just appears.

4. Add phenol solution dropwise until the yellow color just disappears. Use this solution in place of Type I in Step 3.0 above.

Interferences

1. Wipe cuvettes clean prior to reading

2. Turbidity can interfere with the spectrophotometer; follow the steps outlined in the *Turbid samples* section.

Quality control

1. Verify expiration date of all reagents prior to use

2. Verify and clean spectrophotometer quarterly at minimum

3. Analyze samples immediately.

Safety and disposal

1. Ensure appropriate personal protection equipment is used.

2. Sulfide 2 Reagent contains potassium dichromate, and should be handled appropriately. The final solution will contain hexavalent chromium at a concentration regulated as a hazardous waste. Collect final solutions into sulfide waste container and dispose of as hazardous material.

10.5.5 Thermometer calibration SOP

This SOP describes procedures for calibration of digital and mercury thermometers in compliance with the National Environmental Laboratory Accreditation Conference (NELAC) protocol for equipment quality of standards.

Calibration of liquid-in-glass thermometer

A certificate is provided to verify instrument calibration in accordance with NIST. Recalibration of the instrument is to take place annually, and any resulting correction factors will be recorded in the logbook and on the thermometer.

Range: -8 to 32°C in 0.1 graduations.

Accuracy: See reference NIST SP 250-23.

Calibration of laboratory thermometers

Laboratory thermometers are to be calibrated biannually against a thermometer that is traceable to NIST.

Water bath thermometers, digital

1. Three temperature standards are required for accurate calibration of digital thermometers used in water baths, at 12°C, 20°C, and 25°C

2. Place NIST-certified thermometer in protective casing

3. Fully immerse the bulb and liquid column into a water bath set at 12°C

4. Place digital thermometer in same bath as the NIST-certified thermometer

5. Allow thermometers to stabilize

6. Record the correction factor in the Thermometer Calibration logbook and on the thermometer

7. Record the NIST correction factors in the Thermometer Calibration Logbook and on the thermometer.

Refrigerator/freezer thermometers, mercury

One temperature standard, at 4°C, is required for calibration of refrigerators, and at 0°C for calibration of freezers.

1. Place a flask of water (ethanol if calibration is performed in a freezer) in refrigerator 24 hours prior to calibration

2. Place NIST-certified thermometer in the flask

3. Place the mercury thermometer in same refrigerator

4. Allow temperature adjustment with refrigerator doors closed

5. Open refrigerator and record NIST correction factor in Thermometer Calibration Logbook and on the thermometer.

10.5.6 Total ammonia analysis SOP

Theory of operation

The following SOP outlines the procedures for testing ammonia concentrations in wastewater. This spectrophotometric method is based on Hach Method 10205 for ammonia.

Supplies needed

1. Hach spectrophotometer DR/3800.

2. TNT 831 ammonia vials (blue caps), warmed to 20–23°C. TNT 830 (green caps) or TNT 832 (red caps) ammonia vials may be needed for follow-up should the ammonia concentration not fall within the 1–12 mg/L range of the TNT 381 vials.

3. 1 mL pipettor, set to 0.5 mL, and tips.

4. DI water for rinsing vials before discarding.

Procedure

1. Allow samples and reagent vials to warm to 20–23°C. Samples should be unpreserved and measured as soon as possible after receipt. However, if samples arrive above pH 8, they should be adjusted down with small amount of acid to around pH 7. If several samples arrive within a short period of time, it may be convenient to measure them together in one batch.

2. Turn on the spectrophotometer and allow it to self-calibrate.

3. Press the “Barcode Programs” button.

4. Obtain TNT 831 vials. Stand the vials upright in the plastic tray. Peel the foil off the cap of each vial and remove the cap.

5. Add 0.5 mL of sample to a vial. Repeat for each sample, being sure to keep vials and samples in the same order. Change pipettor tips between each sample to avoid contamination.

6. Replace the caps and firmly shake each vial three times. The solution should turn yellow.

7. Press the clock on the spectrophotometer screen and set the timer for 15 minutes (press the 1, 5, 0, 0 buttons). Also be sure to set a portable stopwatch to 14 minutes and keep it with you at all times in case you leave the room. It is essential to return to the

spectrophotometer to take measurements after the reaction is complete. Samples with ammonia will turn green.

8. When the timer beeps, wipe the vial clean with a Kimwipe and make sure there is no debris on the outside of the vial. Gently invert the vial two or three times to mix. Slide the door on top of the spectrophotometer open and gently place the vial in the hole. Release the vial to allow it to turn. Be sure that the arrow-shaped light shield is in place (it should never be removed).

9. The spectrophotometer will automatically read the vial and give a number within a few seconds. If the number is less than 1 mg/L, it will be shown in red as out-of-range, and should be recorded as "< 1" in the log book. If the value is between 1 and 12, the number will be displayed in black and should be recoded as-is. If the value is greater than 12, several red asterisks will be displayed with an out-of-range message.

10. Repeat the above procedures with the remaining samples. The color of the reaction will stay stable only for another 15 minutes after the timer goes off, so it is important to work efficiently to get everything measured quickly.

11. If the sample's value is greater than 12 mg/L, then the process must be repeated using the TNT 832 vial kit (with red caps). The process is identical, except 0.2 mL of sample rather than 0.5 mL is added to the vial. There is no need to change the program, because a TNT 832 vial can be measured with a batch of TNT 831 vials.

12. Typically, samples with a reading of < 1 mg/L do not need any follow-up. For special projects that require the reporting of a precise value less than 1 mg/L, TNT 830 vials (green caps) are used. The process is identical as above, except 5 mL of sample are added to each vial.

13. To dispose of the vials, empty them into the "ammonia test waste" bottle by the spectrophotometer; this waste can be disposed of down the drain once the bottle is full. The vials are made to not spill easily, so it may help to tap them on the inside of the bottle to get them to drain. Rinse the bottle three times with DI water into the waste bottle. Vials can then be disposed of in the sharps container. Caps should be rinsed with DI into the waste bottle, and disposed of into the trash can.

14. Turn off the spectrophotometer when finished, clean it with a wet paper towel (if dirty), cover it, and return any unused vials to the refrigerator for storage. Ensure that the work area around the spectrophotometer is completely cleaned.

10.6 Protocol for Preparation of Spiked Sediment

This protocol was used by GCRL to provide the spiked sediment samples that were used in PER testing.

10.6.1 General guidelines

1. Controls were prepared using the same technique used for spiking sediment, with the exception of adding oil.

2. Each sediment-oil concentration was made separately. For instructions in cleaning and preparing the equipment, refer to the *Decontamination SOP* in the QAPP.

3. For each step during the sample preparation and collection, all appropriate information was entered into the data entry sheets provided by Stratus Consulting; see Section B.4 in Appendix B of the QAPP.

4. Unused prepared sediments were placed into a Ziploc bag and stored in the dark at 4°C (short term) or in freezer at -20°C (long term).

10.6.2 Glassware preparation

Prepare all of the equipment in accordance to the *Decontamination SOP* in the QAPP.

10.6.3 Preparation of sediments

1. Allow the sediment to thaw

2. Remove all debris (grass, shells, etc.) from the thawed sediment and place in a mixer bowl

3. Using a Cuisinart SM-70 7-quart stand mixer, homogenize the sediment by mixing for 2 minutes at low speed (1).

10.6.4 Mixing oil into sediment

1. Weigh out the appropriate amount of oil as outlined below:

   Slick oil should be weighed in a pre-cleaned aluminum weigh boat. Tare a weigh boat and two or three Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat. Using the spatula, transfer the oil onto the sediment in the mixing bowl, placing it in several areas around the bowl. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass of oil transferred.

2. Place the sediment from the mixing bowl over the oil, and lower the mixer paddle into the bowl.

3. Mix the oil into the sediment at medium speed (5) with a Cuisinart SM-70 7-quart stand mixer. Stop the mixer briefly every 2–4 minutes to scrape the sides of the mixing bowl with the putty knife.

4. Once the mixing is complete, scrape down the mixer paddle with the putty knife to remove all of the excess oiled sediment. Transfer the sediment from the mixing bowl into bags for storage using a stainless steel spoon. Store sediment in the dark at 4°C until it is ready for shipment.

References

ASTM. 1997. Standard Guide for Conducting Static 96-hr Toxicity Tests with Microalgae. ASTM Standard E1218-97a. ASTM International, West Conshohocken, PA.

ASTM. 1999. Standard Guide for Conducting 10-day Static Toxicity Tests with Marine and Estuarine Amphipods. ASTM Standard E1367-99. ASTM International, West Conshohocken, PA.

U.S. EPA. 1994. *Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods*. EPA 600/R-94/025. U.S. Environmental Protection Agency Office of Research and Development, Washington, DC.

U.S. EPA. 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. EPA/821/R/02. U.S. Environmental Protection Agency Office of Research and Development, Washington, DC.

A. Testing Protocol 1: 10-Day Sediment Exposure *Leptocheirus plumulosus*

A.1 Biological Testing Procedures

The methods used in conducting these testing followed established guidelines:

- ASTM E1367-99: Standard Guide for conducting 10-day static toxicity tests with marine and estuarine amphipods (ASTM, 1999)
- Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods (U.S. EPA, 1994).

A.2 Solid-Phase Sediment Toxicity Testing

A.2.1 Field-collected sediment preparation

The frozen sediment samples were removed from frozen storage and chunks of sediment were broken off to provide sufficient volume for testing. Once a sufficient volume of sediment had been attained, the remaining bulk sediment was returned to freezer storage; the collected chunks were placed into polyethylene bags and held at 4°C storage and allowed to slowly thaw. The day prior to test initiation, the thawed sediments were removed from cold storage and homogenized in a stainless steel bowl using a stainless steel spatula; at the request of Stratus Consulting, the sediments were not sieved prior to testing.

A.2.2 Spiked sediment preparation

The day prior to test initiation, the sediments were removed from cold storage and homogenized in a stainless steel bowl using a stainless steel spatula; as per instructions from Stratus Consulting, the sediments were not sieved prior to testing.

A.2.3 Test initiation

For each sample, the sediment was transferred into each of nine test replicate 1-L glass beakers to a depth of ~ 2 cm, after which ~ 800 mL of 20 ppt salinity seawater was carefully poured into reach replicate so as to minimize the disturbance and resuspension of the sediment. A negative “Lab Control” control sediment consisting of the same sediment used to culture the test

organisms (provided by the test organism supplier) was similarly processed and tested. The test replicates were then placed into a temperature-controlled room at 25 ± 1°C under continuous illumination.

The tests were initiated the following day. At this time, one of the test replicates was sacrificed for the determination of the initial sediment pore water characteristics and a second replicate was similarly sacrificed for bulk sediment chemical analyses. Immediately prior to test initiation, routine water quality characteristics (pH, temperature, DO, salinity, and total ammonia) were measured in the overlying water in each replicate. The tests were then initiated with the random allocation of 20 amphipods into each test replicate.

Each day of the test, routine water quality characteristics (pH, temperature, DO, and salinity) were measured in the overlying water in one randomly selected replicate for each sediment treatment.

A.2.4 Test termination

The tests were terminated after 10 days of exposure. At this time, one of the remaining test replicates was sacrificed for the determination of the final sediment pore water characteristics and an additional replicate was again sacrificed for bulk sediment chemical analyses. For each of the remaining five replicates, the sediments were rinsed out into a 0.5 mm sieve and wet-sieved, and the number of surviving amphipods in each was determined. The resulting survival data for these sediments were evaluated by PER using the CETIS statistical software (TidePool Scientific, McKinleyville, California). The data were also sent to Stratus Consulting and included in their database.

B. Testing Protocol 2: Characterization of the Acute Toxicity of Oil (WAFs) to the Mysid Shrimp, *Americamysis bahia*

B.1 Biological Testing Procedures

The methods used in conducting these tests followed established guidelines:

- Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition (EPA/821/R/02/012; U.S. EPA, 2002).

B.2 Acute Toxicity Testing

B.2.1 WAF Preparation

The appropriate WAF was prepared by the addition of Slick A or Slick B oil to 25 ppt seawater at a nominal concentration of 1 g/L, after which the oil and 25 ppt seawater were mixed and processed according to the QAPP. The WAF was prepared twice: once to prepare the test solutions used to initiate the test (Day 0); and then again, on Day 2 of the test, to prepare fresh media for the test solution renewals. A WAF "Blank" consisting of Laboratory Water Control medium (25 ppt seawater) was similarly mixed, processed, prepared, and tested.

B.2.2 Test Initiation

The Laboratory Water Control medium and the prepared WAFs were used to create diluted test solutions at desired concentrations (see test-specific TCTs).There were four replicates at each test treatment, each replicate consisting of 200 mL of test solution in a 400-mL glass beaker. The test was initiated by randomly allocating 10 four- or five-day-old *A. bahia* into each replicate beaker. The beakers were randomly positioned in a temperature-controlled room at 20°C (temperature was monitored daily) under a 16 L:8 D photoperiod. The mysids were fed freshly hatched brine shrimp nauplii twice daily.

After approximately 48 hours, fresh WAF solutions were prepared, archived, and characterized as before. The test replicate beakers were removed from the temperature-controlled room and each replicate was examined; any dead animals, uneaten food, wastes, and other detritus was removed. The number of live mysids in each replicate was recorded. Then, approximately 80%

of the test media in each beaker was carefully poured out and replaced with fresh test solution, after which the test beakers were returned to the temperature-controlled room.

Using the test water that had just been removed during the water renewal, water quality characteristics (pH, DO, and salinity) were measured for each treatment on one randomly selected replicate.

B.2.3 Test Termination

After 96 (± 2) hours of exposure, the test was terminated and the number of live mysids in each replicate beaker was recorded. Immobile mysids that did not respond to gentle prodding were considered dead. Live and dead organisms were collected according to the QAPP.

C. Testing Protocol 3: Characterization of the Chronic Toxicity of Oil (WAFs) to the Diatom *Skeletonema costatum*

C.1 Biological Testing Procedures

The methods used in conducting these tests followed established guidelines:

- Standard Guide for Conducting Static 96-hour Toxicity Tests with Microalgae (ASTM E1218-97a; ASTM, 1997).

C.2 Acute Toxicity Testing

C.2.1 WAF Preparation

The appropriate WAF was prepared by the addition of Slick A or Slick B oil to 25 ppt seawater at a nominal concentration of 1 g/L, after which the oil and 25 ppt seawater were mixed and processed according to the QAPP. A WAF "Blank" consisting of a Laboratory Water Control medium (25 ppt seawater) that was similarly mixed and processed, was also prepared and tested.

C.2.2 Test Initiation

The Laboratory Water Control medium and the prepared WAFs were used to create diluted test solutions at desired concentrations (see test-specific TCTs). In some cases, additional auxiliary controls were added to the tests (see test-specific TCTs).There were four replicates at each test treatment, each replicate consisting of a 250-mL glass Erlenmeyer flask containing 100 mL of test solution; an additional replicate was established at each test treatment for the measurement of test solution water quality characteristics during the test and at test termination. Each flask was inoculated to an initial diatom cell density of 20,000 cells/mL from a laboratory culture of *S. costatum* that was maintained in a log-growth phase. These flasks were loosely capped and randomly positioned within a temperature-controlled room at 20°C, under continuous illumination from cool-white fluorescent bulbs.

Each day, the temperature and pH were determined from the designated "water quality" replicate for each treatment; each replicate flask was gently shaken and re-positioned within the temperature-controlled room. All flasks were shaken by hand and re-randomized in the temperature-controlled room daily.

C.2.3 Test Termination

After 96 (± 2) hours exposure, the flasks were removed from the temperature-controlled room and the diatom cell density in each was determined by microscopic analysis. At the end of testing, replicates were pooled by treatment and poured into graduated cylinders. The graduated cylinders were stored in a cold room for 2–3 days, after which the overlying water was decanted and all of the tissue that had settled to the bottom of the graduated cylinder was placed into a freezer-proof container and archived according to the QAPP.

Appendix References

ASTM. 1997. Standard Guide for Conducting Static 96-hr Toxicity Tests with Microalgae. ASTM Standard E1218-97a. ASTM International, West Conshohocken, PA.

ASTM. 1999. Standard Guide for Conducting 10-day Static Toxicity Tests with Marine and Estuarine Amphipods. ASTM Standard E1367-99. ASTM International, West Conshohocken, PA.

U.S. EPA. 1994. *Methods for Assessing the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Amphipods*. EPA 600/R-94/025. U.S. Environmental Protection Agency Office of Research and Development, Washington, DC.

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11. Louisiana State University General Laboratory Procedures and Practices

11.1 Introduction

Louisiana State University (LSU) conducted toxicity tests to identify the toxicological impacts of the 2010 *Deepwater Horizon* oil spill on gulf killifish. Dr. Fernando Galvez served as the Principal Investigator, and all tests were conducted in his laboratory at LSU. This chapter describes General Laboratory Procedures and Practices (GLPP) used at LSU.

11.2 Reporting and Testing Documentation

Experimental data and relevant activities were reported as described in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Hard copies of these data were prepared according to the QAPP, and kept in the Galvez laboratory in a locked filing cabinet after preparation. A duplicate hard copy and an electronic version of the data were prepared according to the QAPP, and sent to Stratus Consulting on a timely basis for quality assurance.

11.3 Equipment Decontamination

All testing equipment used was decontaminated before use following the methods outlined in the *Decontamination SOP* (Standard Operating Procedure) of Appendix A described in the QAPP. After completing the *Decontamination SOP*, equipment was rinsed twice with reverse osmosis (RO) water. Care was taken to avoid any cross-contamination of testing equipment, test exposure chambers, laboratory glassware, and analytical samples. To the extent possible, new, certified, clean materials were used to conduct testing and sampling activities. All equipment and testing materials that could not withstand the decontamination procedure were not reused.

11.4 Exposure Media Preparations

The artificially formulated seawater (AFS) used for water accommodated fraction (WAF) exposures was prepared using RO water mixed with Instant Ocean sea salts to achieve the desired test salinity [see test-specific test conditions tables (TCTs) for the desired test salinity].

RO water was derived from dechlorinated tap water prior to RO in a Culligan Aqua-Cleer water treatment system. Exposure media were prepared as follows:

- WAFs were prepared as outlined in the *Protocols for Preparing Water Accommodated Fractions* found in the QAPP. WAFs were prepared using one of two preparation methods, high energy and chemically enhanced (HEWAF and CEWAF, respectively) and one of two oil types, Slick A (CTC02404-02) and Slick B (GU2888-A0719-OE701).

- Sediment exposures were conducted with clean field-collected sediment spiked with different amounts of the desired oil. Additional details regarding the sediments, the spiking protocol, and oil types used are provided in the testing protocols and test-specific TCTs.

- Oil slick exposures used one of two types of oil: Slick A or Slick B. Additional details regarding the slick formation protocol and oil types used are provided in the Testing Protocols and test-specific TCTs.

  Note: new jars of Slick B oil were pre-mixed over low heat to homogenize contents of the jar before oil was used in oil slick exposures. To mix, the entire content of the oil jar was placed into a large glass bowl, which was then set on a hotplate (FisherBrand Isotemp Model 1160049SH) on the lowest heat setting. The oil was mixed in the bowl on the hotplate until it had the consistency of taffy (this usually took around 5 to 10 min). Oil was then used to prepare polyvinyl chloride (PVC) rings or put back into its original jar for storage at 4°C. This step was only performed once per jar.

11.5 Test Organism Husbandry – Gulf Killifish (*Fundulus grandis*)

All experiments were conducted with fish held at LSU. Fish were held in AFS, prepared as described above, by dissolving Instant Ocean sea salts into water purified by RO.

1. During testing, water quality was monitored as described in the QAPP using the water quality SOPs described in Section 11.9. Dissolved oxygen (DO), pH, conductivity, salinity, total ammonia nitrogen (TAN), and temperature were recorded once every other day from one random container in each treatment. For the initial few tests, conductivity measurements were collected using a YSI 85 meter; for later tests, conductivity was replaced by salinity measurements taken using a refractometer. Information regarding when water quality measurements were taken can be found in the test-specific TCTs. DO, pH, salinity, TAN, and temperature were recorded for all of the brood stock systems at least once weekly.

2. AFS for the adult brood stock was kept at a salinity of 12 ppt. Holding tanks received continuous aeration to maintain DO levels above 6 mg/L. Holding tanks received continuous mechanical, biological, and ultraviolet (UV) filtration through recirculation. Water exchanges of at least 20% were performed, mechanical filter media were cleaned, and the biological bead filtration system was back-flushed with water exchanges on a weekly basis. Tanks were siphoned and scrubbed weekly.

3. Adult gulf killifish were maintained in high-capacity recirculating holding systems in the Life Science Building (LSB) aquatic facility at LSU.

    a. Larval gulf killifish were held in 2.8-L tanks within a Marine Biotech multi-tank zebrafish-style holding system (Figure 11.1).

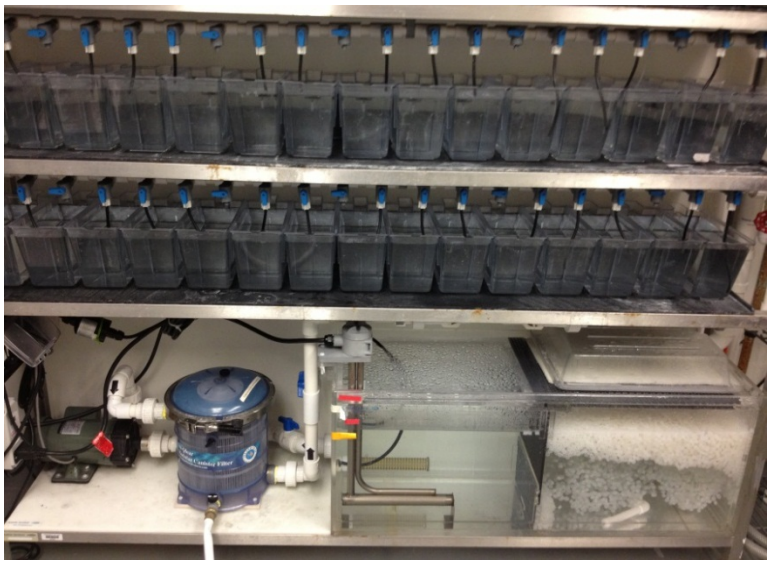

Figure 11.1. Marine Biotech zebrafish-style holding system.

    b. Embryos (pre-hatch) that were not used in larval acute toxicity tests were maintained in 950-mL glass Pyrex dishes under 50% static renewal at 48-hour intervals.

    c. Sexually mature fish used as a brood stock were held in a 1,200-L recirculating system.

4. All fish were maintained at 20°C on a 14-hour light:10-hour dark photoperiod.

5. Feeding:

    a. Adult fish were fed a formulated diet manufactured by AquaFeed (4010) of pellets containing 40% protein and 10% lipid until satiation, once daily.

    b. Larval gulf killifish were fed an Otohime powdered diet daily. The diet was gradually switched to the AquaFeed pelleted food once juvenile fish reached a suitable size to ingest fish pellets.

6. Fish health:

    All tanks were checked for fish mortality or signs of illness and stress daily. Signs of disease and abnormal rates of mortality were treated on a case-by-case basis in consultation with the State Fish Pathologist, Dr. John Hawke, from the Louisiana Aquatic Diagnostic Laboratory.

11.6 Test Organism Sources

11.6.1 Fish collection

1. Adult gulf killifish were collected in Cocodrie, Louisiana, adjacent to the Louisiana Universities Marine Consortium facility, for use as a brood stock (LUMCON; Figure 11.2).

2. Fish were collected using wire minnow traps baited with approximately 1/2 cup of Ol' Roy Puppy Feed. Traps were placed at the marsh edge and allowed to sit undisturbed for at least 45 minutes prior to checking for activity. As fish were collected, they were held in 20-L individually aerated containers at a density of no more than 2 fish per liter for transport back to LSU. Fish were collected for use as a brood stock for embryo production, as described in Section 11.6.2 (*Production of embryos for toxicity experiments*). *In vitro* fertilization occurred onsite at LUMCON or at the LSB following transport of the brood to LSU. If *in vitro* fertilization was performed at LUMCON, embryos were transferred to air-incubation containers (Figure 11.3) immediately upon fertilization, as described in Section 11.6.4.

3. Upon arrival at LSU, adult fish were treated with a buffered active copper treatment of Cupramine for 7–14 days and Praziquantel for 7 days, before being introduced into the brood stock holding tank.

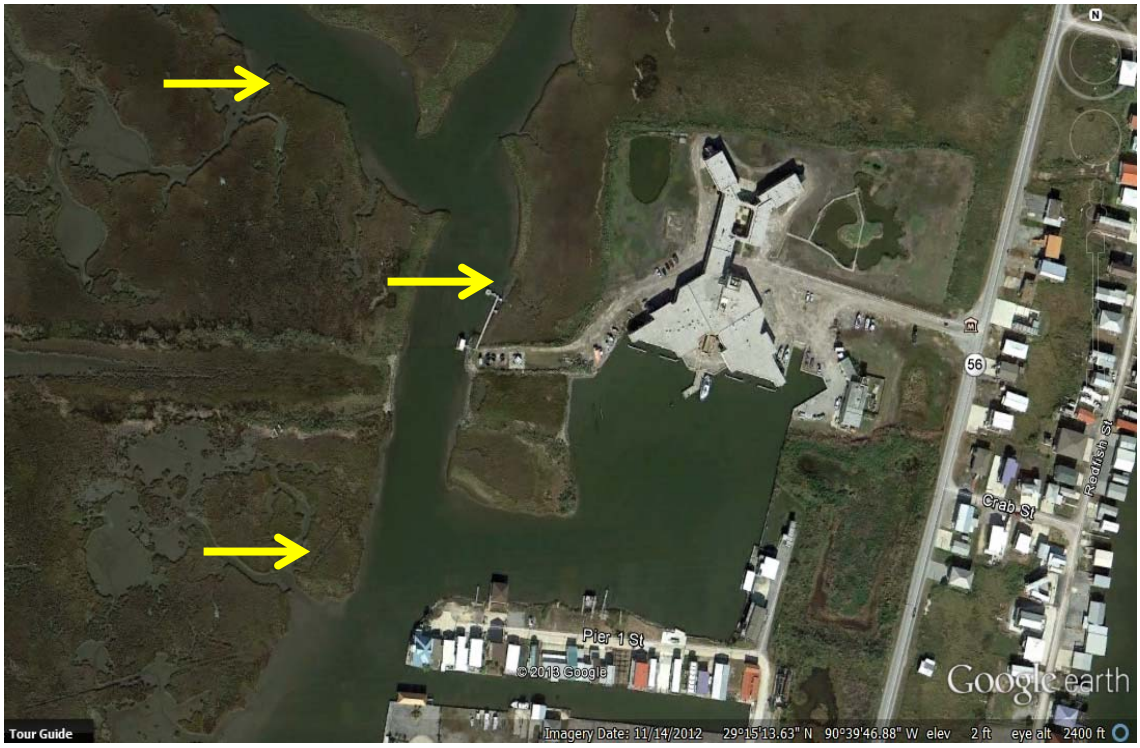

Figure 11.2. Field location of brood stock source population at the Louisiana Universities Marine Consortium facility in Cocodrie, Louisiana. Arrows indicate fish collection locations.

Source: 29°15'13.36" N, 90°39'46.88" W, Google Earth, November 14, 2012.

Figure 11.3. Example of plastic container with polyurethane foam used to air-incubate embryos.

Source: Photograph by Galvez Laboratory – LSU.

11.6.2 Production of embryos for toxicity experiments

1. Embryos were incubated in moist conditions (air incubated), but were not submersed in water until ready for hatching. This allowed for synchronization of hatch time and ensured uniform levels of developmental maturation of larvae upon hatch.

2. Fish selected for spawning were in good health and showed no signs of infection. Male fish that displayed reproductive readiness, as indicated by the darkening of the operculum and yellow coloring; and gravid females were selected to generate embryos.

3. Male and female fish were collected, and held separately in 20-L temporary holding tanks with aeration at a density of no more than 1 fish per liter. Care was taken to collect an adequate number of female fish to generate enough embryos for testing (large females can produce up to approximately 50 eggs per breeding cycle, but may not yield this many, or any at all).

4. To obtain eggs from a potentially gravid female, the female was held over a 950-mL Pyrex dish. A gentle digital pressure was applied at the anterior aspect of the abdominal cavity, behind the opercula, from both sides, sliding fingers posteriorly toward the cloaca. The eggs were deposited from the ovipositor, posterior to the cloaca. The eggs were collected in the 950-mL Pyrex dish. If eggs did not appear, the procedure was repeated twice. If eggs did not appear after the third digital pressure application, the fish was released into a 20-L temporary holding tank until egg collection was complete.

5. To obtain spermatozoa, males were euthanized by cervical severance and then testes were removed. Testes from three males were combined and held in glass Petri dishes covered in 20 mL of Hank's Balanced Salt Solution (350 mOsmol/L, 130.0 mM NaCl, 5.36 mM KCl, 0.08 mM $CaCl_2 \cdot 2H_2O$, 0.81 mM $MgSO_4 \cdot 7H_2O$, 0.42 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.16 mM $NaHCO_3$, 5.55 mM $C_6H_{12}O_6$). To activate and release spermatozoa, testes were immediately forced through a cell sieve. Spermatozoa were rinsed in a 950-mL Pyrex dish with 12 ppt salinity water, and the sieve was also rinsed with 12 ppt water. This created a solution of sperm that was directly added to the eggs. Successful fertilization of the eggs was assessed by monitoring for an elevation of the fertilization envelope.

6. An appropriate number of females (depending on the number of embryos required for the experiment) and 3–5 males were used for each fertilization event.

7. If the yolk comprised greater than 50% of the volume of the egg, the embryo was considered valid for use in experimentation. Embryos with less than 50% yolk were terminated and not used in testing.

11.6.3 Assessing embryo viability for use in test

1. Embryos were obtained using the *in vitro* fertilization technique outlined in Section 11.6.2.

2. Embryonic exposures began either within 24 hours post-fertilization or following neurulation (2–3 days post-fertilization). Until the time of the exposure, embryos were air incubated as described in Section 11.6.4, and monitored once daily to ensure proper development and viability.

3. If the embryos were used for a test within 24 hours post-fertilization, embryo viability was determined by the elevation of the fertilization envelope. This indicated that the eggs had been fertilized.

4. If the embryos were used post-neurulation, embryo viability was determined by the formation of the optic vesicles (Figure 11.4). This signaled that the embryo had completed neurulation. After completion of neurulation, these embryos were either used immediately in exposures to oiled sediment or WAF, or were reared until hatch for use in larval toxicity tests.

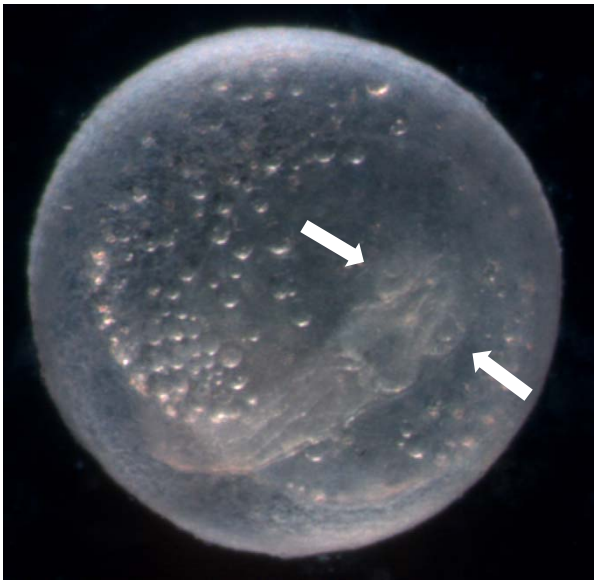

Figure 11.4. Gulf killifish embryo at approximately 2 days post-fertilization. Arrows point to optic vesicles.

Source: Photograph by Galvez Laboratory – LSU.

11.6.4 Air incubation of embryos

1. Embryos were placed in filter baskets and set on top of a disk of polyurethane foam that was saturated with freshly made 12 ppt AFS. The embryos in the basket were then covered with another layer of AFS saturated polyurethane foam prior to replacing the container lid (Figure 11.3; modified from Coulon et al., 2012). The containers were maintained at 21–24°C.

2. Embryo mortality was monitored daily under a stereomicroscope. Prior to the onset of circulation, embryos were classified as mortalities if they were found to be opaque or take on an otherwise cloudy-white appearance. They were considered viable if they were clear and continued to develop.

3. After the onset of circulation, heart function and/or evidence of circulation determined the viability of embryos.

4. Once the embryos were mature, they were hatched by immersion in AFS.

5. To hatch, mature embryos were randomly distributed from air-incubation chambers to glass Petri dishes filled with 50 mL AFS. Each Petri dish contained the number of larvae required per exposure chamber as specified in the test-specific TCTs.

6. The time and date of immersion were recorded. Embryos were given 24 hours to hatch and acclimate prior to being added to the exposure chambers.

11.7 Analytical Chemistry

Water, tissue, and sediment samples were collected as described in the QAPP. Sampling frequency and any additional specific sampling were carried out as described in the testing protocols or test-specific TCTs.

11.8 Water Quality Monitoring

Water quality was monitored as described in the test-specific TCTs using the SOPs in Section 11.9.

11.9 Water Quality SOPs

11.9.1 Total ammonia

Procedure

All samples that were tested for total ammonia were stored at -20°C until assayed. All water samples from a given toxicity test were tested for ammonia at the same time using the same standards. If multiple plates were needed, the same diluted standards were used; however, each plate was treated as a distinct assay that contained a standard curve to account for any plate-to-plate variation.

1. Measure total ammonia using the colorimetric assay described by Verdouw et al. (1978).

2. Run the assay on a flat-bottomed polystyrene 96 well plate (Model # 9017; Corning) with each sample/standard run in triplicate.

3. Prepare the standard curve from an acidified 50 mM stock solution of ammonium sulfate. Make an appropriate standard curve dilution series using AFS.

4. Add 160 µL of each standard/unknown to the respective wells.

5. Using a repeat pipette, add 20 µL of sodium salicylate solution (40 g of sodium salicylate/80 mL Milli-Q) to each well.

6. Using a repeat pipette (different tip), add 20 µL of catalyst citrate solution (0.02 g sodium nitroprusside, 35 g sodium citrate, volume to 100 mL using Milli-Q water) to each well.

7. Using a repeat pipette (different tip), add 20 µL of alkaline hypochlorite solution (4 g NaOH, 14 mL sodium hypochlorite, volume to 100 mL using Milli-Q water) to each well.

8. Allow the samples to develop in a dark location at room temperature for at least 1 hour, but no more than 24 hours.

9. Read the samples at 570 nm using the microplate spectrophotometer. The standard curve should have an R^2 value of greater than or equal to 0.95. Because the assay is run in triplicate, it is acceptable to remove obvious outlying assay replicates, but make careful notes regarding any removed outliers. Keep all raw data files, and also record all mean replicate ammonia values on data sheets, as well as the standard curve R^2 value.

11.9.2 Temperature and DO (YSI ProODO)

Procedure

1. Verify instrument calibration daily. Ensure that the sponge in the storage sleeve is moistened with RO water and store the probe in the sleeve. Check to see that the DO% is reading a calibration value of 101% (associated with a barometric pressure of 30.22 in Hg).

2. Once the instrument is calibrated, remove the storage sleeve and all protective coverings on the probe. Place the probe into each treatment container to be tested. Be sure that the round metallic temperature probe is submerged below the liquid level (continuous movement is not required).

3. Allow temperature and DO readings to stabilize and record readings.

4. Repeat as necessary for each treatment container.

5. After use, decontaminate the probe with mild soap and water and then replace the storage sleeve and all protective coverings.

11.9.3 Conductivity (YSI 85)

Procedure

1. Using the manufacturer's standards, test the range and calibration of the probe. Recalibrate as per manufacturer's instructions if needed.

2. Place the probe in the test stock or control solution and continue to swirl until the reading stabilizes.

3. Record the reading.

4. Clean the probe using detergent and a soft sponge with deionized (DI) water, and store as per manufacturer's instructions.

5. Repeat steps 1 through 4 for each container.

11.9.4 Salinity (YSI 85 or refractometer)

YSI 85 procedure

1. Using the manufacturer's standards, test the range and calibration of the probe. Recalibrate as per manufacturer's instructions if needed.

2. Place the probe in the test stock or control solution and continue to swirl until the reading stabilizes.

3. Record the reading.

4. Clean the probe using detergent and a soft sponge with RO water, and store as per manufacturer's instructions.

5. Repeat steps 1 through 4 for each container.

Refractometer procedure

1. Check the refractometer for calibration at the start of each day using RO water as zero.

2. Place 1–3 drops of water on the viewing window of the refractometer and place the plastic hinged cover over the water.

3. Look through the eyepiece and read the number at the blue line.

4. Repeat as necessary for each treatment to be measured.

5. Decontaminate the refractometer after use with mild soap and water.

11.9.5 pH

Procedure

1. Obtain all pH readings on a Denver Instrument UltraBasic UB-10 pH/mV meter according to the manufacturer's instruction manual. Rinse the probe thoroughly with RO water between each buffer or sample solution and blot excess water lightly from the end of the probe with a Kimwipe.

2. Before each use, calibrate the pH meter with all three reference buffer solutions.

    a. Prepare reference buffer solutions within 1 month; otherwise, prepare a new 50-mL conical tube with the reference pH and the date. Pour 20–40 mL of the corresponding reference buffer into the tube.

    b. To calibrate the pH meter:

        i. Insert the probe into the first reference buffer.

        ii. Press "Standardize" and allow the probe to equilibrate. When the meter has reached equilibrium, the meter reading will stop blinking. Press "Enter" to accept the buffer value. Repeat this step if the meter does not accept the value or if the buffer displays a slope error message.

        iii. Repeat steps a and b for all three reference buffers (pH 4, 7, and 10).

3. During a water change, remove between 30 and 40 mL of water and add to a 50-mL conical tube for testing.

4. Insert the probe into the sample water and allow the probe to reach equilibrium. Record this value.

Repeat steps as necessary for each sample to be tested.

References

Coulon, M.P., C.T. Gothreaux, and C.C. Green. 2012. Influence of substrate and salinity on air incubated gulf killifish embryos. *North American Journal of Aquaculture* 74:54–59.

Verdouw, H., C.J.A. Van Echteld, and E.M.J. Dekkers. 1978. Ammonia determination based on indophenol formation with sodium salicylate. *Water Research* 12(6):399–402.

A. Testing Protocol 1: Gulf Killifish (*Fundulus grandis*) Larval Acute Toxicity Test – Static Exposure

A.1 Before Test Initiation

1. WAFs were prepared with the appropriate treatment oil (see test-specific TCTs) according to the *Protocols for Preparing Water Accommodated Fractions* found in the QAPP.

2. A subsample of the exposure solution for each treatment was collected for chemical analysis by ALS Environmental as specified in the QAPP. Sample collection, labeling, and handling were conducted as specified in the *Analytical Sample Shipping and COC SOP* found in the QAPP.

3. Remaining treatment WAF was disposed of appropriately.

4. Appropriate forms (*WAF Preparation Table, Test Conditions Table, Water Quality Monitoring Table, Tank Identification Table,* etc.) were filled out as described in the QAPP.

5. Glass Pyrex containers (950 mL) were prepared and labeled for the test. Treatments consisted of a control (0% WAF) and a series of WAF dilution treatments. Before larvae were added to exposure chambers, initial water quality measurements were taken for each treatment.

A.2 Larval Exposures

1. Embryos were hatched according to the protocol described in the LSU GLPP.

2. At the end of the 24-hour acclimation period following embryo hatch, excess water and moisture were removed from the glass Petri dishes containing larvae. The glass Petri dishes were then held above replicate treatment containers holding 300 mL of treatment WAF. Using one transfer pipette per replicate treatment container, the glass Petri dish was gently rinsed with treatment WAF to transfer 20 larvae into each treatment container. The time and date were recorded upon transfer of larval groupings to replicate treatment containers. The lid was gently placed on top of each treatment container and then placed

on an orbital shaker set to 20 RPM. Replicates were maintained in an environmental control chamber at 22°C with a 12:12 light/dark photoperiod.

3. Larvae were checked once daily for mortalities and survivors, and observations were recorded. Mortality, missing individuals, and non-test mortalities were recorded on the appropriate bench sheets as described in the QAPP. Dead animals were removed and archived (unless they had decomposed), and all dead animals were retained to the extent possible and archived according to the QAPP. At this time, water was collected to evaluate water quality.

4. At the end of 96 hours, the final number of mortalities and survivors were counted for each treatment and recorded as described in step 3. For each treatment, dead animals were collected and archived according to the sample retention guidelines described in the QAPP.

5. Survivors were transferred to a Marine Biotech multi-tank zebrafish-style holding system maintained at 12 ppt salinity. Tanks were labeled with test number, treatment, and the date when larvae were first added to the tank. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of development. If these fish were not used within 3 months, or if requested by Stratus Consulting, they were sacrificed using approved methods of euthanasia at LSU.

B. Testing Protocol 2: Gulf Killifish (*Fundulus grandis*) Embryo Acute Toxicity Test to Oiled Sediments – Partial Replacements

B.1 Preparation of Exposure Chamber

1. Reference sediments were oiled according to the *Protocols for Preparation of Oil-spiked Sediments* provided by Stratus Consulting and attached below (Section B.2).

2. Sediments were loaded with oil at different concentrations (see test-specific TCTs). Another treatment with no oil added was used as a reference exposure.

3. For each replicate, 280 g (~ 150 mL) of sediment was added to a 950 mL Pyrex container.

4. Nine glass marbles (diameter of approximately 1.5 cm) were placed inside each Pyrex container. The marbles were placed toward the glass wall where they aligned with the rim of the filter basket.

5. The polytetrafluoroethylene (PTFE) basket was placed in the treatment container so that it rested on the glass marbles.

6. Slowly 200 mL of 12 ppt AFS was added into each exposure chamber, taking care to not disturb the sediment. Suspended particles were allowed to settle prior to the addition of the organisms to the filter basket.

B.2 Protocols for Preparation of Oil-spiked Sediments

B.2.1 General guidelines

1. Controls were prepared using the same technique used for spiking sediment, with the exception of adding oil.

2. Each sediment-oil concentration was made separately. For instructions in cleaning and preparing the equipment, refer to the *Decontamination SOP* in the QAPP.

3. For each step during the sample preparation and collection, all information was entered into the appropriate bench sheets provided by Stratus Consulting.

4. Unused sediments were placed in a Ziploc bag and stored in the dark at 4°C (short-term) or in a freezer at -20°C (long-term).

B.2.2 Preparation of sediments

1. A hammer and a chisel were used to break frozen sediment into pieces. The appropriate amount for each treatment was then weighed and placed into a clean glass container and covered (one container/treatment).

2. Sediments were allowed to thaw overnight at room temperature.

3. Once thawed, debris (grass, shells, etc.) was removed and sediment was placed in a mixer bowl.

4. Sediments were homogenized by mixing for 2 min at a low speed (1) using a Cuisinart SM-70BC 7-quart stand mixer.

B.2.3 Mixing oil into sediments

1. Oil was weighed as outlined below.

   A weigh boat and 2–3 Kimwipes were tared on a top loading balance. Using a stainless steel spatula, slightly more than the desired mass of oil was added to the weigh boat. Then, using a clean spatula, the oil was transferred to the mixing bowl by placing it in several areas around the bowl. Any remaining oil was then wiped off the spatula with tared Kimwipes, and the weigh boat and Kimwipes were then reweighed to determine the actual mass of oil transferred.

2. After the oil was added, thawed sediments were added to the mixing bowl.

3. The sediments were then mixed at a medium speed (5) with the Cuisinart SM-70BC 7-quart stand mixer for 30 min. The mixer was stopped every 2–4 min to scrape the sides of the mixing bowl with the putty knife.

4. Once mixing was complete, the mixer paddle and bowl were scraped with a putty knife. The sediments were transferred from the mixing bowl to the test containers using a

stainless steel spoon. If the sediments were not used immediately, they were stored at 4°C until test initiation.

B.3 Embryo Exposures to Oiled Sediments

1. Ten to 20 embryos (number based on available numbers of viable embryos following *in vitro* fertilization; see test-specific TCTs) were transferred to the top of each PTFE mesh on each replicate exposure chamber.

2. Exposure chambers were placed on an orbital shaker at 20 RPM to ensure mixing and prevent formation of stagnant boundary layers around the embryo.

3. Animals were kept at room temperature on a natural light cycle.

4. Every other day, 65 mL of water was removed from the exposure chambers and replaced with new 12 ppt AFS.

5. Embryos were checked once daily for mortalities and hatched embryos. Mortalities, missing individuals, non-treatment mortalities, and hatches were recorded on the appropriate bench sheets as described in the QAPP.

   Note: prior to the onset of circulation, embryos were classified as dead if they were found to be opaque or had an otherwise cloudy-white appearance. Dead animals were removed and archived unless they had decomposed; all dead animals were retained to the extent possible and archived according to the QAPP.

6. At approximately 7 days post-fertilization, embryonic heart rates were measured in 3 embryos per replicate. These animals were selected randomly from the batch of 10–20 animals. To view embryos for heart rate measurements, embryos were carefully removed with a wide-bore transfer pipette and transferred to an empty glass Petri dish. Embryos were then observed under a stereomicroscope and heart beats were counted over a 30-second interval.

7. The number of hatched and unhatched embryos was documented during mortality checks.

8. After counting hatched larvae, they were removed and archived at -20°C according to the QAPP.

9. Embryos were exposed to sediments for a predetermined duration (see test-specific TCTs). At the end of the exposure, all unhatched embryos were counted and then archived according to the QAPP.

Note: sampling baskets were constructed using virgin PTFE pipe stock (4B Plastics, Baton Rouge, Louisiana) and PTFE mesh with 250-µm openings (Macmaster-Carr). Two interlocking rings were machined (4B Plastics, Baton Rouge, Louisiana) and fitted together to clamp the PTFE mesh tightly across the rings to create a filter basket that rested on the sediment for the duration of the exposure, and elevated the embryos approximately 5 mm above the sediment-water interface.

B.4 Water and Sediment Sampling

1. One sediment sample (8-oz sediment jar) per treatment was collected and sent to ALS Environmental for analysis.

2. At each water renewal, a composite water sample (250-mL amber bottle) was collected from each treatment from the water removed during the renewal. The sample was sent to ALS Environmental.

C. Testing Protocol 3: Gulf Killifish (*Fundulus grandis*) Embryo Acute Toxicity Test to WAF – 96-Hour Static Exposures

C.1 Preparation of Exposure Chamber

1. WAFs were prepared as described in the *Protocols for Preparing Water Accommodated Fractions* found in the QAPP.

2. A total of 24 containers (950-mL Pyrex) were used for definitive tests containing 250 mL of 12 ppt salinity AFS (reference), or 250 mL of WAF in 12 ppt salinity AFS at differing concentrations (see test-specific TCT).

C.2 Embryo Exposures to WAF

1. Ten to 20 air-incubated embryos (see test-specific TCT) were transferred per replicate container.

2. Containers were placed on an orbital shaker at 20 RPM to ensure mixing and prevent formation of stagnant boundary layers around the embryo.

3. Animals were kept at room temperature on a natural light cycle.

4. Embryos were checked once daily at 24-hour intervals from the start of exposures for mortalities. Mortalities, missing individuals, and non-treatment mortalities were recorded on the appropriate bench sheets as described in the QAPP.

   Note: prior to the onset of circulation, embryos were classified as mortalities if they were found to be opaque or had taken on an otherwise cloudy-white appearance. Dead animals were removed and archived unless they had decomposed, and all dead animals were retained to the extent possible and archived according to the QAPP.

5. Embryos were exposed to WAF for 96 hours under static conditions.

6. At 96-hours post-exposure to WAF, the embryonic heart rate was assessed in three embryos per replicate and the remaining animals were assessed for viability. To view embryos for heart-rate measurements, embryos were carefully removed with a wide-bore

transfer pipette and transferred to an empty glass Petri dish. Embryos were then observed under a stereomicroscope and heart beats were counted over a 30-second interval.

7. Live embryos were transferred to 250 mL of clean 12 ppt AFS to be monitored for another 16 days. Embryos were first washed by placing them on a nylon mesh, and then passing approximately 100 mL of clean 12 ppt AFS over the top.

8. Embryos were monitored daily for mortality and hatch. The hatching of gulf killifish embryos typically occurred between days 10 and 14 post-fertilization at room temperature. Mortalities, missing individuals, non-test mortalities, and hatched embryos were recorded on the appropriate bench sheets as described in the QAPP.

9. At approximately 7 days post-fertilization, embryonic heart rates were measured in 3 embryos per replicate as described in step 6. These animals were selected randomly from the batch of surviving animals.

10. At test termination, all unhatched embryos were counted and archived according to the QAPP and all hatched larvae were placed in a tank under recirculating conditions for long-term holding. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of development. If not used within 3 months, or if requested by Stratus Consulting, fish were sacrificed using approved methods of euthanasia at LSU.

D. Testing Protocol 4: Gulf Killifish (*Fundulus grandis*) Embryo Toxicity Test to WAF – 20-Day Exposure with 48-Hour Renewals

D.1 Preparation of Exposure Chamber

1. WAFs were prepared as described in the *Protocols for Preparing Water Accommodated Fractions* found in the QAPP.

2. A total of 24 containers (950-mL Pyrex) were used for definitive tests containing 250 mL of 12 ppt AFS (reference) or 250 mL of WAF prepared with 12 ppt AFS at differing concentrations (details in test-specific TCTs).

D.2 Embryo Exposures to WAF

1. Ten to 20 air-incubated embryos were transferred per replicate container (see test-specific TCTs).

2. Containers were placed on an orbital shaker at 20 RPM to ensure mixing and prevent formation of stagnant boundary layers around the embryo.

3. Animals were kept at room temperature on a natural light cycle.

4. Embryos were exposed to WAF for a maximum of 20 days with 48-hour renewals.

5. Embryos were monitored daily for mortality and hatch. Hatching of gulf killifish embryos typically occurred between days 10 and 14 post-fertilization at room temperature. Mortalities, missing individuals, non-test mortalities, and hatched embryos were recorded on the appropriate bench sheets as described in the QAPP.

   Note: prior to the onset of circulation, embryos were classified as mortalities if they were found to be opaque or took on an otherwise cloudy-white appearance. Dead animals were removed and archived unless they had decomposed, and all dead animals were retained to the extent possible and archived according to the QAPP.

6. At approximately 7 days post-fertilization, embryonic heart rates were measured in 3 embryos per replicate. These animals were selected randomly from the batch of surviving animals. To view embryos for heart rate measurements, embryos were carefully

removed with a wide-bore transfer pipette and transferred to an empty glass petri dish. Embryos were then observed under a stereomicroscope and heart beats were counted over a 30-second interval.

7. At test termination, all unhatched embryos were counted and then archived according to the QAPP, and all hatched larvae were placed in a tank under recirculating conditions for long-term holding. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of development. If not used within 3 months, or if requested by Stratus Consulting, fish were sacrificed using approved methods of euthanasia at LSU.

E. Testing Protocol 5: The Effects of Direct Exposure of Gulf Killifish (*Fundulus grandis*) Embryos to an Oil Slick: Assessing the Influence of Time of Direct Slick Exposure

This protocol describes the procedure for exposing embryos directly to an oil slick each day for 2-14 hours per day during a 20-day period.

E.1 Preparation of PVC Rings

To prepare the PVC rings for slick exposures, researchers gathered one PVC ring per replicate test (see test-specific TCTs). PVC rings were 3" PVC pipes cut to lengths of 3.5 to 4.5 cm. The PVC rings were washed with soap and tap water, rinsed with RO water, and then dried before the test.

E.2 Preparation of Oil Slick in Exposure Containers

1. Before preparing the oil rings, oil was placed into a glass bowl and thoroughly mixed by hand at room temperature (see details in test-specific TCTs for the number of treatments and replicates).

2. Approximately 2 g of oil (± 0.2 g) were weighed in an aluminum weigh boat. Using a stainless steel spatula, as much of the oil from the weigh boat as possible was applied to a PVC ring. The oil was applied as a thin layer of oil to the inside of each PVC ring approximately 1 cm from one edge. This step was repeated for each PVC ring. Note that oil was added to the PVC rings no earlier than 2 days before being used in testing. Oiled PVC rings were kept at 4°C until use.

3. To prepare exposure containers, one PTFE basket (containing PTFE mesh from McMaster-Carr with 0.045 x 0.025" openings) was placed into each 950-mL Pyrex dish with 300 mL of 12 ppt seawater (see details in test-specific TCTs for the number of treatments and replicates).

4. One PVC ring was placed on top of the PTFE basket in each Pyrex dish. For oiled treatments, the PVC rings were placed within the PTFE basket with the oiled edge down so that the water surface intersected the oiled layer on the PVC ring. Clean, unoiled PVC rings were used for control treatments.

5. PVC rings were soaked for 4 hours to allow the oil slick to form.

6. After 4 hours, embryos were added to each treatment container (see test-specific TCT). To avoid disrupting the slick, embryos were placed between the outside of the PVC and the inside of the PTFE basket using a laboratory spatula, at an average distance of 1.67 cm from the water surface when resting on top of the PTFE mesh.

7. Immediately after the embryos were added, the oiled PVC rings were removed from the treatment container. This marked the beginning of the exposure. The date and time were recorded on the *Tank Identification Table* as described in the QAPP.

8. Every day at approximately the same time as the start of the exposure, partial water replacements were conducted (see Section E.2), followed by the renewal of the oil slick (steps 2 through 6). PVC rings were removed after a 4-hour soak.

E.3 Partial Water Replacements

1. Partial water replacements in exposure containers were performed daily.

2. During water replacements, the water level was dropped by removing the water, bringing the oil slick in direct contact with the embryos resting on the PTFE mesh. Water replacements were also conducted for the control treatments. Water replacements were conducted as follows:

    a. A 120-mL syringe and a long 18-gauge needle were used to draw water out of the treatment container.

    b. The needle was placed between the side of the Pyrex dish and the PTFE basket.

    c. 120 mL of water was pulled from each exposure chamber. This was enough water to ensure that the water level dropped below the embryos sitting on the PTFE mesh. Embryos were air exposed for 1, 2, 6, or 14 hours, depending on the treatment (see test-specific TCTs). Note that each experiment included control treatments with embryos that were air exposed for corresponding timeframes (i.e., 1, 2, 6, or 14 hours).

    d. After air exposures, water levels were returned to normal levels by adding 120 mL of fresh 12 ppt AFS back into the dish in the same manner that it was removed.

3. Following partial water replacement, the oil slick in each exposure chamber was renewed as described in Section E.2.

E.4 Exposure and Endpoint Measurements

1. During the test, exposure chambers were placed on an orbital shaker set to 20 RPM to prevent the formation of hypoxic boundary layers around the embryos.

2. Animals were kept at room temperature on a natural light cycle.

3. Mortality/hatch checks were performed daily just before water and oil slick renewals. All hatched larvae were removed and placed into a tank under recirculating conditions for long-term holding for possible future experiments. Mortalities, missing individuals, non-test mortalities, and hatched embryos were recorded on the appropriate bench sheets as described in the QAPP.

   Note that before the onset of circulation, embryos were classified as dead if they had an opaque or cloudy-white appearance. Dead animals were removed and archived unless they were decomposed, and all dead animals were retained to the extent possible and archived according to the QAPP.

4. At 7 days post-fertilization, heart rates were measured as follows:

   a. Measurements of heart rates were performed just before the oil slick renewal, so that the embryos could be removed with minimal disruption to the oil slick.

   b. Three animals from each replicate were selected randomly from the batch of surviving animals. To view embryos for heart rate measurements, embryos were carefully transferred to an empty glass Petri dish using a wide-bore transfer pipette. Using a stereomicroscope, heart beats for each embryo were counted for 30 seconds.

   c. Embryos were then returned to their treatment container using a wide-bore transfer pipette.

5. Exposures continued for 20 days.

6. At test termination, all unhatched embryos were counted and then archived according to the QAPP; all hatched larvae were immediately placed in a tank under recirculating conditions for long-term holding. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of

development. If not used within 3 months, or if requested by Stratus Consulting, fish were euthanized at LSU and then archived according to the QAPP.

E.5 Imaging of Oil Slicks

1. Treatment containers were imaged using a digital camera on a solid black surface just after removal of PVC rings and at 3 or 4 additional time points during the test.

2. During imaging, ambient room lighting was turned off. Oil slicks were illuminated using a small lamp (with a Reveal bulb) clamped securely to a ring stand. The light was diffused through an opaque plastic tray to remove glare (see Figure E.1). The distance from the table to the opaque plastic was approximately 20 cm and the distance from the opaque plastic to the light was 10-20 cm.

3. Images were taken at an angle 30 to 60 degrees relative to the light source, approximately 15-25 cm away from the oil slick.

Figure E.1. Set-up for imaging oil slicks indoors.

F. Testing Protocol 6: The Effects of Direct and Indirect Exposure of Gulf Killifish (*Fundulus grandis*) Embryos to an Oil Slick with Subsequent Exposure to UV

This protocol describes the procedure for exposing embryos to an oil slick derived from Slick A or B oil (see test-specific TCTs) and subsequently exposing the embryos to UV light. Embryos that were 1-day post-fertilization were exposed to an oil slick for 14 hours with varying times of direct and indirect exposure to the oil slick (see test-specific TCTs). The oil-exposed embryos were then transferred to clean water and exposed to varying levels of ambient UV light for approximately 6 hours. Embryos were monitored for 20 days following UV exposure for heart rate, mortality, and hatch.

F.1 Preparation of PVC Ring

To prepare the PVC rings for slick exposures, the required number of PVC rings were gathered (1 ring for each replicate was required; see test protocol TCTs). PVC rings were 3" PVC pipes cut to lengths of 3.5-4.5 cm. The PVC rings were washed with soap and tap water, rinsed with RO water, and then dried before the test.

F.2 Preparation of Oil Slick in Exposure Containers

1. Before the preparation of oil rings, oil was placed into a glass bowl and thoroughly mixed by hand at room temperature (see details in test-specific TCTs for number of treatments and replicates).

2. Approximately 2 g of oil (± 0.2 g) were weighed in an aluminum weigh boat. Using a stainless steel spatula, as much of the oil from the weigh boat as possible was applied to a PVC ring. The oil was applied as a thin layer of oil to the inside of each PVC ring approximately 1 cm from one edge. This step was repeated for each PVC ring. Note that oil was added to the PVC rings no earlier than 2 days before being used in testing. Oiled PVC rings were kept at 4°C until use.

3. To prepare exposure containers, one PTFE basket containing PTFE mesh from McMaster-Carr with 0.045 x 0.025" openings was placed in each 950-mL Pyrex dish

with 300 mL of 12 ppt seawater (see details in test-specific TCTs for number of treatments and replicates).

4. One PVC ring was placed on top of the PTFE basket in each Pyrex dish. For oiled treatments, the PVC rings were placed with the oiled edge down so that the water's surface intersected the oiled layer on the PVC ring. Clean, unoiled PVC rings were used for control treatments.

5. PVC rings were soaked for 4 hours to allow the oil slick to form.

6. After 4 hours, embryos were added to each treatment container (see test-specific TCT). To avoid disrupting the slick, embryos were placed between the outside of the PVC and the inside of the PTFE basket using a laboratory spatula at an average distance of 1.67 cm from the water's surface when resting on top of the PTFE mesh.

7. Immediately after the embryos were added, the oiled PVC rings were carefully removed from the treatment container by hand. This marked the beginning of the exposure. The date and time were recorded on the *Tank Identification Table* as described in the QAPP.

F.3 Direct Exposure of Embryos to Oil Slick

For all treatments with direct exposure to the oil slick, the procedures described below were followed. For each oil treatment, a no-oil control treatment was also run using the same methods. For treatments with no direct exposure to the oil slick (i.e., the indirect treatments), see Section F.4. See test-specific TCTs for treatments.

1. Immediately after embryos were added to Pyrex dishes, the water level was dropped, bringing the oil slick in direct contact with the embryos resting on the PTFE mesh. Direct exposure to the oil slick continued for 2–14 hours depending on the treatment (see test-specific TCTs). Water removal was conducted as follows:

    a. A 120-mL syringe and a long 18-gauge needle were used to draw water out of the treatment container.

    b. The needle was placed between the side of the Pyrex dish and the PTFE basket.

    c. 120 mL of water was pulled from each exposure chamber. This was enough water to ensure that the water level dropped below the embryos sitting on the PTFE mesh.

2. After the appropriate amount of time of direct exposure to the oil slick (2–14 hours, depending on the treatment), 120 mL of clean AFS was added back into each Pyrex dish using a clean, 120-mL syringe with an 18-gauge needle. As with water removal, water was added back in by placing the needle between the side of the Pyrex dish and the PTFE baskets.

3. All direct exposures were run for a total of 14 hours.

F.4 Indirect Exposure of Embryos to Oil Slick

1. For indirect exposure treatments, the embryos were never in direct contact with the oil slick during exposure. The purpose of the indirect exposure is to investigate potential differences between direct exposure to slick oil, and exposure to water just beneath. For these exposures, as 120 mL of water was slowly removed from each Pyrex dish containing an oil slick, an equal volume of clean water was added back to the dish at the same rate, keeping the water level constant throughout the exchange. This was done immediately after the embryos were added to the Pyrex dish, using the same methods described above for the removal and addition of water in the direct exposures. However, to carefully control the rate that water was added and removed, two people were required for each water exchange, one to operate a syringe to remove the water, and one to operate a syringe to add water back into the Pyrex dish. For each oil treatment, a no-oil control treatment was also run using the same methods

2. Following water exchange, indirect exposure continued for 14 hours.

F.5 UV Exposure

1. After the oil slick exposure, an equal number of embryos from each Pyrex dish were immediately transferred using a stainless steel spatula to one of three glass crystallizing dishes (corresponding to 10%, 50%, and 100% ambient UV) containing 200 mL of fresh 12 ppt water.

2. Embryos were exposed outdoors to 10%, 50%, or 100% ambient UV light for up to 6 hours for one or more consecutive days (see test-specific TCT). These exposures were performed on the rooftop of the Life Sciences Annex building at LSU. See step 5 below for description of water bath.

3. Crystallizing dishes were covered with fiberglass trays during transport to and from the outdoor water bath for UV exposure.

4. UV and manual temperature measurements were performed at 15-min intervals during embryonic UV exposures. Additionally, water temperature and light intensity were measured using automatic HOBO temperature monitors. These data loggers were placed in 6 locations (listed below) within the water bath or within dummy chambers placed within or outside of the water bath. Dummy treatment containers had an identical amount of water as test treatment containers and were used to monitor temperature in the following locations:

    a. Inside the northwest corner of the water bath
    b. Inside the southeast corner of the water bath
    c. Inside the middle of the water bath
    d. Outside the north side of the water bath to collect air temperature
    e. Inside the dummy treatment container on the east side of the water table
    f. Inside the dummy treatment container on the west side of the water table.

5. A water bath was prepared the day before the start of experiments to ensure that the water cooled overnight. If necessary, during the day a chiller was used to maintain proper water temperature (Figure F.1).

    a. The water table was oriented with its longest dimension facing southward. The water table was placed on cinder blocks to allow for drainage.

    b. The water table was then sectioned into thirds, with different plastic sheeting covering each section to allow 10%, 50%, or 100% of the ambient UV light to reach the crystallizing dishes underneath. See the UNT GLPP for further description of plastic sheeting.

    c. Tap water was used to fill the water table to within 2” of the top.

    d. Tubing (3/4” interior diameter) connected the pump and chiller, and a submersible pump situated inside the northwest corner of the water table pumped water to the chiller. The chilled water was returned to the water table using tubing situated inside the southeast corner of the water table. If needed, an additional pump was located inside the southeast corner of the water table to circulate water inside the water table, maintaining a more uniform temperature throughout the water table during the UV exposure.

Figure F.1. Water table set-up for UV exposure to embryos.

6. Before the crystallizing dishes were moved outdoors, the water in the water bath was brought to the correct temperature.

7. By mid-morning, crystallizing dishes were placed outdoors in the water bath under the different plastic covers corresponding to their UV treatment.

8. At the end of the day (approximately 6 hours of UV exposure), crystallizing dishes were brought indoors and placed on orbital shakers set to 20 RPM, in a room maintained at 20 to 22°C.

9. If repeat UV exposures were conducted, the following day crystallizing dishes were transported back to the outdoors water bath and placed under their respective UV plastic covers by mid-morning for up to 6 hours of UV exposure (see test-specific TCTs).

F.6 Post-exposure Monitoring and Endpoint Measurements

1. Embryos were monitored for heart rate, mortality, and hatch up to 20 days following UV exposure.

2. During this monitoring period, 100% water replacements were performed weekly immediately after water quality monitoring. For each container, embryos were removed using wide-bore transfer pipette to a glass Petri dish for mortality and hatch checks.

While embryos were removed, a total water replacement was performed. Embryos were then placed back into the crystallizing dish wide-bore transfer pipette.

3. Exposure chambers were maintained at 20 to 22°C with a natural light cycle on an orbital shaker set to 20 RPM to prevent the formation of hypoxic boundary layers around the embryos.

4. Mortality/hatch checks were performed daily. All hatched larvae were removed and placed into a tank under recirculating conditions for long-term holding for possible future experiments. Mortalities, missing individuals, non-treatment mortalities, and hatched embryos were recorded on the appropriate bench sheets as described in the QAPP.

   Note that before the onset of circulation, embryos were classified as dead if they had an opaque or cloudy-white appearance. Dead animals were removed and archived unless they were decomposed, and all dead animals were retained to the extent possible and archived according to the QAPP.

5. At 4, 7, and 10 days post-fertilization, heart rates were measured as follows:

   a. Measurement of heart rates was performed just before oil slick renewal, so that the embryos could be removed with minimal disruption to the oil slick.

   b. Three animals from each replicate were selected randomly from the batch of surviving animals. To view embryos for heart-rate measurements, embryos were carefully transferred to an empty glass Petri dish using a wide-bore transfer pipette. Using a stereomicroscope, heart beats for each embryo were counted for 30 seconds.

   c. Embryos were then returned to their treatment container.

6. Exposures continued for 20 days.

7. At test termination, all unhatched embryos were counted, and then archived according to the QAPP; all hatched larvae were immediately placed into a tank under recirculating conditions for long-term holding. These fish were held for possible future experiments to assess the physiological consequences of early-life exposure to oil at later stages of development. If not used within 3 months, or if requested by Stratus Consulting, fish were euthanized at LSU and then archived according to the QAPP.

12. U.S. Army Engineer Research and Development Center General Laboratory Procedures and Practices

12.1 Introduction

The U.S. Army Engineer Research and Development Center (ERDC) conducted sediment toxicity tests using the estuarine amphipod, *Leptocheirus plumulosus*, to identify toxicological effects of the 2010 *Deepwater Horizon* oil spill on benthic species. This chapter describes general laboratory practices and procedures used at ERDC.

12.2 Testing

12.2.1 Test organism sources and husbandry – *Leptocheirus plumulosus*

All experiments were conducted using the amphipod *Leptocheirus plumulosus*. Experimental organisms were cultured on site at the ecotoxicology laboratory of the Environmental Laboratory, ERDC, in Vicksburg, Mississippi, according to Environmental Ecotoxicology and Risk Assessment Team (EERT) standard operating procedure (SOP) M-003: Culture, Care and Maintenance of *Leptocheirus plumulosus*. Cultures were maintained in 42 × 24 × 15 cm polyethylene (PE) tote boxes containing 2 to 3 cm of sediment purchased from Sequim Bay, Washington, and 3 L of artificial seawater at 20 ppt salinity. Cultures were held under a 16:8 hour light:dark cycle at 23°C and with trickle flow aeration. Amphipod cultures were fed ground TetraMin (purchased from Tetra Sales, Blacksburg, Virginia), and 40–60% of the overlying water was renewed three times weekly.

12.2.2 Source water

Artificial seawater at 20 ppt was prepared by mixing Crystal Sea Marinemix Bioassay Laboratory Formula, purchased from Marine Enterprises International, Inc., Baltimore, Maryland, in reverse-osmosis (RO) water.

12.2.3 Oil-spiked sediment preparation

Exposures took place in accordance with test-specific protocols and test conditions tables (TCTs). In all cases, oil-spiked sediments were prepared as outlined in the SOP: *Protocol for Preparation of Spiked Sediment*, in Section A.3. Sediment and slick oil were provided by the National Oceanic and Atmospheric Administration (NOAA).

12.2.4 Biological testing

The chronic effects of oil-spiked sediments on survival, growth and reproduction of the amphipod *Leptocheirus plumulosus* were assessed according to ERDC Testing Protocol 1. The protocol was developed using the following established guidelines:

- ASTM Method E1367-03 (Reapproved 2008): Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates (ASTM, 2008)
- Methods for Assessing the Chronic Toxicity of Marine and Estuarine Sediment-associated Contaminants with the Amphipod *Leptocheirus plumulosus* (U.S. EPA and USACE, 2001)
- Methods for Assessing the Toxicity of Sediment-associated Contaminants with Estuarine and Marine Amphipods (U.S. EPA, 1994).

12.2.5 Overlying water quality monitoring

See the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP; located in Attachment 3) and SOPs listed in Section 12.4 for required monitoring and associated bench sheets.

12.2.6 Analytical chemistry sampling

See QAPP and test-specific TCTs for sampling and processing of sediment and water collected for potential chemical analyses.

12.3 Reporting and Testing Documentation

Reporting and testing documentation was performed as outlined in the QAPP.

12.4 Water Quality

12.4.1 Temperature measurement

1. Temperature measurement in the water baths and environmental chamber was performed with a Thermco Products Model ACC895 certified triple-digit thermometer

2. The water temperature of the overlying water in the test chambers was measured using the YSI model 556 Multiprobe System according to EERT SOP I-002: Procedure for calibration and use of the YSI 556 Multi Probe System (MPS)

3. Temperature in pore water was taken prior to pH and ammonia measurement and performed with the Orion 9107 BN pH low-maintenance triode according to EERT SOP I-006: Calibration and use of Orion 9107 BN pH low maintenance triode with the Orion Dual Star pH/ISE benchtop meter.

12.4.2 Measurement of salinity

1. Salinity in the artificial seawater preparation and in pore water was measured using a temperature compensating refractometer made by Aquamarine, model ABMTC. Using a glass Pasteur pipette, one to two drops of test solution were placed on the measurement window and the lid was closed. The refractometer was pointed toward the light, making sure that it was kept level. The value was recorded.

2. The salinity of the overlying water was measured using the YSI model 556 Multiprobe System according to EERT SOP I-002: Procedure for calibration and use of the YSI 556 MPS.

12.4.3 pH measurement

1. pH in pore water was measured using the Orion 9107 BN pH low-maintenance triode according to EERT SOP I-006: Calibration and use of Orion 9107 BN pH low maintenance triode with the Orion Dual Star pH/ISE benchtop meter

2. The pH of the overlying water was measured using the YSI model 556 Multiprobe System according to EERT SOP I-002: Procedure for calibration and use of the YSI 556 MPS.

12.4.4 Measurement of total ammonia

1. Total ammonia as nitrogen in pore water was measured with the Orion 9512 HPBNWP ammonia probe following the procedures outlined in EERT SOP I-001: Procedure for calibration and use of Orion 9512 HPBNWP Ammonia Probe.

2. The water ammonia in overlying water was measured with the LaMotte model R-5864 colorimetric kit. Five milliliters of sample were added to the colorimetric test tube. Then, eight drops of reagent #1 were added to the test tube. The tube was capped and shaken. Then, eight drops of reagent #2 were added to the tube. The tube was capped and shaken. The tube was placed in the color comparison module. The module was held up to light and the reading closest to color in the test tube was recorded.

12.4.5 Measurement of dissolved oxygen

1. Overlying water-dissolved oxygen (DO) was measured using the YSI model 556 Multiprobe System, according to EERT SOP I-002: Procedure for calibration and use of the YSI 556 MPS.

12.5 Sediment Toxicity Test Sediment and Water Disposal Procedures

1. Overlying water from the tests was disposed of in accordance with ERDC guidelines

2. At the completion of the project, solid waste was disposed of under the chemical safety and disposal regulations of the ERDC.

12.6 General Laboratory SOPs

12.6.1 Culture, care, and maintenance (*Leptocheirus plumulosus*) EERT SOP: M-003

Purpose: To ensure proper handling and care of the estuarine amphipods to yield healthy animals for toxicological testing.

Leptocheirus plumulosus is a gammarid amphipod native to estuarine waters of eastern North America (Bousfield, 1973). This species is cultured for use in acute and chronic sediment toxicity tests (DeWitt et al., 1992).

This SOP outlines procedures for maintaining cultures under relatively uniform conditions. It is divided into the following sections: Culture Overview, Culture Set Up, Culture Breakdown, Food Preparations, Feeding, Water Changes, Water Quality Monitoring, Population Monitoring, and Cadmium Reference Testing. The SOP uses the following definitions:

"Size or size class"	Unless otherwise indicated, determined by sieve pore diameter, rather than animal length
"Neonate"	Animals $\geq$ 300 µm but $\leq$ 425 µm in size
"Juvenile"	Animals $\geq$ 425 µm but $\leq$ 600 µm in size
"Sub-adult"	Animals $\geq$ 600 µm but $\leq$ 1 mm in size
"Adult"	Animals $\geq$ 1 mm in size
"Culture sediment"	300 µm sieved sediment from Sequim Bay, Washington; this sediment was frozen prior to initial sieving
"RO"	Reverse-osmosis water
"Culture water"	5 or 20 ppt salinity artificial salt water generated from Crystal Sea and RO water (aged $\geq$ 5 days)
"Culture container"	PE tote box [45 cm (L) ×24 cm (W) × 15 cm (D) (approximate internal dimensions); surface area approximately 1,073 cm^2]
"Cultures"	Culture containers containing populations of *L. plumulosus*

Culture overview

The ERDC *L. plumulosus* culture was initially derived from animals collected from James Pond on the Magothy River of the Chesapeake Bay, Maryland, in October 1994. To increase diversity, additional laboratory cultures from other commercial vendors were added to the laboratory cultures in February 1997 and approximately every 2 years afterward. The culture was maintained in the ERDC culture facility at 20 ± 2 ppt, 23 ± 2°C, at a radiation intensity of approximately 6 µE S^{-1} m^{-2} and under 16:8 hours light:dark cycle. Animals were cultured in plastic tote bins placed in temperature-regulated water baths. All animals were maintained on culture sediment with overlying culture water. Culture containers were routinely broken down and set up again to avoid undue crowding and to provide animals for testing purposes. Culture "health" or sensitivity was monitored through cadmium reference tests. Water quality was monitored weekly and maintained by frequent water changes. Cultures were fed three times per week.

Culture set up

Overview

Culture containers were set up to provide a regular supply of animals for test purposes. If a constant culture was desired, the number of new culture containers was approximately equal to the number of culture containers broken down. New culture containers were generated on the same day as culture containers were broken down.

Materials

- Squirt bottle
- Culture book
- Indelible black ball-point pen
- Pasteur pipets
- Pipet bulb
- Culture container
- ~ 3 L culture sediment
- Air stone and air line
- ~ 8 L culture water
- Dispensing container (100–250 mL glass beaker)
- 200 animals (67 juveniles, 67 sub-adults, 66 adults)
- *L. plumulosus* food (see below for composition).

Methods

1. Rinse culture container with culture water.

2. Add culture sediment to each culture container to a depth of 2.0 ± 0.5 cm.

3. Add ~ 8 L culture water (to $\geq$ 6 cm depth). Aerate with air stones.

4. Label each culture with the date of the sediment addition and the sediment code.

5. Allow the sediment to settle for > 12 hours and < 3 days.

6. Before adding animals, and also within 12 hours after adding animals, replace 60% of the culture water. This action may reduce ammonia and hydrogen sulfide levels in the overlying water.

7. Stock culture containers with animals generated from breakdowns conducted the same day (i.e., do not leave animals overnight before adding them to a culture container).

8. Pool animals from each size class; pooling animals from separate culture containers may increase homogeneity of animals in the new culture containers.

9. For each new culture container, count 67 juveniles, 67 sub adults, and 66 adults into a dispensing container. If insufficient animals are available from any size class, substitute animals from another class. Record deviations in the culture record book.

10. Gently add the 200 animals into the culture container. Be sure to add animals from below the water level of the culture container. Rinse the dispensing beaker thoroughly with culture water to ensure that no animals are retained in the dispensing container. Gently push animals trapped on the water surface into the water column using drops of culture water from a pipet or squirt bottle. If the water in the dispensing container is > 2°C different from the culture container, then the dispensing container should be allowed to equilibrate with the culture water before the animals are added. This can be accomplished by placing the dispensing container in the culture container for 1 hour.

11. See *Feeding*, below, for feeding instructions. Only feed cultures on regular feeding days.

12. Replace the culture container label with a label containing the following culture code "L" followed by: a number, one number larger than the one on the previously created culture container; the number of animals added, usually "200;" size class, or "mixed;" sediment code; and the date animals were added.

13. Record culture code, number animals added and size class, animal source (i.e., culture codes from which the animals were derived), culture container (if not standard), salinity (20 ppt), temperature (23°C), module number, and any additional comments in the culture record book.

Culture breakdown

Overview

Culture containers were broken down when they were 28 to 35 days old. Older culture containers were broken down before younger culture containers. When culture containers were used to generate animals for testing purposes, including reference tests, juveniles from three culture containers were counted; if only one or two culture containers were broken down, juveniles in all containers were counted. During the culture breakdown process, animals received air exposure of only 3 to 4 minutes and remained moist during the entire air exposure; animals were never allowed to dry out.

Materials and personnel

- One 12-in. sieve of each of the following sizes: -300, 425, and 600 µm; 1 mm
- One 5 L or equivalent pitcher labeled "20‰"
- RO water
- Two squirt bottles each labeled "20‰"
- One fire-polished Pasteur pipet with narrow end > 1.5 mm diameter; wide end inserted in pipet bulb
- "Catch-pan" to place under bucket
- Laboratory apron
- *L. plumulosus* culture record book
- One person for culture breakdown; if culture animals were counted, a second person to assist with counting; if multiple breakdowns in one month, animals counted only once
- Mechanical counters
- In addition, for each culture to be broken down:
    - One 5-gal bucket with outer diameter slightly less than 12-in. sieve
    - *L. plumulosus* culture container
    - 12 L culture water
    - Glass culture bowls (4 and 8 in.).

Methods

1. Place catch-pan in a convenient working area and next to a work bench; place the bucket inside the pan. Stack the sieves sequentially on the bucket: 300, 425, 600 µm, then the 1 mm sieves – smaller-diameter sieve on bottom, largest on top.

2. Fill a pitcher and squirt bottles with culture water. Label four culture bowls with the four sieve sizes. For counting juveniles, label an additional three culture bowls with "425 µm" and the culture identification number.

3. Don a laboratory apron.

4. Move the culture container to work bench.

5. Using a gloved hand, gently stir the sediment into the overlying water for approximately 30 seconds. Once overlying water becomes a relatively thick suspension of sediment, pour the suspension gently and from as low a height as possible onto the stacked sieves. Do not pour more than 200 mL of suspended sediment onto sieves at one time.

6. Examine the 300-µm sieve to ensure it is not retaining water. Correct a vapor lock by gently lifting one side of the 300-µm sieve away from the bucket. Correct accumulation of sediment by tapping the underside of the 300 µm sieve with your fingers until water passes through sieve.

7. If sediment remains in the culture container, pour approximately 1 L culture water into the culture container. Repeat steps 5 and 6.

8. Repeat step 7 until < 200 mL of unsuspended sediment remains in the culture container.

9. Using a squirt bottle, remove sediment attached to walls of culture container and pour onto stacked sieves. The culture container should now be free of sediment.

10. If there is a need to count juveniles from a particular culture container, ensure that sediment is not spilled directly onto the catch-pan or allowed to leak from any sieve other than from the 300-µm sieve. This will help prevent the loss of individual animals.

11. Using a squirt bottle, gently rinse the sediment through the top sieve, avoiding undue pressure on the animals. Do not use a brush to push sediment through the sieve; this method would injure animals by forcing them against the metal sieve.

12. Using a squirt bottle, transfer animals from the 1-mm sieve to the appropriate culture bowl. Squirt water from behind the sieve, and venially downward along the sieve plane; this method will help ensure that animals are transferred to the culture bowl rather than to the work bench. If the water in the culture bowl appears too cloudy for counting, animals may be sieved again using a clean 300-µm sieve; use a 300-µm sieve for all but the initial sieves to ensure that all animals are retained within the original size class.

13. Repeat steps 10 and 11 for the remaining sieves, using the appropriately labeled culture bowl.

14. Label the bucket containing the sieved sediment with sediment type (e.g., “SC9501a”), source (*L. plumulosus* culture), sieve diameter (300 µm), date, and generator’s initials.

15. Rinse sieves and tray thoroughly with RO water.

16. Using fire-polished pipet, count juveniles from the designated cultures. Record counts and breakdown dates in the *L. plumulosus* culture record book.

17. Animals not needed for new culture containers or for tests are euthanized by exposing them to hot tap water and pouring them down a drain.

Food preparation

Materials

- TetraMin
- 0.5-mm screen
- Food mill from Cyclone Sample Mill, UDY Corp., Fort Collins, Colorado
- ~ 100-mL sample vial
- Sealable, water-impermeable food container.

Methods

1. Attach sample vial to mill output. Place the 0.5-mm screen in the food mill. Firmly attach mill cover and turn on mill. Pour TetraMin flakes slowly into the food mill.

2. When desired amount of food is grinded, clean mill. Clean detachable components with paper towel or soap and water. Attach clean sample vial to mill output.

3. Place food in food container and refrigerate.

Feeding

Materials

- Food (see above)
- Weighing balance
- Weighing boat
- Beaker (size determined by volume of food solution)
- Stir bar
- Stir plate
- Culture water
- 10-mL pipet.

Methods

1. Round the number of culture containers plus three to a convenient number. Multiply by 0.8 g. (For example, you count 10 culture containers: 10 + 3 = 13; 13 culture containers × 0.8 g/culture = 10.4 g). Weigh this amount of food into the weighing boat.

2. Multiply the number of culture containers by 10 mL to give the total dilution volume (for example, 13 culture containers × 10 mL/culture = 130 mL). Add this volume of culture water to a beaker. Place the beaker on the stir plate and add the stir bar.

3. Add the food in the weighing boat to the beaker. Stir for ≥ 30 minutes.

4. Pipette 10 mL food suspension into each culture. Attempt to evenly distribute food across culture container surface. Ensure that food particles remain suspended during the pipetting process by stirring the food suspension with the pipet tip before and during the pipetting process.

Water changes

Overview

Water quality in cultures containers is likely improved by frequent water changes.

Materials

- Culture water at 20 ± 2 ppt salinity and at 23 ± 2°C
- Siphon hose of ≥ 1 m length and containing a ≤ 300 µm screen over one end
- Bucket
- 5-L or equivalent pitcher
- RO water.

Methods

1. Three times a week, on Monday, Wednesday, and Friday, siphon 40–60% of overlying water from all culture containers into a bucket or other container. Pour the water into a drain.

2. Test the salinity and temperature of culture water. If salinity is 20 ± 1 ppt and the temperature is 23 ± 3°C, proceed to step 4. Otherwise, adjust culture water salinity or temperature.

3. Gently pour new culture water from a pitcher directly into the culture container.

4. Rinse the siphon hose, pitcher, and bucket with RO water.

Water quality monitoring

Overview

To ensure that water quality remains within prescribed limits, water quality parameters are measured once per week.

Materials

- DO and temperature meter and probe
- pH meter and probe
- Refractometer
- Culture book.

Methods

1. On a new page in the culture book, create a table for entry of water quality parameters.

2. Calibrate meters.

3. Measure DO, pH, temperature, and salinity in culture containers, record data in the water quality table mentioned above. If high ammonia levels > 1 mg/L are suspected, take a 30-mL sample of the overlying water and check it for ammonia.

4. The following actions should be taken by the water quality measurer when water quality parameters deviate from the following "norms:"

Parameters	Norm	Action by measurer if parameters differ from norm	Contact laboratory QA/QC officer if:
DO	≥ 6 mg/L	Increase aeration	> 10% of culture containers ≤ 4 mg/L
Temperature	23 ± 2°C	Carefully adjust Remcor temperature control	> 2 culture containers are ± 5°C from norm; > 50% culture containers are ± 3°C from norm
pH	6.5–9	Contact QA/QC Officer	pH differs from norm
Salinity	20 ± 2‰	Change water and, if aeration and salinity are both high, reduce aeration	Salinity in > 50% of culture containers is outside normal limits for > 2 weeks
Ammonia	< 5 mg/L (total ammonia)	Increase aeration	Ammonia level in > 10% culture containers is > 5 mg/L

QA/QC: quality assurance/quality control.

12.6.2 Procedure for calibration and use of the YSI Model 556 MPS EERT SOP: M-002

Purpose

- To ensure accurate and reliable water quality measurements (DO, conductivity, temperature, and pH) in aqueous solutions.

Materials

- YSI 556 MPS
- pH buffers in small specimen cups (4.01, 7.0, and 10.0)
- Conductivity standards in small specimen cups (1.413, 12.9, 15, 28 ms/cm^2)
- 2-mL PE (blue) bonded DO membrane caps (YSI # 5909)
- DO electrolyte filling solution
- Rinse bottle with RO or deionized (DI) water
- Kimwipes
- Safety equipment (rubber gloves, safety glasses, laboratory coat).

Summary

The YSI Multi probe should be calibrated each day before initial use and calibrations should be logged into the calibration book for that instrument.

Procedure

1. ***Probe setup:*** When the probe is first received, install each of the sensors. Periodically a sensor or DO membrane may need to be replaced. Follow these instructions for installing sensors and DO membranes. For information on setting up the meter for the proper sensors installed and the measurement units reported, refer to the YSI 556 MPS Operations Manual (YSI, 2002).

    a. Remove the transport/calibration cup and thoroughly dry the entire probe. Water must not get into the sensor ports during the changing of the sensors. Use compressed air to dry the probe before removing old sensors.

    b. Using the sensor installation tool supplied in the maintenance kit, unscrew and remove the old sensor or the sensor port plugs.

    c. Locate the port with the connector that corresponds to the sensor that is to be installed. Apply a thin coat of o-ring lubricant to the o-ring on the connector side of the sensor.

d. To prevent the o-ring from leaking, make sure there are no contaminants between the o-ring and the sensor. Again, be sure the probe is free of moisture and dry further if necessary with compressed canned air.

e. Align the sensor with the sensor port so that the two connectors will fit together properly. With the connectors aligned, screw down the sensor nut using the sensor installation tool. Tighten the nut until it is flush with the face of the probe module bulkhead. Do NOT over tighten.

f. Caution: Do not cross thread the sensor nut. This may damage the port threading, causing the entire probe to leak and malfunction.

g. Repeat the above procedures for each sensor to be installed.

h. Install the DO membrane cap onto the DO sensor by removing the old cap. Filling a new cap half full of electrolyte solution, tap the cap lightly on the table to remove any bubbles from the walls of the cap and screw the membrane cap onto the sensor moderately tightly. A small amount of electrolyte should overflow. Observe the probe for bubbles inside the cap. If bubbles are present, remove the cap and repeat the process.

2. ***Calibrations:*** Each of the sensors in the probe must be calibrated separately. The typical order of this calibration is: DO, conductivity, and then pH.

a. *DO calibration:* To begin the calibration of the DO sensor, obtain information on barometric pressure from the following websites. http://www.wunderground.com/weather-forecast/39180 will provide the current Vicksburg barometric pressure. This measurement is provided in inches of mercury and must be converted to mm of mercury. Enter the measurement from the weather underground site into the conversion program at http://www.onlineconversion.com/pressure.htm and select inches of Hg to mm of Hg. Subtract 5 from the value provided to correct for the elevation of the ERDC laboratory and use that number in the calibration set-up. The aforementioned process must be completed each time the instrument is calibrated.

i. Turn on the instrument by pressing the power button (green).

ii. Access the calibration menu by pressing the <Esc> button, and using the down arrow key to scroll down to the line that says Calibrate. Press the Enter button that looks like a left facing arrow <←>.

iii. From the Calibration Menu, scroll down using the down arrow button to the DO line and press the Enter <←–> button.

iv. Scroll down in the DO calibration menu to DO% and press the Enter <←–> button.

v. Using the keypad, enter the local barometric pressure obtained from the websites and press the Enter <←–> button.

vi. Make sure that the probe is in the calibration cup and is loosely threaded in an upright position; the cup should contain approximately 1/8 in. of water. Also be sure that no water is on the DO membrane from rinsing.

vii. Observe the DO readings and when they show no observable change in 30 seconds, press the Enter <←–> button.

viii. Press the Enter <←–> button again to return to the DO calibration screen and then the <Esc> button to return to the main calibration screen. The DO sensor is now calibrated.

b. *Conductivity calibration:* To calibrate the conductivity sensor, pour one or more conductivity standards into small specimen cups with lids. To calibrate the probe properly, volumes must be sufficient to cover the opening at the side of the sensor.

i. Remove the storage/calibration cup from the probe and submerge the probe in the lowest conductivity standard, making sure that the opening at the side of the sensor is submerged in the standard

ii. From the main calibration menu, select Conductivity and press the Enter <←–> button

iii. From the Conductivity calibration menu, select Specific Conductance and press the Enter <←–> button

iv. Enter the specific conductance of the first (lowest) standard to be measured and press the Enter <←–> button

v. Observe the conductivity readings and when they show no observable change in 30 seconds, press the Enter <←–> button

vi. Press the Enter <←–> button again to return to the Conductivity Calibration Menu

vii. If more than one standard is to be measured, repeat the above process for each standard

viii. Be sure to rinse the probe with RO or DI water in between standards and dab dry with a Kimwipe

ix. Once all standards are measured, the conductivity sensor is calibrated. Press the <Esc> button to return to the Main calibration menu.

c. *pH calibration:* To calibrate the pH sensor, one or more pH buffers should be poured into small specimen cups with lids. Volumes must be sufficient to cover the bottom of the pH sensor to calibrate the sensor properly. Three buffer calibrations are typically performed: 4.01, 7.0, and 10.0.

i. From the main calibration menu, use the down arrow button to scroll down to the pH line and press the Enter <←> button.

ii. Place the rinsed and dried probe into the pH 7 buffer and select "3-point calibration" from the menu; press the Enter <←> button.

iii. Using the key pad, enter the pH of the buffer and press the Enter <←> button.

iv. Observe the pH reading and when it shows no change for at least 30 seconds, press the Enter <←> button to accept the reading.

v. The screen will ask for the pH of the second buffer. Using the key pad, enter 4.01, remove the probe from the pH 7.0 buffer, rinse and dab the probe dry with a Kimwipe, and submerge it in the 4.01 buffer; press the Enter <←> button.

vi. Observe the pH reading and when it shows no change for at least 30 seconds, press the Enter <←> button again to accept the reading.

vii. The screen will ask for the pH of the third buffer. Using the key pad, enter 10.0, remove the probe from the pH 4.01 buffer, rinse and dab dry the probe with a Kimwipe, and submerge it in the 10.0 buffer; press the Enter <←> button.

viii. Observe the pH reading and when it shows no change for at least 30 seconds, press the Enter <←> button again to accept the reading.

ix. Once the third buffer has been accepted, press the <Esc> button twice to exit all of the calibration menus and return to the run screen.

3. ***Measurements:*** Following calibration, make the measurements. Make sure the meter is in the run mode by hitting the <Esc> button until all the probes sensor measurements are displayed. Submerge the probe into the solution to be measured. Keep the probe moving slowly to achieve accurate DO readings. When the readings stabilize, record them on a data sheet or press the Enter <↵> button to record them into memory.

   a. The readings can be recorded into a list of available files that will appear on the screen or, a new file can be set up using the enter information screen. Refer to the YSI 556 MPS Multi Probe Operations Manual (YSI, 2002) for instruction on setting up the files.

   b. If no files exist, the enter information screen should appear. Using the key pad, enter a file name for the measurement. If you would like to enter optional site or sample descriptions, highlight that field and use the keypad to enter the information. Press Enter <↵>, highlight OK, and press Enter <↵> again. If logging one sample, the instrument will confirm that the data point was successfully logged.

   c. Remove the probe from the sample, rinse with RO or DI water, briefly shake excess water off the probe, and place it into the next sample to be measured. Repeat the process above to record the data.

4. ***Viewing and recording stored data and calibration information:***

   a. From the run mode, Press the <Esc> button to go to the main menu. Highlight the File line and press the Enter <↵> button.

   b. From the File menu, select View File and press Enter <↵>.

   c. A list of files will be displayed, including one that ends in .glp. This is the calibration file. Highlight this file and press the Enter <↵> button. Scroll down to the date the calibration was performed and use the right arrow button to scroll the page to the right. Record the information on the DO gain, the DO local gain, conductivity gains from each of the standards measured, as well as the pH gains and offsets that are displayed into the calibration/maintenance log for that instrument. Note: The conductivity gains and the pH gains and offsets are all displayed with the same designation. Be sure to record them as the conductivity standards and pH buffers in the concentration and in the order in which they were measured.

d. Press the <Esc> button to return to the list of files and highlight any files you wish to record on data sheets. Highlight the file name, press the Enter <←> button and scroll to the date of the measurement. The right arrow will take you to other pages with data beyond the date and temperature that is displayed on the first page. When all files are recorded, press the <Esc> button until you return to the Run mode.

Maintenance

1. ***Probe storage:*** Store the probe in its calibration/transportation cup in an upright position in the holding vessels attached to the shelf. A small amount of tap water should be left in the bottom of the calibration/transportation cup to keep the probe environment moist. Do not allow the probe to dry out and do not store the probe with the sensor in water.

2. ***Membrane life:*** According to the instruction manual, the DO membranes will last for weeks but should be changed at least every 30 days, or more frequently if the samples measured have high levels of suspended solids. Follow the instructions above to change out the DO membranes.

If problems occur that this SOP does not address, refer to the YSI 556 MPS Operations Manual (YSI, 2002) or the technical support section at www.YSI.com.

Training

1. All personnel who perform this task should first read this protocol and then operate under supervision until the proper technique and accuracy of measurements is ensured.

Safety

1. pH buffers can be acidic or caustic liquids. Care should be taken to avoid contact with skin or clothing. Should contact occur, quickly flush affected area with water. A sink is present along the wall of the wet lab in 6009, and an eye flushing station is near the sink. The samples measured may be test waters, effluents, discharges, or other water samples that may contain contaminants. Care should be taken to avoid contact with samples by donning gloves, laboratory coats, and other necessary safety equipment.

12.6.3 Procedure for calibration and use of the Orion 9107 BN pH low maintenance triode with the Orion Dual Star pH/ISE benchtop meter EERT SOP: I-006

Purpose

- To ensure accurate and reliable water quality measurements (pH) in aqueous solutions.

Materials

- Orion DUAL STAR pH/ISE benchtop meter or similar
- Orion model 9107BN gel-filled low-maintenance pH/ATC Triode
- Electrode holding arm
- pH electrode storage bottle with electrode storage solution
- pH buffers in small scintillation vials (4.01, 7.0, and 10.0)
- Stir plate with small stir bars
- Rinse bottle with RO or DI water
- Kimwipes
- Safety equipment (rubber gloves, safety glasses, laboratory coat).

Summary

1. Calibrate the Orion pH probe each day before initial use and log calibrations into the calibration book for that instrument. Recalibration may be necessary throughout the day if measurements against standards deviate more than 5%. This probe and meter are typically used for measuring pH and temperature of samples before ammonia measurements are taken.

Procedure

1. ***Meter setup:*** Consult the Thermo Scientific Orion DUAL STAR User Guide (Thermo Fisher Scientific, 2009) for information on setting up the meter's features, including measuring modes, resolution, and units displayed.

   The meter is typically set up for the activation of "hold and ready" function. Once the meter registers a stable reading of the probe, the "hold and ready" function locks in a reading on the display screen.

   a. The display will briefly display RDY and then HLD when the reading is ready to be recorded

   b. The probe is kept in the electrode holding arm to keep it upright in the sample vials on the stir plate.

2. ***Calibration:***

   a. Turn meter on by pressing the power button.

   b. Press the <Channel> button to display the screen for pH measurement.

   c. Press the <F2> button at the top to begin the calibration.

d. Rinse the exterior of the probe with DI or RO water with a squirt bottle and dab dry with a Kimwipe.

e. Place the probe into the pH 7.0 buffer on the stir plate and stir at 500 RPM.

f. Press the <F3> button to start and wait for a stable reading. The meter will lock in the reading once it becomes stable. Press <F2> if the reading is the correct concentration; otherwise, enter the correct concentration with the keypad and then select <F2>.

g. Press <F3> to move to the next buffer, remove the probe from the vial, rinse and dab dry the probe, and place the next buffer (4.01) onto the stir plate.

h. Press <F2> to begin measuring the next buffer; wait for a stable reading.

i. Repeat this procedure for the remaining pH 10.01 buffer. When the final buffer is measured and the correct concentration is entered, select <F3> to end the calibration. The meter will display the calibration average slope and all the millivolt readings from each of the standards. If the slope is acceptable (95–105%), then select <F2> to log/print the calibration and return the meter to the measurement mode. Record the slope and time in the instrument calibration log.

j. Allow the last calibration standard measurement to lock into the meter, remove, rinse, and dab dry the probe. The probe is now ready for measurement.

3. ***Adjusting the calibration curve:*** If the calibration slope is not in the acceptable range, the entire calibration curve can be re-measured or individual points can be re-measured or deleted from the curve. Consult the Thermo Scientific Orion DUAL STAR pH/ISE Meter user Guide (Thermo Fisher Scientific, 2009, pp. 40–41) for instructions.

4. ***Measurements:*** Following calibration, measure the concentration of at least one standard buffer solution for verification. If the value is not within 2% of the expected value, repeat the calibration procedure. Once the verification is complete, measure the various samples by placing at least 10 mL of sample into a clean scintillation vial with a magnetic stir bar, place it on the stir plate, place the probe in the sample, and stir at 500 RPM. Press the <1/measure> button to begin measurement of the sample. When the auto read feature senses a stable reading, the measurement will lock in. Record the pH and temperature measurement on the data sheet for the project. Remove the probe from the sample, rinse with DI or RO water, and dab dry before placing it in the next sample.

5. ***Measuring hints:***

    a. Replace buffers at least once a week
    b. Properly seal vial of buffer after each use to prevent evaporation
    c. Do not allow the probe to dry out between measurements
    d. Rinse the probe between each sample and dab dry with a Kimwipe to prevent cross contamination.

Maintenance

1. ***Probe storage:*** Store the pH probe in the electrode storage bottle containing electrode storage solution in an upright position. Perform periodic inspection of the storage solution; replace it if it has evaporated or has become moldy.

2. ***Cleaning:*** Clean the pH probe by soaking in a pH 4.01 buffer or by soaking in an electrode cleaning solution available from multiple vendors. Thoroughly rinse the probe with DI following cleaning, before storing or using. Calibrate the probe after the cleaning procedures but before using them.

For problems encountered that this SOP does not address, refer to the Thermo Scientific Orion DUAL STAR User Guide (Thermo Fisher Scientific, 2009) and the Low Maintenance Gel-Filled pH Electrodes User Guide (Thermo Fisher Scientific, 2007b).

Training

1. All personnel who perform this task should first read this protocol and then operate under supervision until proper technique and accuracy of measurements is ensured.

Safety

1. pH buffers can be acidic or caustic liquids. Care should be taken to avoid contact with skin or clothing. Should contact occur, quickly flush affected area with water. A sink is present along the wall of the laboratory in building 6000, room 65, and an eye flushing station is near the sink. The samples measured may be pore waters, test waters, effluents, discharges, or other water samples that may contain contaminants. Care should be taken to avoid contact with samples by donning gloves, laboratory coats, and other necessary safety equipment.

12.6.4 Procedure for calibration and use of Orion 9512 HPBNWP Ammonia Probe EERT SOP: I-001

Purpose

- To ensure accurate and reliable ammonia measurements in aqueous solutions.

Materials

- Orion model 9512 HPBNWP high performance ammonia probe
- Orion DUAL STAR pH/ISE benchtop meter or similar
- Magnetic stirrer and small magnetic stir bars
- Bonded membrane caps (Orion #951205) or loose membranes (Orion #951204)
- Electrode internal filling solution (Orion #951202)
- Standard Solution, NH_4Cl, 1,000 mg/L as nitrogen (Orion # 951007) or prepared as described below
- Ionic Strength Adjuster (ISA; Orion #940011)
- Ammonia pH Adjusting ISA (Orion # 951211)
- Rinse bottle with RO or DI water
- Kimwipes
- Safety equipment (rubber gloves, safety glasses, laboratory coat).

Summary

1. Calibrate the ammonia probe each day before initial use and every 2 hours if necessary as determined by checks against standards.

Procedure

1. ***Electrode setup:*** When the electrode is first received or after it has been stored dry, soak the inner body in internal filling solution for at least 2 hours before assembling the electrode. For best results, soak the inner body overnight. Then, follow these steps:

a. Unscrew top cap and remove electrode inner body; drain old filling solution. Set cap with inner body aside carefully.

b. Remove bottom cap from electrode outer body. If using bonded membrane caps, screw cap in end of electrode until finger tight and proceed to step 1.e. If using loose membranes, proceed with instructions in step 1.c.

c. Using flat-end tweezers, carefully grasp a white membrane from between paper separators. Hold the membrane at the edge with the tweezers. Hold the electrode outer body in a free hand, loosely stretch the membrane across the opening in the bottom of the probe, holding the ends against the threads with your thumb and forefinger. Avoid excess handling of the membrane, since this may affect its hydrophobic properties and reduce its life.

d. Replace the cap on the probe and screw it on until it is finger tight. The membrane should be smooth with no wrinkles.

e. Add internal filling solution up to the embossed line on the outside of the electrode outer body. If measuring low ammonia concentrations (e.g., < 0.06 mg/L ammonia nitrogen), the filling solution can be diluted by 1/10 to increase response time.

f. Replace inner body into the outer body and screw on upper cap.

g. Place a finger over the hole in the upper outside of the outer body and shake probe as if it were a clinical thermometer to remove internal bubbles.

h. Record date of membrane and internal filling solution change in the instrument calibration log.

i. Soak the assembled probe in a 10 mg/L NH_4Cl standard for at least 2 hours before attempting calibration and making measurements.

2. ***Preparation of standards:*** A 1,000-mg/L ammonia as nitrogen stock standard can be purchased (Orion #951007) or prepared. Prepare by adding 3.82 g of reagent grade NH_4Cl to 500 mL of Milli-Q DI water in a 1,000 mL volumetric flask, stir to dissolve and dilute to the full 1,000-mL volume with Milli-Q DI water. Make or purchase again before shelf life expiration, approximately six months.

a. Prepare 100-, 10-, 1.0-, and 0.1-mg/L standard solutions by serial dilution from the 1,000-mg/L stock solution using Milli Q water to which 2 mL of ionic

strength adjuster (Orion #940011) has been added to each 100 mL. These can be made in scintillation vials:

1 mL 1,000-mg/L standard + 9 mL ionic adjusted water = 100-mg/L standard
1 mL 100-mg/L standard + 9 mL ionic adjusted water = 10-mg/L standard
1 mL 10-mg/L standard + 9 mL ionic adjusted water = 1-mg/L standard
1 mL 1-mg/L standard + 9 mL ionic adjusted water = 0.1-mg/L standard.

3. ***Perform calibrations using standard solutions:*** For detailed instructions for setting up the Thermo Scientific Orion DUAL STAR pH/ISE Meter consult the Thermo Scientific Orion DUAL STAR User Guide (Thermo Fisher Scientific, 2009).

   a. Turn meter on by pressing the power button.

   b. Press the <Channel> button to display the screen for NH_3 measurement.

   c. Press the <F2> button at the top to begin the calibration.

   d. Rinse the exterior of the probe with DI or RO water with a squirt bottle and dab dry with a Kimwipe.

   e. Place five drops of Ammonia adjusting ISA (Orion #951211) and a small stir bar into the scintillation vial containing the lowest concentration standard and place the vial on the stir plate and submerge the tip of the probe into the sample, while stirring the sample at approximately 500 RPM.

   f. Press the <F3> button and wait for a stable reading. The meter will lock in a reading once it becomes stable. Press <F2> if the reading is the correct concentration; otherwise, enter the correct concentration with the keypad and then select <F2>.

   g. Press <F3> to move to the next standard, remove the probe from the vial, rinse, and dab dry the probe and place the next standard onto the stir plate. Be sure to add five drops of Ammonia pH adjusting ISA (Orion # 951211) and a stir bar to the vial before placing it on the stir plate.

   h. Press <F2> to begin measuring the next standard and wait for a stable reading.

   i. Repeat this procedure for the remaining standards beginning at the lowest and going to the highest concentration. When the final standard is measured and the correct concentration is entered, select <F3> to end the calibration. The meter will display the calibration average slope and all the millivolt readings from each of

the standards. If the slope is acceptable (-54 to -60), then select <F2> to log/print the calibration and return the meter to the measurement mode. Record the slope and time in the instrument calibration log.

j. Allow the last calibration standard measurement to lock into the meter, remove, rinse, and dab dry the probe. The probe is now ready for measurement.

4. ***Adjusting the calibration curve:*** If the calibration slope is not in the acceptable range, the entire calibration curve can be re-measured or individual points can be re-measured or deleted from the curve. Consult the Thermo Scientific Orion DUAL STAR pH/ISE Meter user Guide (Thermo Fisher Scientific, 2009, pp. 40-41) for instructions.

5. ***Measurements:*** Following calibration, measure the concentration of at least one standard solution for verification. If the value is not within 2% of the expected value, repeat the calibration procedure. Once verification is complete, measure the various sample concentrations by placing five drops of the pH ammonia adjusting ISA and a stir bar into each sample in a scintillation vial just before placing the probe in the sample on the stir plate; stir at 500 RPM. Press the <1/measure> button to begin measurement of the sample. When the autoread feature senses a stable reading, the measurement will lock in. Record the measurement on the data sheet for the project. Remove the probe from the sample, rinse with DI or RO water and dab dry before placing it in the next sample.

6. ***Measuring tips:***

a. Rinse the electrode with DI or RO water between measurements.

b. Check the electrode for bubbles on the membrane. If present, bubbles can be removed by shaking the electrode.

c. Magnetically stir all samples during measurement. Magnetic stirrers may generate some heat, and a layer of Styrofoam can be placed below the sample to help limit any change in temperature.

d. The temperature must be consistent for all samples and standards. This ion-selected electrode is not temperature compensated. A 1°C difference will introduce a 2% measurement error.

e. If the electrode response is slow, the membrane may contain a surface layer of contaminants. To restore performance in this case, soak the electrode in DI water for about 5 minutes and then soak in a standard solution for about 1 hour before use or replace the membrane and soak in a 10 mg/L NH_4Cl standard for 2 hours.

For large numbers of "dirty" samples, more than one probe may be assembled and used to reduce or eliminate the waiting time for probe equilibration.

f. If samples cannot be measured on the same day, they can be acidified with 1 M HCl to a pH of less than 2 and held refrigerated for up to 28 days without significant loss of ammonia.

Maintenance

1. ***Electrode storage:*** Between measurements, do not allow the electrode tip to dry. For overnight or week-long storage, the electrode tip should be immersed in a 1,000 mg/L standard solution. For storage over 1 week or if the electrode is stored indefinitely, disassemble completely and rinse the inner body, outer body, and bottom cap with DI water. Allow parts to dry, then reassemble the electrode without internal filling solution or a membrane.

2. ***Membrane life:*** According to the instruction manual, membranes will last from 1 week to several months depending on usage. At ERDC, membranes may last less than 1 day when measuring samples with contaminants or high ammonia levels. Membrane failure is characterized by a shift in the electrode potential, drift, or poor response time. Membrane failure may be apparent on visual inspection as dark spots or discoloration of the membrane or carbonate deposits on the membrane and end cap. Follow the above procedure step 1 (*Electrode Setup*) to replace the membrane and add internal filling solution.

For problems encountered that this SOP does not address, refer to the model 9512 HPBNWP high-performance ammonia probe user guide (Thermo Fisher Scientific, 2007a) and the Thermo Scientific Orion DUAL STAR pH/ISE Meter User Guide (Thermo Fisher Scientific, 2009).

Training

1. All personnel who perform this task should first read this protocol and then operate under supervision until proper technique and accuracy of measurements is ensured.

Safety

1. Ammonia pH adjusting ISA is a highly caustic liquid. Care should be taken to avoid contact with skin or clothing. Should contact occur, quickly flush affected area with water. A sink is present along the wall of room 65, and an eye flushing station is near the sink. The samples handled may be pore water, effluent, discharges, or other water samples that may contain contaminants. Care should be taken to avoid contact with samples by donning gloves, laboratory coats, and other necessary safety equipment.

References

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YSI. 2002. YSI 556 MPS Multi Probe System Operations Manual. YSI Incorporated, Yellow Springs, OH. 153 pp.

A. Testing Protocol 1: 28-Day Chronic Sublethal Sediment Tests Using *Leptocheirus plumulosus*

A.1 Testing Procedures Overview

This protocol provides guidance for conducting 28-day chronic sediment toxicity tests using the estuarine amphipod *Leptocheirus plumulosus*. The protocol is written in basic accordance with the guidance provided in *Methods for Assessing the Chronic Toxicity of Marine and Estuarine Sediment-Associated Contaminants with the Amphipod* Leptocheirus plumulosus (EPA 600-R-01-020) and *Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates* (ASTM 1367-03). The ERDC modified this procedure specifically for evaluating sediments for NOAA. In some instances, reburial was recorded as an endpoint (see test-specific TCTs). In those cases, reburial of surviving adult *Leptocheirus plumulosus* following removal from sediment treatments was assessed. According to ASTM Method E1367-03 (ASTM, 2008) and (U.S. EPA, 1994), this assessment is optional for use in association with the standard 10-day sediment toxicity testing. Use of this approach in association with the standard 28-day protocol is described below.

A.2 Sediment Preparation

A.2.1 Receipt of sediment

Sediments were shipped frozen to the ERDC in a plastic bag. Sediments were kept frozen until testing.

A.2.2 Preparation of sediment

Sediments were prepared for testing following the guidance provided in the latest revision of the sediment preparation SOP entitled *Protocols for Preparation of Spiked Sediment*.

A.3 SOP: Protocol for Preparation of Spiked Sediment

A.3.1 General guidelines

- Prepare all sediments using the *SOP: Protocol for Preparation of Spiked Sediment;* however, do not add oil to the control sediments.

  *Note: Prior to 2014, the ERDC control was not mixed in the stand mixer.

- Prepare each sediment-oil concentration separately. For instructions in cleaning and preparing the equipment, refer to the *Decontamination SOP* in the QAPP.

  *Note: Prior to 2014, the mixing paddle was not rinsed with hexane and dichloromethane (DCM); only acetone was used.

- Store unused prepared sediments in a 1-L glass jars and store in the dark at 4°C (short term) or in freezer at -20°C (long term).

A.3.2 Glassware preparation

Prepare all of the equipment in accordance to the *Decontamination SOP* in the QAPP.

A.3.3 Preparation of sediments

- Allow the sediment to thaw
- Remove all debris (grass, shells, etc.) from the thawed sediment and place in a mixer bowl
- Using a Cuisinart SM-70 7-quart stand mixer, homogenize the sediment by mixing for 2 minutes at low speed (1).

A.3.4 Mixing oil into sediment

- Weigh out the appropriate amount of oil as outlined below:

  Weigh slick oil in a pre-cleaned aluminum weigh boat. Tare a weigh boat and a stainless steel spatula on the top loading balance. Using the stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat. Using the spatula, transfer the oil onto the sediment in the mixing bowl, placing it in several areas around the bowl.

Reweigh the weigh boat and spatula to calculate and record the actual mass of oil transferred.

- Place the sediment from the mixing bowl over the oil, and lower the mixer paddle into the bowl.
- Mix the oil into the sediment at medium speed (5) with a Cuisinart SM-70 7-quart stand mixer. Stop the mixer briefly every 2–4 minutes to scrape the sides of the mixing bowl with a stainless steel spoon.
- Once the mixing is complete, scrape down the mixer paddle with the putty knife to remove all of the excess oiled sediment. Transfer the oiled sediment from the mixing bowl into 4-L glass beakers for storage using a stainless steel spoon. Store sediment in the dark at 4°C until it is ready for test initiation.

A.4 Methods

A.4.1 Collection of testing organisms

1. Obtain *Leptocheirus plumulosus* from in-house cultures. Obtain individuals that are in the 250 – 600 µm size class. Be sure to collect plenty of individuals for the test. To ensure a large selection pool of individuals, obtain an additional 1/3 of the total number of organisms that are need for the test from the in-house culture (see test-specific TCTs).

2. Transfer organisms to a holding container and gradually bring them to testing temperature. *Leptocheirus plumulosus* can easily become trapped in the water surface tension and will float. Use a dropping pipette to break surface tension and allow floating amphipods to swim to the bottom of the container. Supply aeration using an air stone or glass pipette. During the acclimation period, hold the animals at 25°C with a photoperiod of 16:8 hours light:dark. Proceed with laboratory acclimation, at testing conditions, without sediment for ~ 24 hours before initiating the test. This period is designated to gradually acclimate organisms to laboratory/testing conditions and ensure that test organisms are healthy before distribution into test chambers.

A.4.2 Test chamber and sediment preparation

1. Use pre-cleaned 1-L beakers (~ 10 cm diameter) during testing. Prepare five beakers per treatment to use as testing chambers and prepare two additional beakers per treatment to use as "dummy beakers." Treat the dummy beakers the same as the testing chambers, but do not collect biological data from these beakers. One dummy beaker is for measuring pore water quality at day 0 and one dummy beaker is for measuring pore water quality and sediment chemistry at day 28. Do not place organisms in the day 0 dummy beaker. Label each beaker with an assigned tank ID designation.

2. Fill beakers with 175 mL (~ 2 cm) of sediment prepared as described in Section A.3, *Protocol for Preparation of Spiked Sediment*. Add the sediment carefully to minimize contact with the sides of the beakers; level the sediment by tapping the sides of the beakers. Fill chemistry sample jars incrementally.

3. Slowly add the overlaying reconstituted seawater (20 ppt), using a turbulence reducer to minimize re-suspension of the sediment. Fill beakers to the 850-mL line. This will allow room for the additional water added with the organisms at test initiation. Once the organisms are added, fill the beakers to the 900-mL mark.

4. Randomly place all treatment replicates in the specified controlled water bath at 25 ± 1°C and cover each beaker with a plastic cover.

5. After the sediment has partially settled, gently aerate ~ 2 to 3 cm from the sediment surface and allow full settling overnight. Provide aeration using plastic tubing connected to an air supply and inserted into a glass pipette; insert the pipette through a hole in the plastic cover. The aeration rate should be adequate to keep DO at ~ 90% saturation but not disturb or re-suspend the test sediment.

6. Measure pore water quality from a dummy beaker for all treatments at day 0 and at day 28.

7. Take pore water quality measurements by obtaining a sediment sample and placing it in a centrifuge tube. Centrifuge the sediment and sample the pore water from the overlying water in the centrifuge tube.

8. Take 10 mL of overlying water (pore water from the centrifuge tube) and place into a 20 mL scintillation vial. See Section 12.4 for the pore water quality guidelines.

A.4.3 Test initiation

1. Remove and count the dead organisms in the holding containers. If the total mortality/inactivity exceeds 5% at day 0, the entire collection is unsuitable for testing.

2. Measure pore water ammonia concentration and other pore water quality parameters (see Section 12.4) in one dummy beaker from each treatment. Testing cannot be initiated unless total ammonia is less than 60 mg/L.

3. Take overlying water quality measurements (temperature, salinity, pH, and DO) in all chambers and record. Measure and record overlying water total ammonia in one composite sample of all replicates within a treatment. Initiate the test if parameters are within the specified ranges according to the U.S. Environmental Protection Agency guidelines.

4. Place *Leptocheirus plumulosus* neonates (250 – 600 µm) in a culture bowl or counting tray. Count ten organisms randomly into high-density polyethylene (HDPE) cups (counting chambers) so that counts and QA/QC are manageable. Transfer organisms using wide-bore pipettes, fully submerging the pipettes to minimize injury; do not use forceps. Add two counting beakers containing 10 organisms each to each test chamber so that the total number of amphipods per replicate is 20. Only select apparently healthy individuals at random for testing; amphipods typically swim or curl up. Do not use any organisms that are dropped or that contact a dry surface in testing. After all the designated counting chambers are filled, verify that each container has 10 individuals. Include an additional six counting chambers for initial weight measurements. During the addition of the two counting chambers to the test replicates, stop aeration to test beakers and gently pour the two counting chambers into a test beaker. Additionally, ensure that a second technician is present to verify that two counting chambers are added to each beaker and that all individuals are removed from the counting chambers. Use wash bottles containing 20 ppt seawater to dislodge amphipods that stick to the sides of the counting chamber. Submerge floating amphipods using a drop of test water from the test beaker using a pipette.

5. Upon the addition of test organisms, record the time of test initiation on the appropriate bench sheet. Re-supply aeration 1 hour after test initiation to allow time for neonates to burrow.

6. Place 20 neonates from two counting chambers on each of three pre-weighed pans for initial weight determination.

7. Approximately 1 hour following the addition of test organisms, observe chambers for injured amphipods and individuals that did not burrow. At this time, replace injured or

unhealthy individuals, or individuals that failed to burrow, if the response does not appear to be specific to the particular sediment treatment. Use a dropping pipette to submerge floating amphipods.

A.4.4 Test monitoring and maintenance

1. For the first week of testing, observe each test chamber once daily for floating amphipods. Submerge any amphipods observed floating using water droplets dropped gently from a pipette. Record behavioral observations, floaters, and other notes on appropriate test bench sheets.

2. Water quality: Record the exposure chamber temperature (min/max) daily. Record the overlying water temperature, salinity, pH, and DO three times per week on Mondays, Wednesdays, and Fridays before water exchange in at least one replicate per treatment using the provided bench sheets.

3. Exchange the water (400 mL) in all chambers, including dummy chambers, by siphoning the overlying water using a 1.5-cm ID siphon hose and refilling with 20 ppt seawater using a pitcher and a turbulence reducer to minimize sediment disturbance.

4. Add 20 mg of TetraMin to each test chamber by preparing a seawater TetraMin slurry that contains 20 mg of TetraMin per mL. Deliver 1 mL to each chamber three times per week (Mondays, Wednesdays, and Fridays), following water exchange and water quality monitoring. After 2 weeks, deliver 40 mg of TetraMin by adding 2 mL per chamber.

5. Check light cycle and ensure that each test chamber is adequately aerated daily.

A.4.5 Setup of reburial evaluation chambers

If the test requires the evaluation of the reburial endpoint (see test-specific TCTs), add ERDC control sediment to five replicate pre-cleaned beakers. Add overlying water and place in a temperature controlled chamber or water bath on trickle-flow aeration.

A.4.6 Test termination and breakdown

1. Water quality: Record temperature, salinity, pH, and DO in all replicate exposure chambers for the 28-day test and reburial evaluation beakers. Take total ammonia measurements of the overlying water total ammonia in one composite sample of all replicates within a treatment and record. Also, take pore water quality measurements from one dummy beaker for each treatment.

2. Survival, reproduction, and growth endpoint determination

   a. Gently pour off all but 200 mL of overlying water through stacked 8- or 12-in. diameter 0.425-mm and 0.25-mm ASTM testing sieves to isolate adults and neonates. Adults are retained on the 0.425-mm sieve and neonates on the 0.25-mm sieve. Swirl and suspend sediment in the remaining overlying water for easier passing of sediment through the sieves. If using an 8-in. sieve, a 12-in. diameter sieve (1-mm mesh) can be placed over the bucket receiving the waste.

   b. Using a squirt bottle with 20 ppt seawater, transfer the material retained on the 0.425-mm sieve to a counting bowl or tray for examination.

   c. Rinse and consolidate the material on the 0.25-mm sieve, containing the neonates, and transfer it to a 1-L wide mouth glass jar. Add 70% ethanol/Rose Bengal stain solution to the jar until the original volume is doubled.

   d. Count recovered *Leptocheirus plumulosus* adults. Remove obviously dead adults and transfer to a labeled vial containing 70% ethanol and retain under chain of custody (COC). If the reburial endpoint is to be evaluated (see test-specific TCTs), transfer living adults to a labeled 1-L beaker and hold for reburial evaluation. If the reburial endpoint is not being evaluated, transfer living adults to a pan for the evaluation of the growth endpoint.

   e. Remove any neonates contained with the adults and place them into the wide mouth glass jar for that treatment/replicate. If the test includes the reburial endpoint (see test-specific TCTs), place surviving adults from each replicate into a corresponding 1-L reburial evaluation beaker and record the number added and the time. After 1 hour, make observations of the beakers and record the number of animals not buried. Remove unburied animals and record number alive and dead. After all reburial data has been collected and recorded, sieve the adults from the reburial evaluation beakers following guidance provided in steps a–d above. Record the number of adults recovered and place all surviving amphipods from each replicate on a pre-dried/pre-tared pan. Dry in oven at 60°C for 24 hours. If there is no evaluation of reburial (see test-specific TCTs), then place all surviving amphipods from each replicate on a pre-dried/pre-tared pan. Dry in oven at 60°C for 24 hours.

A.4.7 Biomass and weight endpoint quantification

Remove pans from oven and allow them to cool for 1 hour in a desiccator. Record the total weight of each pan. Retain pans with dried animals under COC.

A.4.8 Reproduction endpoint quantification

Allow the stain to interact with the organisms for at least 2 days. Rinse the preserved material with tap water through a 0.25-mm sieve and transfer the material placed in the wide mouth glass jars into an 8-in. culture bowl or counting tray and record the number of neonates recovered. Neonates should be pink in color. It may be necessary to transfer small amounts of the preserved material from the 8-in. culture bowl into a 4-in. culture bowl for observation under a dissecting microscope. Transfer preserved neonates to a 20-mL labeled scintillation vial containing 70% ethanol and retain under COC. Record the number of neonates counted on the appropriate bench sheet.

A.4.9 Acceptability

1. The test is not acceptable if any of the following occur.

    a. Less than 80% survival in the ERDC control/reference (less than 60% survival in any individual replicate) or growth not observed in a control replicate

    b. If DO dropped below 40% saturation in any treatment replicate.

A.4.10 Recording data

1. Record all specified data on the appropriate bench sheets

2. If any parameters are not within the specified range, record on the appropriate bench sheet and make a note in the comments.

Appendix References

ASTM. 2008. Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates. ASTM Method E1367-03 (Reapproved 2008).

U.S. EPA. 1994. *Methods for Assessing the Toxicity of Sediment-associated Contaminants with Estuarine and Marine Amphipods.* EPA/ 600/R-94/025, Office of Research and Development, Washington, DC.

13. Northwest Fisheries Science Center General Laboratory Procedures and Practices

13.1 Introduction

The Northwest Fisheries Science Center (NWFSC) conducted exposure studies with zebrafish, southern bluefin tuna, amberjack, yellowfin tuna, mahi-mahi, and red drum to determine the cardiotoxic effects of *Deepwater Horizon* MC252 crude oil on developing fish embryos [zebrafish were exposed to either Alaska North Slope crude oil (ANSCO) or *Deepwater Horizon* MC252 crude oil]. These tests were often conducted in collaboration with other researchers. The NWFSC General Laboratory Procedures and Practices (GLPP) chapter describes the zebrafish, southern bluefin tuna, and amberjack tests, while other tests are described in the collaborator's chapters of the GLPP (see Section 13.2; *Test Organism Sources and Husbandry*).

13.2 Test Organism Sources and Husbandry

Zebrafish (*Danio rerio*)

Experiments were conducted using embryos from a zebrafish (*Danio rerio*) colony (AB strain) maintained at the NWFSC, in Seattle, Washington. All zebrafish husbandry protocols, including spawning and water quality, are described in Linbo (2009).

Southern bluefin tuna (*Thunnus maccoyii*)

Bluefin tuna embryos were obtained from the Clean Seas hatchery in Arno Bay, Australia. The embryos were collected approximately 4–8 hours after fertilization and then transported to the Lincoln Marine Science Centre in Port Lincoln, Australia, where tests were started immediately upon arrival. The husbandry techniques used by Clean Seas to spawn bluefin tuna are proprietary.

Amberjack (*Seriola lalandi*)

Amberjack embryos were obtained from the Clean Seas hatchery in Arno Bay, Australia. The embryos were then transported to the Lincoln Marine Science Centre in Port Lincoln, Australia, where tests were started immediately upon arrival.

Yellowfin tuna (*Thunnus albacares*)

See *University of Miami Rosenstiel School of Marine and Atmospheric Science (RSMAS) GLPP* for information on yellowfin tuna husbandry and testing protocols.

Mahi-mahi (*Coryphaena hippurus*)

See RSMAS GLPP for information on mahi-mahi husbandry and testing protocols.

Red drum (*Sciaenops ocellatus*)

See *Stratus Consulting Red Drum and Speckled Seatrout GLPP* for information on red drum husbandry and testing protocols.

13.3 Exposure Media Preparations

For testing protocols included in the NWFSC GLPP, exposure media were prepared according to established protocols with slight modifications. See *Protocols for Preparing Water Accommodated Fractions* in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Water accommodated fractions (WAFs) were made using ANSCO and the four *Deepwater Horizon* oil types: Slick A (CTC02404-02), Slick B (GU2888-A0719-OE701), source oil (072610-03), and weathered source oil (072610-W-A). The slight modifications included the following:

- Rather than using a gastight syringe, a Rainin positive displacement pipette with positive displacement tips was used to transfer the liquid oil samples.
- For tests that used the Slick A and B oils, a small amount of the viscous oil was transferred to an amber vial that was then heated in a 65°C water bath for approximately 8 minutes, reducing the viscosity to allow the use of the Rainin positive displacement pipette. This oil was pipetted onto the surface of the blade in a three-speed commercial food blender with a 1-gal stainless steel container, before water was added to mix the oil into the water column.
- For tests in which the high-energy WAFs (HEWAFs) were filtered, the exposure media was put through a 2.7-µm glass fiber filter, followed by a 0.7-µm glass fiber filter.
- The water used in all zebrafish exposure studies was prepared with 1-ppt Instant Ocean Salt mixed with reverse-osmosis deionized treated water. The water used in the southern bluefin tuna and amberjack exposure studies was natural seawater obtained from the Clean Seas hatchery; the water matched the water quality parameters of the seawater in

which the embryos were spawned. The techniques used to treat the natural seawater were conducted by Clean Seas and are proprietary.

13.4 Water Quality Monitoring

Water quality was not measured for zebrafish tests. For other tests, water quality measurements were completed following the guideline in the QAPP and the *Hopkins Marine Station of Stanford University and NWFSC GLPP*.

13.5 Analytical Chemistry

For zebrafish tests, analyses of polycyclic aromatic hydrocarbons (PAHs) and other compounds were conducted by NWFSC (Sloan et al., 2014). For all other tests, analyses of PAHs and other compounds were conducted by ALS Environmental, with some samples analyzed at NWFSC. For the collection procedure, see the QAPP.

13.6 Reporting and Testing Documentation

For zebrafish tests, results were recorded in laboratory notebooks and on Excel spreadsheets. Images, videos, and all electronic data were stored on secure hard drives. For all other tests, reporting of the data followed procedures described in the QAPP.

13.7 General Testing Standard Operating Procedures

13.7.1 Preparation of HEWAF for NWFSC GLPP testing protocols

- Glassware/mixer decontamination procedure

  Zebrafish tests

  Rinse all glassware and HEWAF mixer components with three rinses of acetone, followed by three rinses of dichloromethane (DCM) between each preparation. Allow sufficient time for full evaporation of final solvent rinse. Inspect the inside parts of the blender and wipe down the blades with solvent-soaked Kimwipes. Use gloved hands throughout all preparation steps, and use common-sense laboratory safety for handling solvents, especially DCM. Refer to appropriate material safety data sheets if necessary.

Other tests

Decontaminate glassware following the *Decontamination SOP* found in the QAPP.

- Preparation of oil HEWAF for Alaska North Slope Crude and *Deepwater Horizon* source and artificially weathered source oils

1. Measure appropriate volume of water into pre-cleaned Waring blender (1 or 2 L).
2. Measure desired volume of oil. These oil samples have a low enough viscosity so that a Rainin Displacement Pipette can measure out volumes effectively.
3. Common dilution of oil: 1:10,000 = 100 µL oil into 1 L of water.
4. Seal blender with aluminum-wrapped lid.
5. Blend for 30 seconds on low.
6. Transfer to glass separatory funnel and allow to settle for 1 hour.

- Preparation of oil HEWAF for *Deepwater Horizon* Slick A and Slick B oils

1. Warm an aliquot of the oil at 65°C in a water bath for 8 minutes to decrease the viscosity.
2. Measure the desired volume of oil using the Rainin Displacement Pipette. Dispense oil directly onto blades of pre-cleaned Waring blender.
3. Add the appropriate volume of water into blender (1 or 2 L).
4. Seal blender with aluminum-wrapped lid.
5. Blend for 2 minutes on low.
6. Check the oil solution; if the oil did not blend well, scrape down the sides of the blender with metal spatula and reblend for 1 or 2 more minutes.
7. Transfer to glass separatory funnel and allow to settle for 1 hour.

- Filtering of HEWAF

1. If a filtered HEWAF is required, set up filter apparatus.

2. While running at low vacuum, drain the bottom layer of the settled HEWAF from the separatory funnel through the filtration apparatus, which should be fitted with a Whatman GF/D filter (0.27 mm). Do not drain the top ~ 100 mL of the HEWAF.

3. Remove the filter apparatus and replace the dirty filter with a Whatman GF/F filter (0.7 mm). Pour first filtrate through the second filter using the low vacuum.

13.7.2 General morphological analysis of fish embryos and larvae

1. Assess gross morphological defects by inspecting images and videos captured for each animal. Include an overall assessment of all visible organs, including but not limited to, eye, brain, otic capsule, gastrointestinal tract, urinary pore, notochord, myotomes, finfolds, pigment, lateral line neuromasts. Only record abnormalities (i.e., the absence of a score for a particular structure indicates there is no evidence of a defect).

2. Record axial defects (dorsal or upward curvature of the trunk/tail) and tail finfold defects (reduction, irregularities, blisters) based on a comparison to control animals. Only record abnormalities (i.e., the absence of a score for a particular structure indicates there is no deviation from the controls).

13.7.3 Viewing video files for cardiac endpoint measurements

1. Open the ".MOV" or ".JPG" file in ImageJ (download for free at http://rsbweb.nih.gov/ij/download.html; on a Mac computer, drag and drop the filename onto an alias of ImageJ).

2. If it is a ".MOV" file, use "virtual stack" or convert the file to 8-bit grayscale; if it is a ".JPG," proceed to Step 3.

3. Under "Analyze" on the menu bar, select "Set Measurements." In the pop-up box, check the "Display Label" and "Area" box (deselect any other checked boxes). Type "3" into the box following "Decimal Places."

4. Confirm that the resolution of the file located in the upper left corner of the window is 800 x 600 pixels (red drum) or 640 x 480 pixels (mahi-mahi and amberjack); record the resolution of the file located in the upper left corner of the picture. For a ".JPG" file, record the resolution of the file located in the upper left corner of the picture.

5. Continue to the protocol for specific endpoint to be measured.

13.7.4 Heart rate, rhythm, and heart rate variability measurements: Blue fin tuna, yellowfin tuna, and amberjack larvae

1. Open and prepare the video clip according to *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Play the entire video at half-speed, counting the number of heart beats that occur during the video.

3. Calculate the heart rate in beats per minute.

4. For arrhythmia measurements, scroll slowly through the video, counting the number of individual video frames that encompass the onset of contraction (systole phase) and relaxation (diastole phase) for cardiac chambers. Using the number of individual frames, calculate the mean duration of the systole and phase diastole phase for each fish. The duration of the systole and the diastole phase cannot be determined for yellowfin tuna because of their higher heart rates.

5. For heart rate variability (i.e., beat-to-beat variability), count the number of video frames between the initiation of contractions for an entire video clip (Incardona et al., 2009).

13.7.5 Atrial contraction measurements – Red drum

1. Open and prepare video clips according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the line tool from the tool bar.

3. Scroll through the video. Watch the walls of the ventricle closest to the jaw line (towards where the blood would leave the ventricle) through a couple of heartbeats. Look for indentations or distinguishing characteristics that are visible both during contraction (systolic) and relaxation (diastolic). Pause the video when the atrium is fully contracted and the ventricle is fully dilated/relaxed. Use the line tool to draw a line between the outer edges of the atrium, trying to keep as perpendicular to the atrial walls as possible. Keep the line as close to the jaw line as possible (Figure 13.1).

4. Select “Measure” under “Analyze” in the menu bar. This is the diastolic measurement.

5. Continue scrolling through the video until the ventricle fully contracts and the atrium is relaxed. At full contraction, the entire length of the atrium forms a chute, with approximately the same diameter throughout. Using the same distinguishing characteristics of the atrial walls that were noted in Step 3, draw a line between the outer edges of the atrium. The resulting line should still be roughly perpendicular to the atrial walls.

6. Select “Measure” under “Analyze” in the menu bar. This is the systolic measurement.

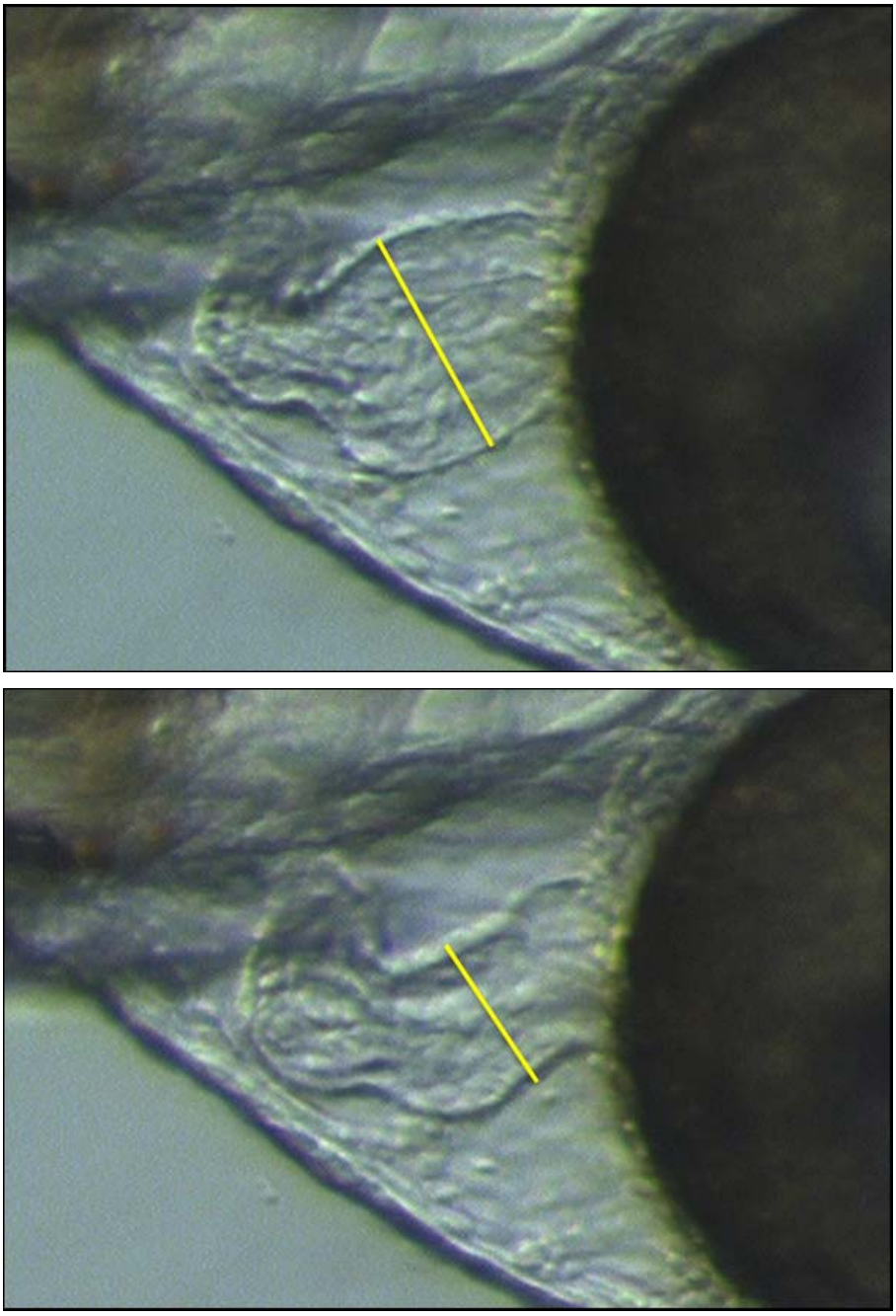

Figure 13.1. Images showing full atrium (top) and contracted atrium (bottom) for diastolic and systolic measurements.

7. Repeat Steps 3 through 6 two more times and save the three sets of measurements for each fish.

   NOTE: Sometimes the atrium is difficult to measure if the orientation of the heart is incorrect or the video is out of focus. In these cases, do not take a measurement.

8. From your three sets of diastolic/systolic measurements, calculate the mean diastolic and the mean systolic measurements for each fish. Then use these means to calculate the percent atrial contractility ((diastolic-systolic)/diastolic * 100) for the fish.

   NOTE: If the first three sets of diastolic and systolic measures differ by greater than 10%, repeat measuring until the values are consistently within 10% of each other. The mean value should be taken from the first three measures within 10%.

9. Take notes throughout the process when necessary.

10. See specific testing protocols and test-specific test conditions tables (TCTs) for number of larvae collected per replicate for each measurement.

13.7.6 Ventricular contraction measurements – Red drum

1. Open and prepare video clip according to *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the line tool from the tool bar.

3. Scroll through the video. Watch the walls of the ventricle closest to the jaw line (towards where the blood would leave the ventricle) through a couple of heartbeats. Look for indentations or distinguishing characteristics that are visible both during contraction (systolic) and relaxation (diastolic). Pause the video when the atrium is fully contracted and the ventricle is fully dilated/relaxed. See Figures 13.2 and 13.3 for diastolic and systolic measurements, respectively.

4. Use the line tool to draw a line between the outer edges of the ventricle, trying to keep as perpendicular to the ventricle walls as possible. Keep the line as close to the jaw line as possible.

5. Select “Measure” under “Analyze” in the menu bar. This is the diastolic measurement.

6. Continue scrolling through the video until the ventricle fully contracts and the atrium is relaxed.

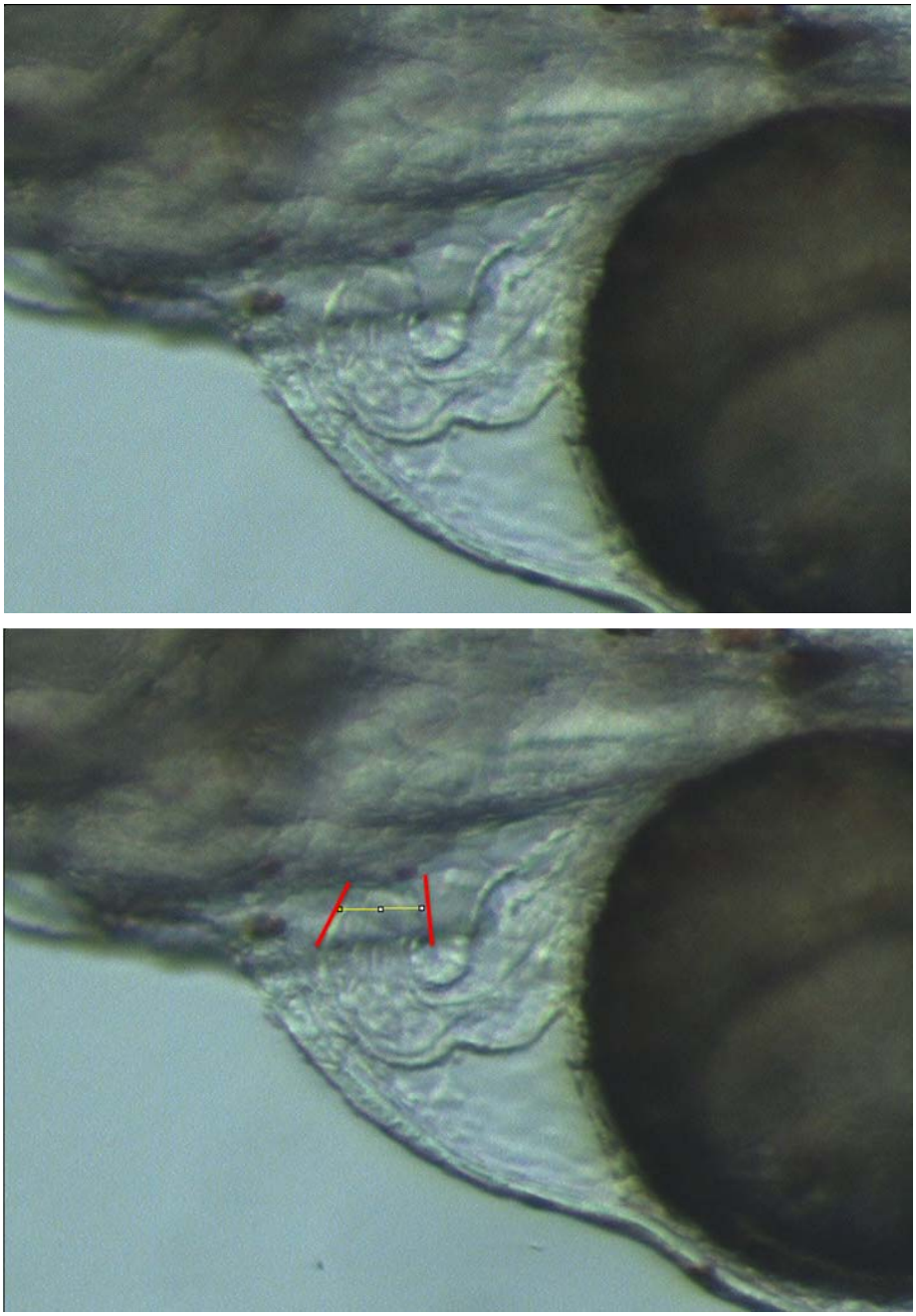

Figure 13.2. Images showing diastolic measurement. Scroll through video until the atrium is fully contracted and the ventricle is relaxed. Draw a line between the outer edges of the ventricle, trying to stay as perpendicular to the ventricle walls as possible. Red lines highlight the angle at which the diastolic measurement line meets ventricle walls.

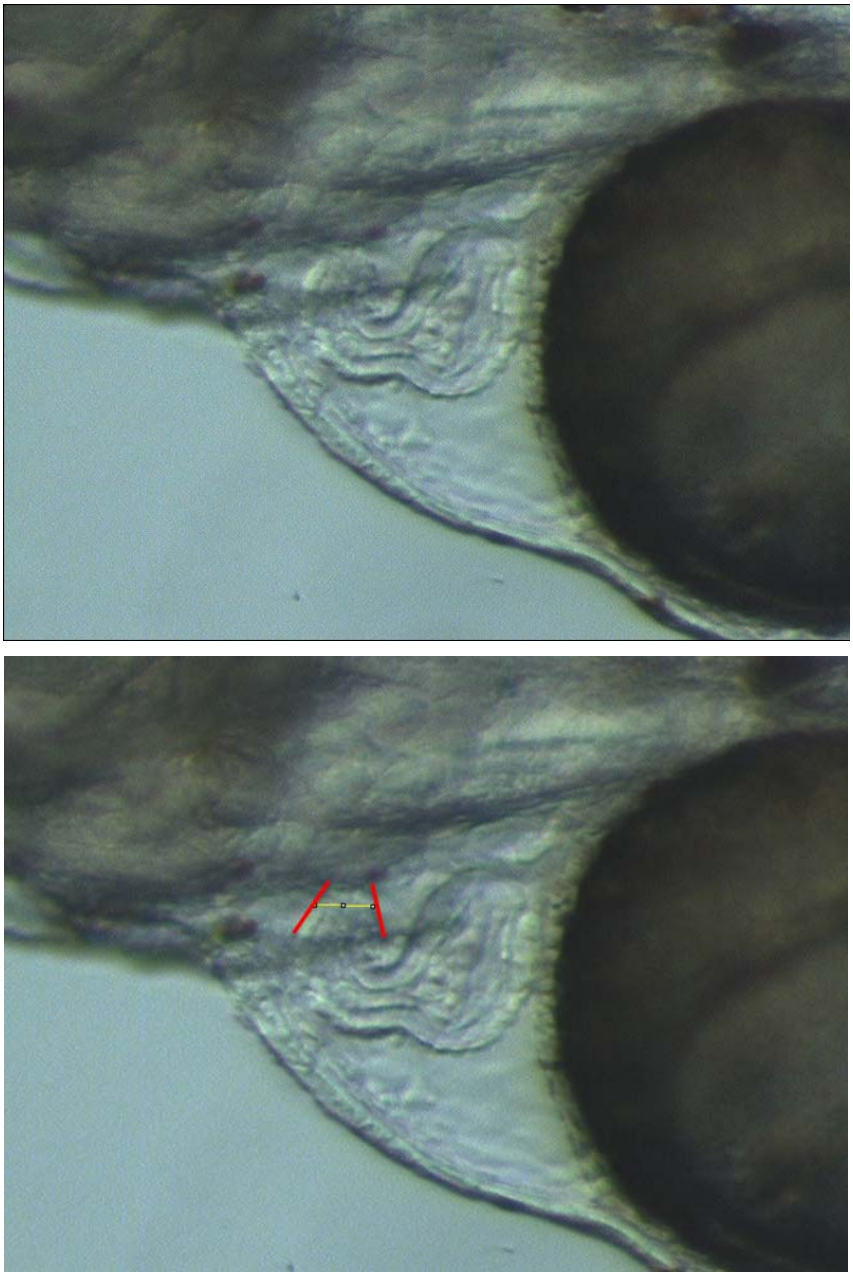

Figure 13.3. Images showing systolic measurement. Scroll through video until the ventricle is fully contracted and the atrium is relaxed. Draw a line between the outer edges of the ventricle, trying to stay as perpendicular to the ventricle walls as possible. Red lines highlight the angle at which the systolic measurement line meets ventricle walls.

7. Using the same distinguishing characteristics of the ventricle walls that were noted in Step 3, draw a line between the outer edges of the ventricle. The resulting line should still be roughly perpendicular to the ventricle walls.

8. Select "Measure" under "Analyze" in the menu bar. This is the systolic measurement.

9. Repeat Steps 3 through 8 two more times and save the three sets of measurements for each fish.

   NOTE: Sometimes pigment or the atrium may obstruct the ventricle wall. In these cases, do not take a measurement for the fish.

10. Transfer measurements (three sets of diastolic and systolic measurements for each fish) to an Excel spreadsheet. For each fish, calculate the percent ventricular contractility using the following equation: (diastolic-systolic)/diastolic * 100).

    NOTE: If the first three sets of diastolic and systolic measurements differ by greater than 10%, repeat the measures until measurements are consistently within 10% of each other. The mean value is taken from the first three measures within 10% of each other.

11. Calculate the means of the diastolic and systolic measurements for each fish. Use the individual fish median to calculate the mean ventricular contraction for a replicate beaker (equation in Step 10).

12. Take notes throughout the process, if necessary.

13. See specific testing protocols or test-specific TCTs for the number of larvae collected per replicate for each measurement.

13.7.7 Pericardial area measurements (as defined in Incardona et al., 2014 and Incardona and Scholz, 2015)

1. Open and prepare video clip or photo according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the free draw tool from the tool bar.

3. Outline the pericardial sac. This area will change, sometimes drastically, depending on the presence or absence of edema and on the severity of edema. For the analyses of red drum images, note that the area of the pericardial sac may exceed the visual field in severely edematous larvae. Make a note of the excluded area with appropriate label

options: "Ventral Cutoff," "Dorsal Cutoff," or "Ventral/Dorsal Cutoff." For mahi-mahi and amberjack analyses, no areas should extend past the oil droplet present at the posterior end of the yolk sac.

a. ***In a fish without edema,*** the outline typically encloses the area anterior to the yolk sac and ventral to the lower jaw (see Figures 13.4 and 13.5).

b. ***In a fish with edema,*** the outline encloses a fluid-filled area that can be seen pushing against the yolk sac. For red drum analyses, sometimes the yolk sac is pushed outside the frame of the picture. Also note that in some red drum, the pericardial sac may curve under the yolk and out of visual range, in which case the label "Ventral Cutoff" should be applied (Figure 13.4). For mahi-mahi and amberjack, only, draw outlines within the boundaries of the yolk sac if a sharp, angular, defined, dark line is present pushing against the yolk sac (Figure 13.5).

4. After the area has been drawn on the image, select "Measure" under "Analyze" in the menu bar.

5. After all measurements have been taken, transfer data to an Excel spreadsheet.

6. Remember to take succinct notes throughout the process, if necessary (e.g., "blurry" for an out-of-focus image).

7. See specific testing protocols or test-specific TCTs for number of larvae collected per replicate for each measurement.

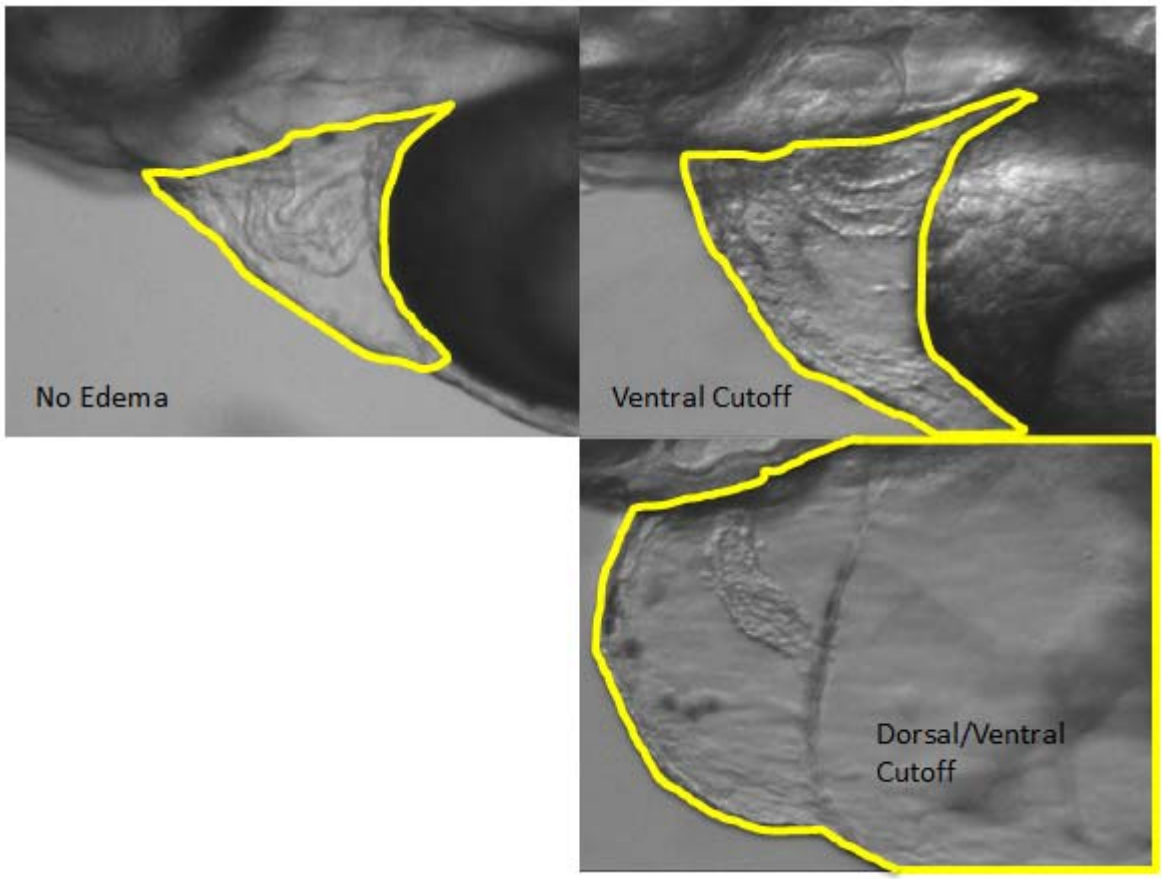

Figure 13.4. Images showing examples the pericardial measurement of red drum with no edema (upper left) and fish with two different severities of edema (upper and lower right).

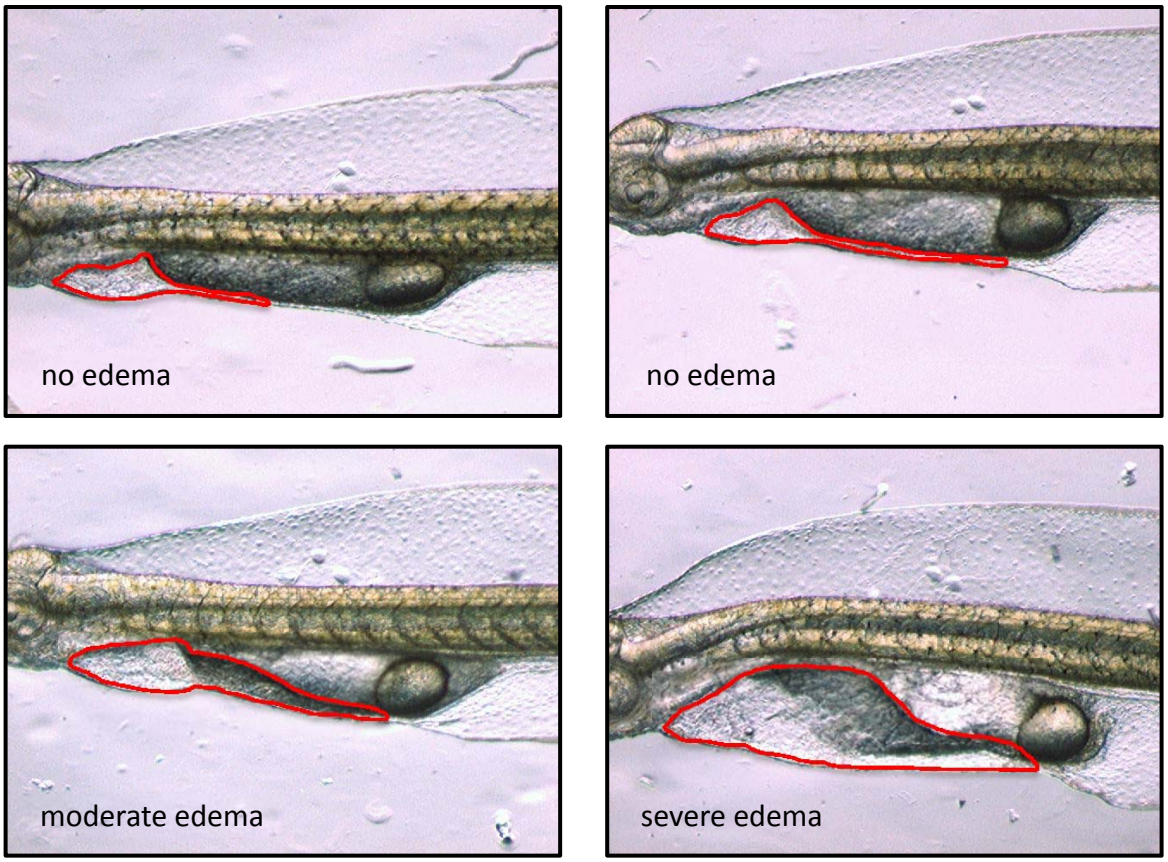

Figure 13.5. Images showing examples the pericardial measurement of mahi-mahi with no edema (upper row) and fish with two different severities of edema (lower row).

13.7.8 Atrioventricular angle measurements of red drum larvae

1. Open and prepare the video clips according to *the Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the angle tool from the tool bar.

3. Scroll through the video and pause the video when the following conditions have been met: the atrium is full, a narrow chute has formed through the ventricle, and the heart has pinched between the atrium and ventricle. For consistency, use the frame where the

atrium is at its fullest right before it starts to contract. Scroll until the atrium just starts to contract and reverse one frame (Figure 13.6).

4. For red drum, start the line by clicking on the outer dorsal edge of the heart, where the ventricle and bulbus arteriosus meet at a pinch. Complete the first segment of line by clicking on the outer dorsal edge of the heart where the AV node is located. Finally, create the second segment of line (and therefore an angle between the two lines) by clicking on the outer dorsal edge of the heart where the atrium and sinus venosus meet (Figures 13.7 and 13.8). For mahi-mahi, draw a line through the center of the ventricle, starting at the anterior end of the ventricle and moving toward the AV node. Stop drawing your line and VISUALIZE an additional line being drawn through the center of the atrium (moving from posterior toward anterior). Now with the visualized atrium line in mind, continue the line that you are drawing through the ventricle by extending it until it would meet the visualized line (even if it extends beyond the wall of the ventricle). At this point, change directions (create the angle) and extend the line through the lumen of the atrium.

   NOTE: If the fish's head is angled downward relative to the viewer, then the measured AV angle will decrease. An indication of this is when the atrium overlaps the ventricle. In Figure 13.8, an example is shown. The yellow outlines the ventricle and the red outlines the anterodorsal edge of the atrium. In this case, the fish cannot be used for the AV angle measurement.

5. Select "Measure" under "Analyze" in the menu bar.

6. Repeat Steps 2 through 4 three times and save the mean value.

   NOTE: If the first three measurements differ by greater than 10%, repeat the measures until the values are consistently within 10% of each other. Take the mean value from the first three measurements that are within 10% of one another.

7. After all measurements have been taken, transfer data to an Excel spreadsheet.

8. Calculate the mean AV angle measurements for each fish.

9. Remember to take notes throughout the process if necessary.

10. See specific testing protocols or test-specific TCTs for number of larvae collected per replicate for each measurement.

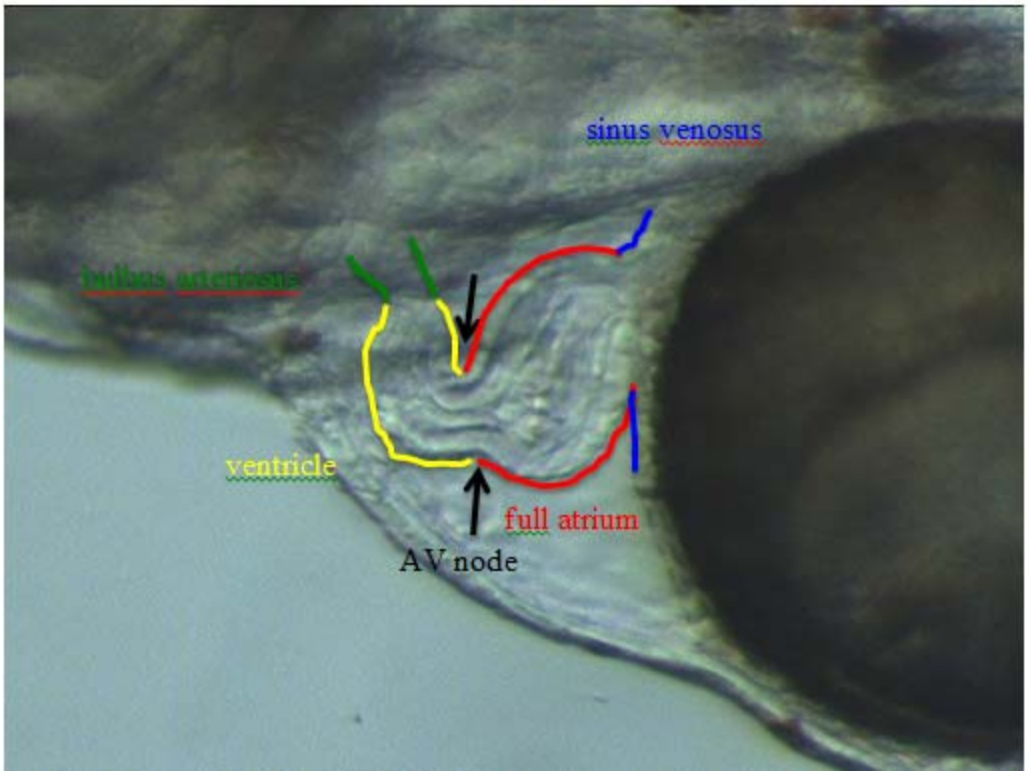

Figure 13.6. Image showing an example frame where the atrium is full, a narrow chute has formed through the ventricle, and the heart has pinched between the atrium and ventricle. This image was obtained by scrolling through the video until the atrium just started to contract and then reversing one frame.

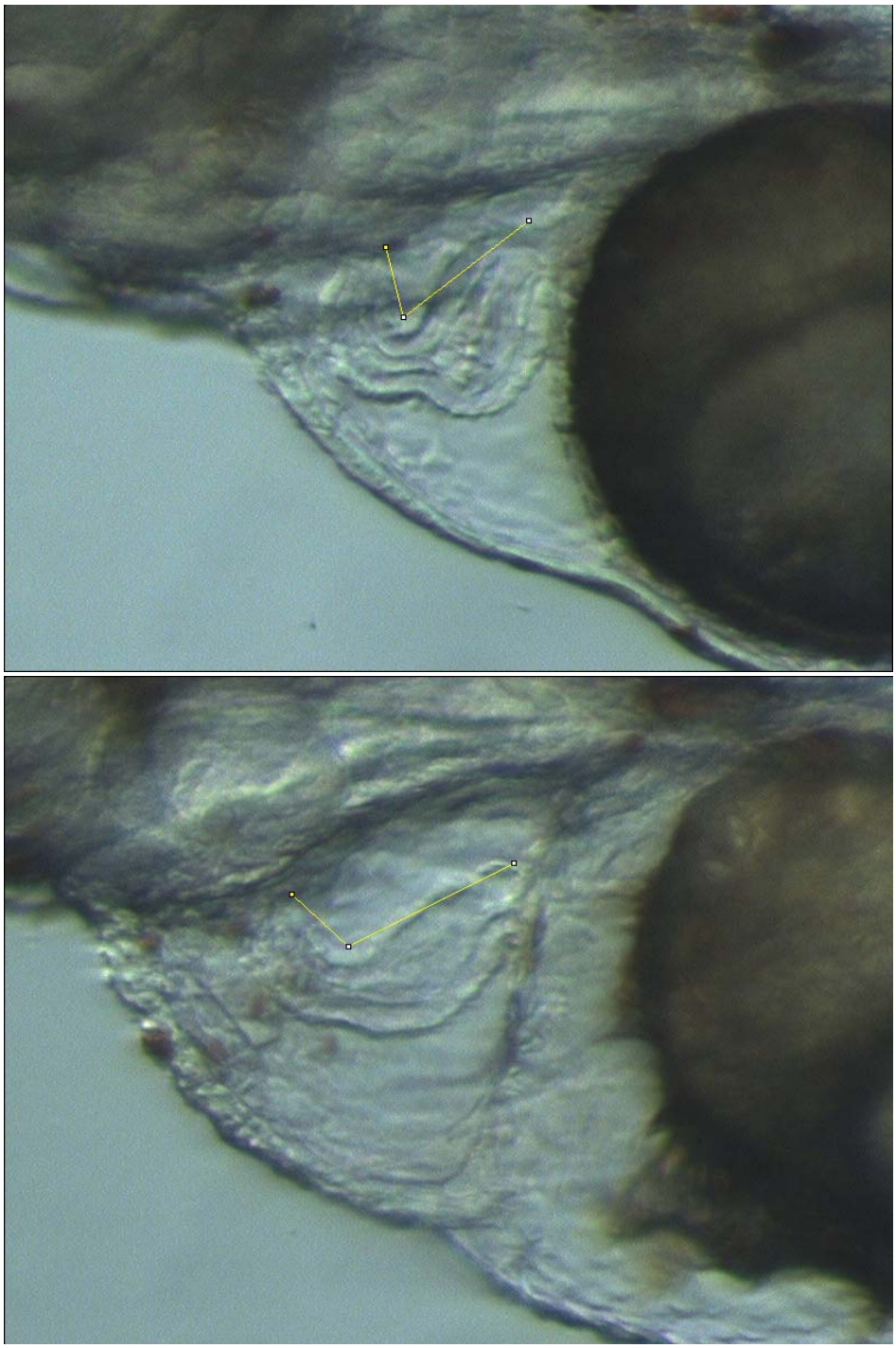

Figure 13.7. Example images of the AV measurement.

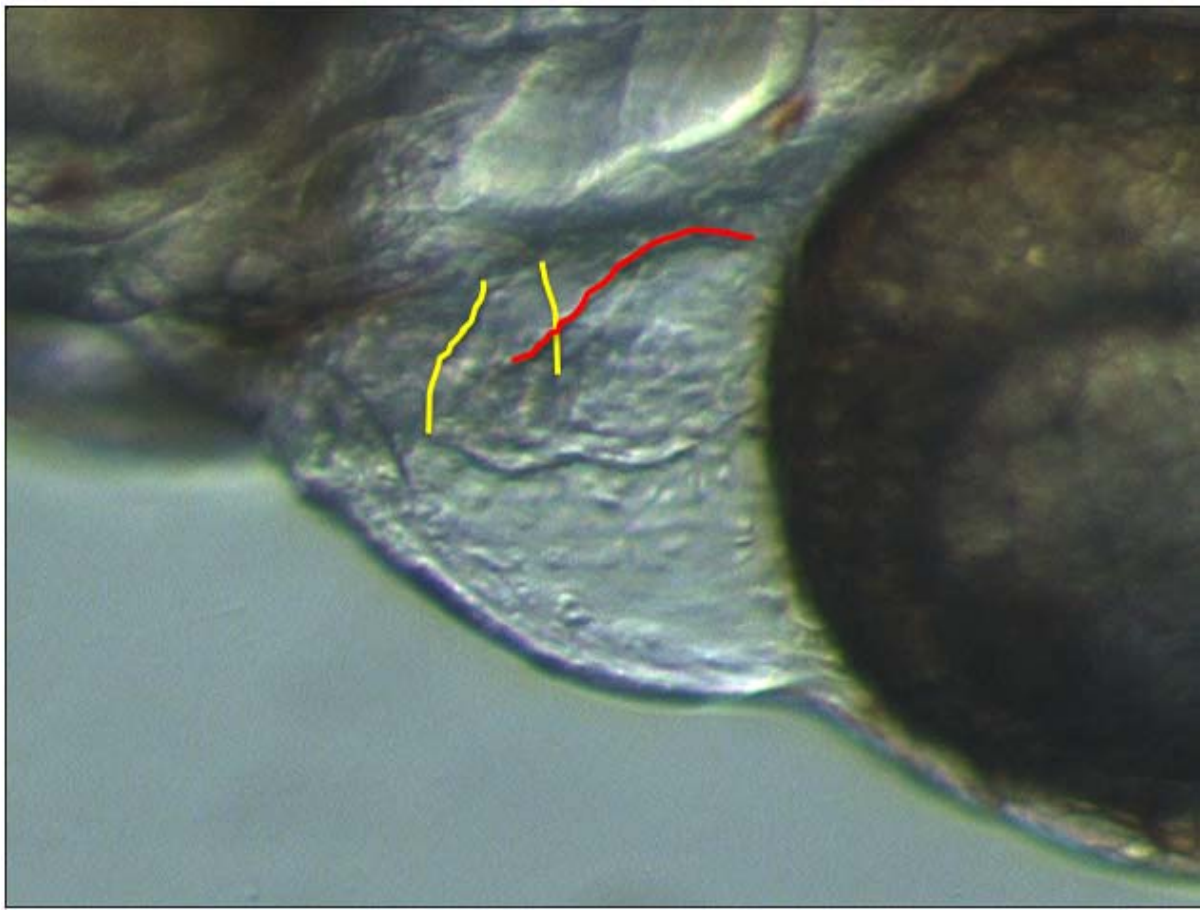

Figure 13.8. Example image where fish is tilted in video and thus AV measurement from the video is not possible because the ventricle and atrium overlap, biasing the measurement. Yellow lines show the outline of the ventricle and the red line outlines the anterodorsal edge of the atrium to show overlap.

13.7.9 Identifying presence or absence of edema (as defined in Incardona et al., 2014 and Incardona and Scholz, 2015)

1. Open and prepare the video clip according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Examine the shape of the yolk sac and pericardial area.

3. Record “edema” or “no edema” based on the definitions below.

Record "no edema" if the anterior portion of the yolk sac is smooth and rounded with a bullet-shaped tip and if there are no obvious indentations on the yolk sac because of pressure from fluid buildup in the pericardial area. See examples below.

Record "edema" if the anterior portion of the yolk sac is concave or pushed to a sharp point or if indentations indicated by dark, angular lines are seen pushing on the yolk sac because of pressure from fluid buildup in the pericardial area (Figure 13.8).

Note: There is a range of yolk sac shapes for normal (non-edema) fish. Sometimes, the yolk sac does not have a perfect rounded, bullet shape, but the fish is still within the range of normal. The rounded, bullet shape can be a bit blunt or can come to a semi-point and still be considered normal (Figure 13.9).

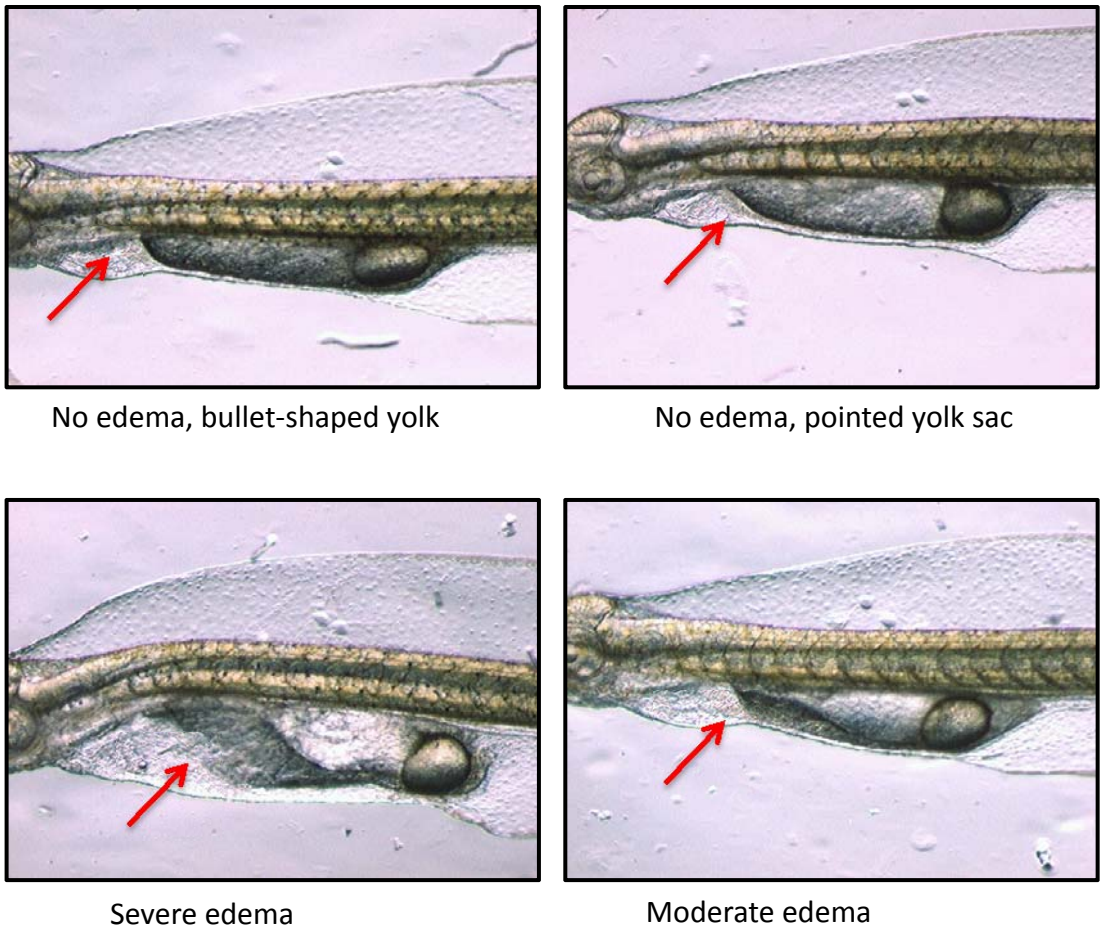

Figure 13.9. Example images of mahi-mahi fish with and without edema.

13.7.10 Atrial yolk mass gap

1. Open and prepare video clip according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP.

2. Select the line tool from the tool bar.

3. Play the video and pause the video when a line becomes visible at the posterior edge of the atrium where it connects to a wall of blood/fluid anterior to the yolk sac. This is typically when the posterior part of the atrium is in contraction.

4. Measure the thickness of this wall of blood/fluid using the line tool. To be consistent, start the line where the atrium meets the wall, centered between the dorsal and ventral sides of the atrium. Draw your line through the wall of blood/fluid until it meets the yolk sac. See examples below (Figure 13.10).

5. Select "Measure" under "Analyze" in the menu bar.

6. Repeat Steps 4 through 8 three times and save the mean value.

   NOTE: If the three measurements differ by greater than 10%, repeat the measures until measurements are consistently within 10% of each other. The mean value is taken from the first three measures within 10% of one another.

7. After all measurements have been taken, transfer data to an Excel spreadsheet.

8. Remember to take notes throughout the process if necessary.

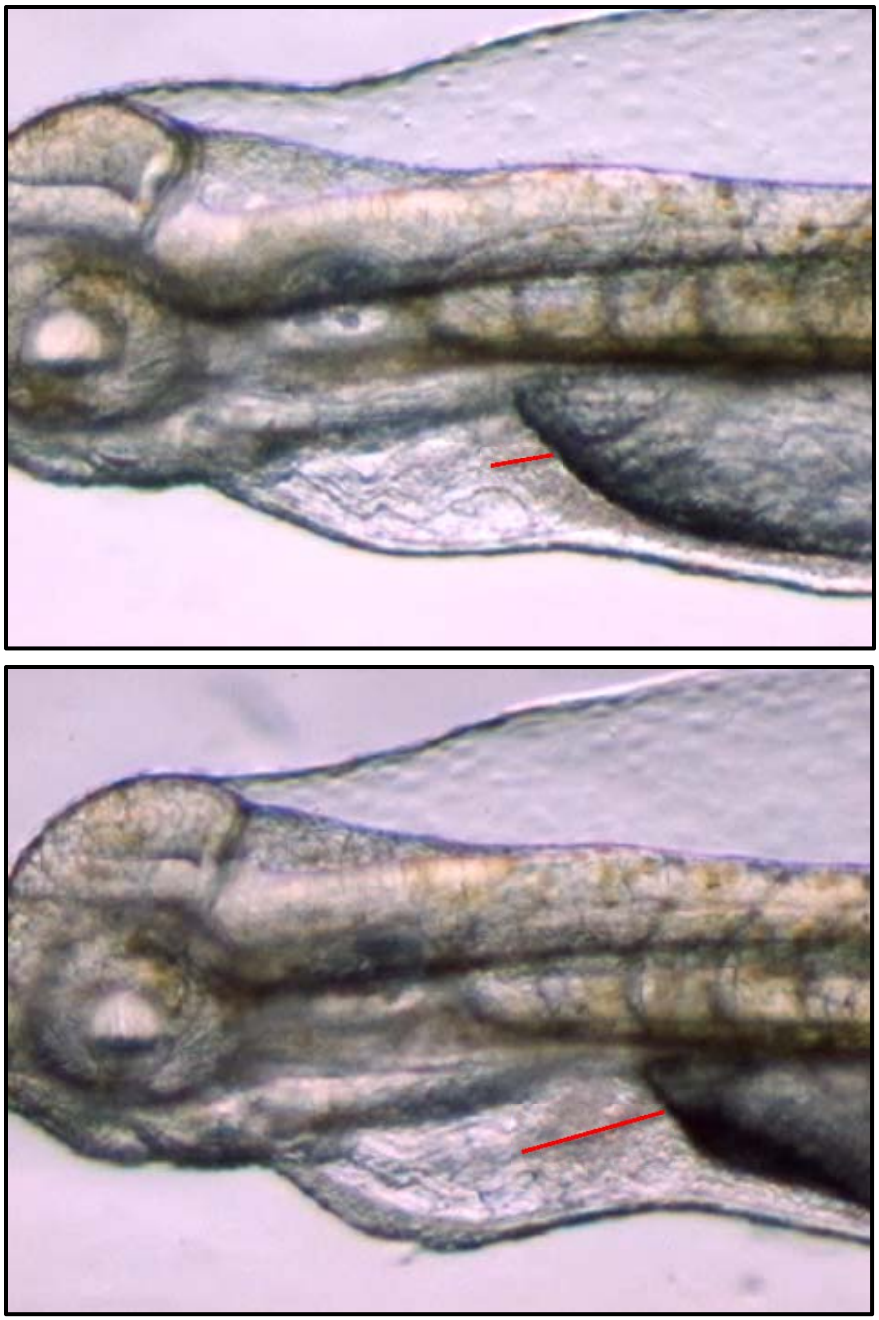

Figure 13.10. Example images of atrial-yolk mass gap measurement. The red lines measure the width of the edema space located posterior to the atrium and anterior to the yolk sac. The video should be paused when the most posterior part of the atrium is contracting.

13.7.11 Molecular assessment of crude oil cardiotoxicity in larval fish

Total RNA extraction

1. To clean homogenizer between samples, prepare three Eppendorf tubes (1.5–2 mL) for each sample, one with 1 mL of RNaseZap Solution (Ambion, USA) and two with 1 mL of nuclease-free water.

2. Clean homogenizer (e.g., Ultra-Turrax T8; Kia, USA) by running at high speed in RNaseZap Solution and 2x water washes for 10 seconds each. Dry with a Kimwipe between washes.

3. Remove sample tube from -80°C freezer, weigh, and record weight in grams (e.g., 1.722 g).

4. Add 800 µL of TRIzol (Invitrogen, USA) or TRI-Reagent (Sigma, USA) to cryovial and homogenize for 10 seconds. Thoroughly clean homogenizer between samples (see Steps 1 and 2 above).

5. For samples snap-frozen in 2-mL Eppendorff SafeLock tubes: add stainless steel bead (Qiagen) and 800 µL of TRIzol or TRI-Reagent. Homogenize using TissueLyser (Invitrogen).

6. Following homogenization, let the samples stand at room temperature for ≥ 5 minutes (up to 6 hours).

7. Add 80 µL of BCP (1-Bromo-3-Chloropropane; Sigma-Aldrich, USA) to each sample, shake vigorously for 15 seconds, incubate at room temp for 3 minutes, and then spin: 12,000 g at 4°C for 15 minutes.

8. During the centrifugation step, label one 1.5-mL Eppendorff tube per sample and place the tube on ice.

9. Remove one sample at a time from chilled 4°C centrifuge, taking care to not disturb phase-separated solution, and transfer the supernatant (~ 500 µL) to a chilled 1.5-mL Eppendorff tube via pipette (e.g., Rainin P200). Avoid touching the interphase; leave a small visible layer of supernatant above the interphase.

10. Return the labeled Eppendorff tube containing the supernatant (RNA) to ice; proceed to the next sample.

11. Purify and on-column the DNAse supernatant (i.e., total RNA) using a Direct-zol RNA MiniPrep Kit (Zymo Research, USA), per the manufacturer's instructions, as follows:

    a. Mix an equal volume of supernatant with absolute ethanol and vortex
    b. Load 700 µL of mixture onto column and spin ("soft"): 16,000 g for 1 minute
    c. Repeat to finish loading all of the supernatant/ethanol mixture
    d. Add 400 µL of RNA Wash Buffer and spin: 16,000 g for 1 minute
    e. Add 80 µL of DNAse master mix directly to the column

        i. DNAse I = 5 µL per sample
        ii. 10x DNAse I Reaction Buffer = 8 µL per sample
        iii. Nuclease-Free Water = 3 µL per sample
        iv. RNA Wash Buffer = 64 µL per sample

    f. Incubate at 37°C on a heat block for 15 minutes and then spin at 16,000 g for 30 seconds
    g. Add 400 µL of RNA PreWash to the column and spin (16,000 g for 1 minute); empty the effluent
    h. Repeat: add 400 µL of PreWash and spin (16,000 g for 1 minute); empty the effluent
    i. Add 700 µL RNA Wash Buffer and spin (16,000 g for 1 minute); empty the effluent
    j. Spin the column for an additional 2 minutes at 16,000 g to dry
    k. Transfer the column to a new, labeled, RNAse-Free tube
    l. Add 26 µL directly to the column (1 tip per sample)
    m. Incubate at room temperature for 1 minute, then spin ("soft") at 16,000 g for 1 minute
    n. Remove the column and save until RNA quantity is verified
    o. NOTE: Alternative DNAse treatment is available.

12. Quantify all samples using NanoDrop 1000 (Invitrogen, USA)

    a. Record quantity (ng/µL) and purity ratios (260/280 nM and 260/230 nM)

13. Begin cDNA synthesis or store RNA (25 µL) on ice overnight in a refrigerator at 4°C.

cDNA Synthesis

Option 1 (High-Capacity RNA-to-cDNA Kit):

1. Prepare cDNA synthesis reaction ON ICE.

2. Calculate the RNA volume need for 2,000 ng per sample (i.e., 2000/[RNA concentration]).

    a. NOTE: cDNA synthesis can be performed using lower RNA input (e.g., 200 ng or 50 ng), depending on the amount of RNA available (i.e., individual embryo extractions generally permit 50–100 ng RNA input).

    b. All calculations below are based on 2,000 ng per sample input, which results in a final cDNA concentration of [100 ng/µL].

    c. All calculations below can be modified according to the RNA input used (experiment specific) by adjusting the stock cDNA concentration accordingly (e.g., if only 50 ng of RNA was used, then the stock cDNA is [2.5 ng/µL].

        i. NOTE: Buffering cDNA with tRNA is not done for individual embryo cDNA because the stock concentration (or 1:2) is required for qPCR.

3. Calculate the water volume needed for a final volume of 10 µL (i.e., 10 µL – (2000/[RNA])); the reaction can be scaled to 2x (e.g., final volume of RNA + water = 20 µL).

4. Calculate the volume of master mix needed for the desired number of reactions as follows:

    a. 10x Buffer: 1x = 2 µL (2x = 4 µL)
    b. 10x Random Primers: 1x = 2 µL (2x = 4 µL)
    c. 25x dNTP Mix: 1x = 0.8 µL (2x = 1.6 µL)
    d. Reverse Transcriptase (RT): 1x = 1 µL (2x = 2 µL)
    e. RNAse-Free Water: 1x = 4.2 µL (2x = 8.4 µL).

5. In strip-tubes or a 96-well plate, combine the RNA and water to a final volume of 10 µL for 1x or 20 µL for 2x reaction size.

6. Add 10 µL of master mix to each well for 1x reaction or 20 µL master mix to each well for 2x reaction.

7. For negative RT (–RT) control, combine 0.2 µL of each sample and add all master mix constituents, except RT enzyme. NOTE: be sure to add water to make up the appropriate volume.

8. Seal, vortex, and spin down.

9. Run on a standard thermal cycler according to manufacturer's conditions:

    a. 25°C for 10 minutes
    b. 37°C for 120 minutes
    c. 85°C for 5 minutes
    d. 4°C bath.

Option 2 (SuperScriptIII Kit):

1. Use a SuperScriptIII Reverse Transcription Kit (Invitrogen, USA) with oligo dT_{20} primers to synthesize [100 ng/µL] complementary DNA (cDNA), per manufacturer's instructions.

2. Use post-DNAse quantifications (ng/µL) to calculate the RNA and water volumes required:

    a. For half reaction (10 µL):

        i. RNA volume (µL) = 1000 ng / sample quantity (ng/µL)
        ii. Water volume (µL) = 3 µL – X µL RNA
        iii. 0.5 µL Annealing Buffer + 0.5 µL oligo dT_{20} + 5 µL 2x Buffer + 1 µL SSIII.

    b. For full reaction (20 µL):

        i. RNA volume (µL) = 2000 ng / sample quantity (ng/µL)
        ii. Water volume (µL) = 6 µL – X µL RNA
        iii. 1 µL Annealing Buffer + 1 µL oligo dT_{20} + 10 µL 2x Buffer + 2 µL SSIII.

    c. NOTE: Reaction can be scaled to 2x.

3. For –RT control, combine 0.2 µL of each sample and add all constituents except SSIII.

4. Store strip-tubes or 96-well plate at 4°C overnight (in cycler or on ice in fridge).

Standard curve generation

1. Determine the volume required to pool for "standards" (e.g., 100/10/1/0.1 ng or 20/10/5/2.5 ng):

    a. Calculate the total µL needed to run a total number of target genes (e.g., 15 genes)

        i. 2 µL per replicate x 2 technical replicates = 4 µL of each standard (STD) needed per gene

ii. 4 µL per STD for 15 genes = 60 µL needed for each STD (30 µL per duplicate well)

iii. Add the volume needed for serial dilution, plus ~10% for "slop:"

1. Log_{10} requires 7 µL be taken for each serial dilution, so make 70 µL per STD
2. Log_2 requires 60 µL be taken for each serial dilution, so make 130 µL per STD

iv. Multiply the total volume needed for top STD (e.g., 100 or 20 ng) by the concentration of cDNA required (e.g., [50 ng/µL] or [10 ng/µL]; NOTE: assays use 2 µL of cDNA):

1. For 100 ng top STD (Log_{10}): 3,500 ng of cDNA are needed in 70 µL
2. For 20 ng top STD (Log_2): 1,300 ng of cDNA are needed in 130 µL

v. Divide the total number of nanograms needed by the concentration of stock cDNA ([50 ng/µL]), then divide that number by the total number of samples to be included in the STD curve pool (e.g., 20 samples available across control and treatment replicates):

1. For 100 ng, top STD (Log_{10}): 65 aliquots of 50 ng/µL are needed

    a. Pool 3.5 µL from each of 20 samples into a final volume of 70 µL

2. For 20 ng top STD (Log_2): 26 aliquots of 50 ng/µL are needed

    a. Pool 1.3 µL from each of 20 samples into a final volume of 130 µL

vi. Start with top STD and dilute serially (1:10 or 1:2) into 3–4 subsequent tubes:

1. For 100 ng top STD (Log_{10}): Add 7 µL into 63 µL water for each dilution
2. For 20 ng top STD (Log_2): Add 65 µL into 65 µL water for each dilution

vii. Load duplicates of each STD into wells A1–A12 on cDNA Plate (if applicable).

"cDNA Plate" generation

1. Dilute stock tRNA 1:100 in water (to [100 ng/µL]; "tRNA-water")
2. Dilute stock cDNA ([100 ng/µL]) 1:1 with tRNA-water
    a. Add 20 µL tRNA-water into each 20 µL cDNA reaction well = [50 ng/µL]
3. Seal, vortex, and spin down the stock cDNA plate
    a. Each well now contains 40 µL of [50 ng/µL] cDNA
4. Label individual Eppendorff tubes for each cDNA sample (including RT)
5. Dilute cDNA 1:5 (20 ng assay) or 1:50 (2 ng assay) with tRNA-water
    a. 20 µL [50 ng/µL] cDNA into 80 µL tRNA-water for 100 µL cDNA at [10 ng/µL]
    b. 2 µL [50 ng/µL] cDNA into 98 µL tRNA-water for 100 µL cDNA at [1 ng/µL]
6. Vortex and spin down each individual Eppendorff tube containing diluted cDNA
7. Split diluted cDNA volume in Eppendorff tube (100 µL) into 2 wells on 96-well plate
    a. If running an STD curve, load STDs first (A1-A12), then individual assays (B1 onward)
        i. See "Standard Curve Generation," above
    b. If not running STD curve, load individual assays starting at well A1
    c. Load 50 µL per technical replicate into adjacent wells (A1 + 2, A3 + 4, A5 + 6, etc.)
    d. Treat the RT sample the same and load diluted –RT into two wells after last treatment sample
    e. Add 50 µL water into the final two wells after –RT wells for a "no template control" (NTC).

"qPCR Plate" generation

1. Dilute the gene-specific primers (F & R) from 100 µM stock to the desired assay concentration:

    a. For 150 nM: 3 µL of 100 µM primer into 97 µL water for 100 µL at 3.75 µM
    b. For 250 nM: 5 µL of 100 µM primer into 95 µL water for 100 µL at 5 µM

2. Generate master mix based on the number of wells used on the cDNA Plate (see above):

    a. Water = 2 µL each
    b. SYBR Green = 5 µL each
    c. Forward Primer (3.75 or 5 µM) = 0.5 µL each
    d. Reverse Primer (3.75 or 5 µM) = 0.5 µL each
    e. cDNA (1 ng/µL) = 2 µL each

3. Load 8 µL of gene-specific master mix into the required number of MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems, USA) wells using electronic dispensing pipette (e.g., Rainin E4 XLS).

4. Add 2 µL of diluted cDNA from the cDNA Plate (see above) into each corresponding well using a 12-channel pipette, for a 10 µL total reaction volume. Repeat if multiple genes can be run on the same plate (e.g., if total wells on the cDNA Plate is less than 24 or 48, or A1–B12 or A1–D12).

5. Seal the qPCR Plate with MicroAmp Optical Adhesive Film (Applied Biosystems, USA).

6. Vortex for 30 seconds (Level 6).

7. Centrifuge at 3,000 g for 30 seconds.

8. Load onto a Viia7 Real-Time Detection System (Applied Biosystems, USA).

9. Use manufacturer's pre-set thermal cycling conditions for either fast or slow chemistry.

References

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A. Testing Protocol 1: Morphological Assessment of Crude Oil Cardiotoxicity of Zebrafish Embryos

A.1 Test Preparations

1. Prior to beginning toxicity tests, all glassware was cleaned by triple rinsing with acetone and DCM and allowed to dry between rinses and before use.

2. Adult zebrafish were spawned according to standard procedures (Linbo, 2009).

3. Desired WAFs were prepared on the morning of the exposure with slight modifications from the QAPP (see Section 13.2.2, *Exposure media preparations*).

4. Subsamples of WAFs were placed in 250 mL pre-cleaned, amber glass bottles for chemical analysis by NWFSC. For preservation, 10% methyl chloride was added to each water sample and kept at 4°C.

A.2 Embryo Exposure

1. Embryos were collected shortly after fertilization.

2. Embryos were exposed at either 4–6 hours (sphere to shield developmental stage) or 28–30 hours post-fertilization (hpf) in replicate groups of 3 or 6 using either 60- or 100-mm glass Petri dishes (n = 25 or 35 embryos per replicate, respectively).

3. Embryos were cleaned and sorted by stage, then divided into Petri dishes via controlled-drop Pasteur pipettes in a small volume of water (< 1 mL); 15 mL or 40 mL of HEWAF or control water was added to the Petri dishes, and HEWAFs and control water were not renewed for the duration of the exposures.

4. Embryos were incubated in covered Petri dishes at 28.5°C in the dark for 48 hours.

5. Dead embryos were removed every 24 hours. Mortalities were recorded.

A.3 Morphological Assessment of Embryos

1. Embryos were screened for abnormalities at 48 or 72 hpf based on observations using the stereomicroscope or digital still images and videos.

2. All observations were made under a Nikon SMZ800 stereomicroscope or Nikon Eclipse E600 compound microscope. Images were collected using a SPOT imaging system (Diagnostic Instruments, Inc., Sterling, MI; camera model 2.3.1; software version 4.5.9.9) or Fire-i400 camera (Unibrain, San Ramon, CA).

3. Presence and absence scores for pericardial edema were based on observations in the live embryos or digital video clips. The presence of edema was scored as a lack of external epidermal pulsation adjacent to the heart (i.e., no movement of pericardial sac across contractions).

4. Intracranial hemorrhage was scored visually in either live embryos or in lateral images and video clips.

5. For quantification of pericardial edema and detailed imaging, embryos were mounted in 2% methylcellulose. Pericardial area was measured in still frames extracted from digital video, as described previously (Incardona et al., 2006).

6. For cardiac dimensional measurements, embryos were mounted without anesthesia in 2% methylcellulose and digital video collected using differential interference contrast optics with a 20x objective lens on the Nikon E600 compound microscope. Diastolic and systolic chamber diameters were measured, as was fractional shortening (contractility) as described in Bendig et al. (2006) and Incardona et al. (2011).

7. For CYP1A expression, approximately 20 embryos from each treatment were dechlorinated and fixed in 4% paraformaldehyde. Standard protocols were used to fluorescently stain the heart for CYP1A and myosin heavy chain protein expression (Incardona et al., 2006).

B. Testing Protocol 2: Morphological Assessment of Crude Oil Cardiotoxicity of Zebrafish Embryos – Phototoxicity

B.1 Test Preparations

1. Prior to beginning toxicity tests, all glassware was cleaned by triple rinsing with acetone and DCM and allowed to dry in between rinses and before use.

2. Adult zebrafish were spawned according to standard procedures (Linbo, 2009).

3. Desired WAFs were prepared the morning of the exposure following the modified QAPP.

4. Subsamples of WAFs were placed in 250 mL pre-cleaned, amber glass bottles for chemical analysis by NWFSC. For preservation, 10% methyl chloride was added to each water sample and kept at 4°C.

B.2 Embryo Exposure

1. Embryos were collected shortly after fertilization.

2. Embryos were exposed at 4–6 hpf (sphere to shield developmental stage) in replicate groups of 3 using 60-mm glass Petri dishes (n = 25 embryos per replicate).

3. After being cleaned and sorted by stage, embryos were divided into Petri dishes via controlled-drop Pasteur pipettes in a small volume of water (< 1 mL); 15 mL of HEWAF or control water was added to the Petri dishes, and HEWAFs and control water were not renewed for the duration of exposures.

4. Embryos were incubated in covered Petri dishes at 28.5°C in the dark for 72 hours.

5. Dead embryos were removed every 24 hours and mortalities were recorded.

6. Phototoxicity tests were carried out after exposing embryos to HEWAFs from 4 to 72 hpf as detailed previously (Hatlen et al., 2010).

7. Larvae were transferred into new Petri dishes with clean water, and exposed outdoors to full sunlight for 30 minutes.

8. Larvae were returned to the laboratory for immediate imaging.

B.3 Morphological Assessment of Embryos

1. Embryos were screened for abnormalities after exposure to sunlight based on observations using the stereomicroscope or digital still images and videos.

2. All observations were made under a Nikon SMZ800 stereomicroscope fitted with a Fire-i400 camera (Unibrain, San Ramon, CA).

3. Presence and absence scores for pericardial edema were based on observations in the live embryos or digital video clips. The presence of edema was scored as a lack of external epidermal pulsation adjacent to the heart (i.e., no movement of pericardial sac across contractions).

4. Intracranial hemorrhage was scored visually in either live embryos or in lateral images and video clips.

5. For caudal finfold measurements, 10 larvae were randomly selected from each replicate, anesthetized with tricaine mesylate and mounted in 2% methylcellulose for imaging. Using ImageJ, area measurements were taken by tracing a roughly half-circular line using the end of the notochord as a landmark along the posterior edge of the caudal finfold.

C. Testing Protocol 3: Morphological Assessment of Crude Oil Cardiotoxicity of Zebrafish Juveniles

C.1 Test Preparations

1. Prior to beginning toxicity tests, all glassware was cleaned by triple rinsing with acetone and DCM and allowed to dry in between rinses and before use.

2. Adult zebrafish were spawned according to standard procedures (Linbo, 2009).

3. Desired WAFs were prepared on the morning of the exposure, following the modified QAPP.

4. Subsamples of WAFs were placed in 250 mL pre-cleaned, amber glass bottles for chemical analysis by NWFSC. For preservation, 10% methyl chloride was added to each water sample and kept at 4°C.

C.2 Embryo Exposure and Larval Rearing

1. Embryos were collected shortly after fertilization.

2. Embryos were exposed at 4–6 hpf (sphere to shield developmental stage) in replicate groups of 6 using 60-mm glass Petri dishes (n = 30 embryos per replicate).

3. After being cleaned and sorted by stage, embryos were divided into Petri dishes via controlled-drop Pasteur pipettes in a small volume of water (< 1 mL); 15 mL of HEWAF or control water was added to the Petri dishes, and HEWAFs and control water were not renewed for the duration of exposures.

4. Embryos were incubated in covered Petri dishes at 28.5°C in the dark for 48 hours.

5. Dead embryos were removed every 24 hours and mortalities were recorded.

6. At 48 hpf, embryos were scored for pericardial edema. The presence of edema was scored as a lack of external epidermal pulsation adjacent to the heart (i.e., no movement of pericardial sac across contractions).

7. HEWAF-exposed embryos were pooled and divided into three replicate groups (n = 30 each) with edema and three replicate groups (n = 30 each) without edema.

8. In control embryos, rates of edema were low enough to provide only a single group with edema (n = 30), while embryos without edema were pooled into five replicate groups (n = 30 each).

9. The new replicate groups were transferred to clean water, incubated at 28.5°C in 100-mm plastic Petri dishes until feeding stage and then raised until juvenile stage (2 weeks post-fertilization) as detailed in Linbo (2009), with mortality assessed daily.

C.3 Morphological Assessment of 2-Week Juveniles

1. At 2 weeks post-fertilization, fish were anesthetized with tricaine mesylate, assessed for morphological defects, and measured for length.

2. Representative individuals were imaged using a Nikon SMZ800 stereomicroscope fitted with a Fire-i400 camera (Unibrain, San Ramon, CA).

3. A subset of individuals from each treatment were randomly selected, fixed, and assessed for craniofacial skeletal defects using an alcian blue and Alizarin Red S stain.

C.4 Alcian Blue and Alizarin Red S Stains

1. Following morphometric data collection, fish were fixed in 4% paraformaldehyde overnight at 4°C on a rocker, dehydrated through a gradient of methanol/phosphate-buffered saline (PBS) (25%/75%, 50%/50%, 75%/25%; 5 minutes each) and stored at -20°C in 100% methanol.

2. For staining, samples were rehydrated through methanol/PBS series (75%/25%, 50%/50%, 25%/75%; 10 minutes each) and washed in water (3 times; 1 hour each).

3. Fish were then stained with alcian blue solution (0.01% in 7:3 ethanol/glacial acetic acid; 24 hours), destained (7:3 ethanol/glacial acetic acid; 30 minutes), transferred into 100% ethanol (30 minutes), rehydrated through ethanol/water series (75%/25%, 50%/50%, 25%/75%; 30 minutes each) and washed in water (2 times; 30 minutes each).

4. Following rehydration, alcian-stained fish were subjected to trypsin digestion (50% saturated aqueous sodium borate, 1.7% trypsin powder; 10 minutes) before 0.5% potassium hydroxide (KOH) enzyme deactivation (5 minutes) and 0.5% KOH washes (2 times; 5 minutes each).

5. Alizarin Red S solution (0.04%) was added to samples (in 0.5% KOH) until solution turned deep purple. Alizarin staining proceeds for 12 hours before clearing through 0.5% KOH/glycerol series (3:1, 1:1, 1:3; 6 hours each) and storage in 100% glycerol.

6. Dissected craniofacial skeletons were mounted in glycerol and imaged using the Nikon Eclipse E600 compound microscope and SPOT imaging system.

7. Multiple images encompassing all focal planes (n = 10) were then aligned and Z-stacked using Hugin version 2011.4.0 (http://hugin.sourceforge.net/) and Enfuse version 4.0 (http://enblend.sourceforge.net/) to generate an extended depth of field.

D. Testing Protocol 4: Assessment of Crude Oil Cardiotoxicity in Southern Bluefin Tuna (*Thunnus maccoyii*) Yolk-Sac Larvae – Static Exposure

These tests were performed in collaboration with the NWFSC/University of Miami RSMAS/ Hopkins Marine Station of Stanford University.

Testing protocol of southern bluefin tuna cardiotoxicity tests followed the same protocol as described in the RSMAS GLPP Testing Protocol 3, with the following deviations:

- Embryos were screened at 8–10 hpf and exposed until 8–10 hours post-hatch (~ 36 hours).
- Embryos were kept in a temperature controlled room set at 25°C.
- Exposures were done without any agitation (i.e., without a reciprocating shaker).

E. Testing Protocol 5: Assessment of Crude Oil Cardiotoxicity in Amberjack (*Seriola lalandi*) Larvae – Beaker Exposure

These tests were performed in collaboration with the NWFSC/RSMAS/Hopkins Marine Station of Stanford University.

Testing protocol of amberjack cardiotoxicity tests followed the same protocol as described in the RSMAS GLPP Testing Protocol 3, with the following deviations:

- Embryos were exposed in 1-L beakers at a density of 80 embryos/L.
- Embryos were kept in a temperature controlled room set at 25°C.
- Beakers were agitated on a horizontal shaker water bath.

F. Testing Protocol 6: Assessment of Crude Oil Cardiotoxicity in Amberjack (*Seriola lalandi*) Larvae – Bucket Exposure

These tests were performed in collaboration with the NWFSC/RSMAS/Hopkins Marine Station of Stanford University.

Testing protocol of amberjack cardiotoxicity tests followed the same protocol as described in the RSMAS GLPP Testing Protocol 3, with the following deviations:

- Embryos were exposed in 10-L buckets with an exposure volume of 7 L and stocked at a density of 80 embryos/L.
- Embryos were kept in a temperature-controlled room set at 25°C.
- Buckets were agitated with magnetic stir bars on stir plates.
- Due to the large number of embryos per bucket (> 500), hatching rates were not precisely quantified.
- 10 hatched larvae were randomly captured from each treatment and imaged within 1 hour.

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14. Stratus Consulting/Abt Associates General Laboratory Procedures and Practices – Red Drum, Speckled Seatrout, and Pacific White Shrimp

14.1 Methods

Stratus Consulting/Abt Associates[1] conducted the tests described below in collaboration with the University of North Texas (UNT) and the Northwest Fisheries Science Center (NWFSC) at the Texas Parks and Wildlife Department (TPWD) Sea Center Texas marine hatchery in Lake Jackson, Texas. This chapter describes General Laboratory Procedures and Practices (GLPP) that Stratus Consulting/Abt Associates used at TPWD.

14.1.1 Source water

The natural seawater that was used during toxicity testing was the same as the source water used at TPWD. To obtain source water, TPWD pumps water from Galveston Bay and runs the water through a drum filter. Source water was pulled directly from distribution lines that ran throughout the TPWD hatchery. For static renewal tests, testing staff used source water stored in a 90-gal carboy that was covered in dark plastic and maintained under aeration at ambient temperature (a sample of this 90-gal "batch" was collected and sent to ALS Environmental for chemical analyses). Testing staff used water from the carboy to prepare test exposure solutions. A sample of every batch that was used during testing was sent to ALS Environmental for analyses.

TPWD hatchery source water was also used during flow-through tests. Testing staff plumbed water lines from the TPWD hatchery distribution lines directly to head tanks used for flow-through exposure systems. The amount of source water needed for flow-through tests precluded storing and sampling batches of water. However, a series of water samples were collected from head tanks during each flow-through test and sent to ALS Environmental for chemical analyses.

1. The name of the company changed while tests were ongoing.

14.1.2 Fish culturing

All of the fish used in these studies came from the TPWD hatchery. The TPWD hatchery rears juvenile red drum and speckled seatrout for the purposes of stock enhancement. Mature adults live indoors and spawn in large circular brood stock tanks maintained by a large recirculating filtration system. TPWD hatchery staff regulate spawning by manipulating the water temperature and photoperiod in the brood-stock tanks. During a successful spawning event, female fish release eggs that are externally fertilized by sperm. Fertilized embryos float to the surface of brood-stock tanks. The hatchery staff use skimming devices on the edges of the brood tanks to concentrate embryos in the overflow filter box on the side of each tank. During the spawning season, late each night, early the following morning, or both, TPWD hatchery staff collect embryos from each tank's overflow box to stock into 100-gal circular incubation tanks maintained by a flow-through system. Then they volumetrically enumerate the number of embryos stocked into each incubation tank. The hatchery staff rear embryos in incubation tanks until the embryos hatch and the resulting larvae absorb their yolk sack, which takes approximately 3 to 4 days. After this rearing period, TPWD hatchery staff transfer larvae to one-acre outdoor ponds. Fish at the hatchery live in outdoor ponds for approximately 1 month before being stocked as juveniles (> 15 mm) in State waters. Larvae are fed granulated prepared feed (Rangen, Angleton, TX) twice daily and live food (natural, native zooplankton) are available throughout larval and juvenile development.

For early life stage toxicity testing, testing staff collected embryos and larvae directly from the hatchery system described above. For juvenile life stage toxicity testing, test organisms were collected directly from outdoor rearing ponds just before stocking.

Red drum (*Sciaenops ocellatus)* and speckled seatrout (*Cynoscion nebulosus*)

Testing staff obtained red drum and speckled seatrout embryos, larvae, and juveniles from the TPWD hatchery for use in toxicity testing. Embryos were collected directly from the brood stock tank's overflow filter box between 20:00 and 24:00, a few hours after fertilization. Before initiating a test, testing staff examined a sample of embryos in Petri dishes or welled slides using a stereo or compound microscope to determine fertilization status. Spawns with low fertilization rates were not used for definitive testing.

Testing staff collected red drum or speckled seatrout larvae from stocked incubation tanks a few hours after hatching [see test-specific test conditions tables (TCTs)]. Testing staff collected newly hatched larvae by dipping a beaker into incubation tanks. Larval morphology and behavior were grossly assessed by looking through the sides, bottom, and top of the beaker. Only healthy-looking, vigorous larvae were used to stock test exposure vessels.

Testing staff obtained red drum juveniles from outdoor rearing ponds located on the TPWD hatchery property. Juveniles used for testing were collected approximately three weeks post-

hatch, just before being stocked in State waters. Testing staff used a long-handled dip net to capture and transfer fish from rearing ponds to buckets of pond water aerated with pure oxygen. Once enough fish were collected, they were immediately transported to the TPWD hatchery incubation room for acclimation and testing.

Juvenile red drum were held in two 100-gal, circular incubation tanks for approximately 48 hours before testing. Water quality was maintained via flow-through conditions and monitored throughout the holding period. Red drum juveniles were fed ad libitum with prepared feed (Rangen, Angleton, TX) and newly hatched *Artemia* nauplii until stocked into the testing exposure system. Just before stocking the exposure tanks, testing staff randomly captured juvenile red drum from the incubation tanks using a large net and transferred them to an aerated bucket of clean source water. Fish were taken from this aliquot, inspected, measured, weighed, and stocked into exposure tanks. Only healthy-looking, normal fish were used in toxicity tests.

New aliquots of fish were collected from the incubation tanks throughout the stocking procedures. Remaining fish not used to stock exposure tanks were retained and reared in incubation tanks during the course of respective testing. Juvenile red drum tests were conducted under the UNT Institutional Animal Care and Use Committee (IACUC) animal use protocols.

14.1.3 Pacific white shrimp

In addition to red drum and speckled seatrout, Pacific white shrimp (*Litopenaeus vannamei*) were also used during TPWD hatchery toxicity testing. Post-larva (PL) 8 to 10 mm shrimp were obtained from the Shrimp Improvement Systems (SIS) LLC Nucleus Breeding Center in Islamorada, Florida, USA. Although SIS is a specific pathogen-free aquaculture facility, we had an aliquot of SIS shrimp shipped to the University of Arizona Aquaculture Pathology Laboratory for additional diagnostic microbiology and bacteriology screening. Once shrimp were determined to be free of pathogens, SIS sent shrimp to the TPWD hatchery for use in testing. Before importing live shrimp to the TPWD hatchery, we obtained an Exotic Species Permit from the TPWD Exotic Species program (No. RES 07 15-112). Shrimp rearing, testing, and disposal were conducted within permit specifications.

Once shrimp were received, they were slowly acclimated to the TPWD hatchery source water. This was done by slowly dripping source water into the shrimp shipping water until it had the same water quality as the source water. Shrimp were maintained in a 100-gal circular incubation tank under flow-through conditions. Holding/incubation tank outflow water was directed through a series of filters into the flow-through exposure system sump where it was filtered and pumped to a 1,275-gal holding tank. At least once daily, the contents of the shrimp water holding tank were mixed with TPWD hatchery tap water and pumped into a sink drain that ran to the Lake Jackson sanitary sewer system.

Shrimp were fed ad libitum with prepared feed (Raceway Plus from Zeigler, MN) and newly hatched *Artemia* nauplii until stocked into the testing exposure system. Artemia cysts were obtained from INVE Aquaculture (Salt Lake City, UT) and hatched in a series of 10-L hatching cones. Remaining shrimp not used to stock exposure tanks were retained and reared in incubation tanks during the course of respective testing.

14.1.4 Media preparation

The testing media used during toxicity testing included both water accommodated fractions (WAFs), oil slick, and oil spiked sediment exposures. For WAF and slick testing, staff prepared exposure media using source water stored in the 90-gal carboy. The protocols for WAF preparations listed below appear in the "Protocols for Preparing Water Accommodated Fractions" standard operating procedure (SOP) in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. The protocols for Slick A and Slick B oils appear in Appendix D *Testing Protocol 4: Red Drum Oil Slick Exposures*. Sediment exposure preparation methods are included in the individual testing protocols listed in the Stratus Consulting GLPP. The types of toxicity testing media preparations were as follows:

- Three different WAF preparations – high-energy, low-energy, and chemically enhanced WAFs (HEWAF, LEWAF, and CEWAF, respectively) – using one of two oil types: Slick A (CTC02404-02) or Slick B (GU2888-A0719-OE701).

- Oil slick exposures using either Slick A (CTC02404-02) or Slick B (GU2888-A0719-OE701) oil.

- Slick B (GU2888-A0719-OE701) oil-spiked sediment exposure. Sediment was obtained from Point aux Pins near Bayou La Batre, Alabama (30.379079, -88.305423). A composite sample of collected sediment was analyzed for a large suite of hydrocarbons, metals, pesticides, and semi-volatile organic compounds, none of which were detected at concentrations that would be deleterious to sensitive aquatic organisms.

14.1.5 Exposure systems

Both static and flow-through exposure systems were used throughout TPWD hatchery testing activities.

For static tests using fish embryos and larvae, staff filled pre-labeled exposure vessels with final-dilution exposure media and placed them into a temperature-controlled water bath. Staff removed the exposure vessels from the water bath, stocked them with fish, and returned them to the water bath one at a time. The water bath temperature was maintained with heaters and flow-

through hatchery water. A small pump was used to recirculate water in the water bath tank, and staff adjusted water depth using standpipes at the water bath outflow.

For flow-through tests, two rack systems were constructed in the TPWD hatchery incubation room. Each rack systems consisted of a 45-gal head tank; 24 individual, covered, 2.5-gal exposure tanks; a 45-gal sump; and a three-stage canister filter system (Figure 14.1). Air lines were run to each exposure tank and air flow was adjustable using gang and inline values. Head tanks were also aerated. The source water distribution system was constructed using 0.25-in. ball valves that adjusted inflow to each tank. Exposure tank effluent flowed through individual, screened overflow bulkheads into an effluent collection pipe. Before entering the sump, effluent was filtered through a 200-micron filter sock at the end of the effluent collection pipe. A float valve was used to turn on the filter pump when the sump was approximately half full of effluent. The filtration system consisted of one 20-micron pleated filter, one 5-micron pleated filter, and an activated carbon filter run in a series. Filter cartridges were changed at least once daily during the experiment. Filtered effluent was either directed to a 1,275-gal holding tank for shrimp or the floor drain for red drum.

Figure 14.1. Shrimp and red drum flow-through exposure systems. Photograph taken by I. Lipton on 7/8/2015.

14.1.6 Water-quality monitoring

Testing staff monitored water quality in exposure vessels at specific time points throughout testing activities. See "Water Quality Protocols" in Section 14.3, below, for detailed water quality methods used throughout testing activities. See the QAPP and TCTs for required monitoring schedule.

In addition to time-point-specific temperature measurements, HOBO data loggers (Onset Computer Corporation, Pocasset, MA) were sometimes used to continuously monitor the temperature in water baths or dummy exposure beakers. When deployed, pendant-type temperature/alarm (UA-001-64) loggers were used in conjunction with the Pendant Base Station (BASE-U-1) and HOBOware Lite version 3 software. Logger data files were named with the test identification (ID), location (water bath or dummy beaker), and date and time deployed, and saved as comma delimited Microsoft Excel files on the project server.

14.1.7 Analytical chemistry sampling

See the QAPP, related testing protocols, and test-specific TCTs for sampling and processing of water and tissue collected for chemical analyses.

14.2 Reporting and Testing Documentation

Reporting and testing documentation was performed as outlined in the QAPP. Samples that we collected were labeled according to the QAPP, using "ST-" for the first sample ID segment.

14.3 Water Quality Protocols – General Laboratory SOPs

Testing staff used commercially available water quality meters, probes, and colorimetric test kits to measure various water quality parameters throughout testing activities. Testing staff measured pH, dissolved oxygen (DO), and conductivity directly in the exposure chambers using meters and probes. Water samples for ammonia measurements were collected for analysis at the beginning and end of the tests. Temperature measurements were taken within the water bath and during the in-chamber pH or DO measurements. Salinity/conductivity was measured by either using a hydrometer or a salinity meter. When using a hydrometer, water samples were collected for measurements at the beginning and end of a test. When using a salinity meter, the measurements that were taken at the beginning and the end of a test were taken directly in the exposure chambers.

To limit the possibility of cross-contamination from the probes, measurements began with the controls and then proceeded in order of treatment concentration. Probes were also rinsed with reverse osmosis (RO) water between exposure chambers. After use, testing staff decontaminated probes and acrylic vials using a non-toxic mild detergent solution and light scrubbing, followed by an RO water rinse.

Prior to use, meters were bench-calibrated for their accuracy and, if possible, the calibrations were verified against standards. Testing staff followed each meter**'**s manufacturer calibration frequency and protocols.

14.3.1 pH and temperature – YSI EcoSense PH100A pH meter/temp probe

1. Calibrate the instrument according to the operations manual using neutral (pH 7) and basic (pH 10) calibration solutions.

2. Rinse the probe with RO water and place the probe into the treatment container to be tested. Confirm that the probe's electrode elements are submerged below the liquid level; continuous movement is not required.

3. Allow the temperature and pH readings to stabilize and record the readings on the "Water Quality Monitoring" datasheet.

4. Repeat steps 2 through 3 as necessary for each treatment container.

5. After using the probe, decontaminate it with mild soap and water and then place the probe into the proper storage container in the electrode storage solution.

14.3.2 DO – YSI ProODO

1. Verify the instrument calibration according to the operations manual.

2. Rinse the probe with RO water and place the probe into the treatment container to be tested. Confirm that the probe's electrode elements are submerged below the liquid level; continuous movement is not required.

3. Allow DO readings to stabilize and record readings on the "Water Quality Monitoring" datasheet.

4. Repeat steps 2 through 3 as necessary for each treatment container.

5. After using the probe, decontaminate it with mild soap and water and then place the probe into the proper storage container.

14.3.3 Salinity – Instant Ocean Hydrometer or Pinpoint Salinity Meter

Instant Ocean Hydrometer

1. Ensure that the black plug is inserted into the flow outlet at the bottom of the hydrometer.

2. Add water into the hydrometer until it is full. Remove any air bubbles by lightly tapping the hydrometer until no bubbles are visible. Note that air bubbles on the pointer will cause incorrect readings.

3. Place the hydrometer on a flat surface. Do not tilt the hydrometer as that will cause water to spill from the water inlets.

4. Record the reading for salinity on the "Water Quality Monitoring" datasheet.

5. After using the hydrometer, flush it with warm, fresh tap water to prevent salt deposits from forming on the swing arm, which can distort future readings.

Pinpoint Salinity Meter

1. Verify instrument calibration according to the operations manual.

2. Wash the probe with RO water and place the probe into the treatment container to be tested. Be sure that the probe elements are submerged below the liquid level and gently swirl the probe.

3. Allow salinity readings to stabilize. The salinity meter measures conductivity in millisiemens (mS). Record conductivity results on the "Water Quality Monitoring" datasheet and correct the conductivity units on the datasheet in the respective entry heading. To convert the conductivity measurements to parts per thousand (ppt), use the table provided by the meter manufacturer. Record that value in the datasheet.

4. Repeat steps 2 through 3 as necessary for each treatment container.

5. After using the probe, decontaminate it with mild soap and water and then place the probe into the proper storage container.

14.3.4 Total ammonia (NH_3/NH_4^+) – API Ammonia Test Kit

1. Collect 5 mL of test solution and place it into the glass test tube provided in the API test kit. The test tube has a white 5-mL graduation line.

2. Add eight drops of test solution #1 to the test tube.

3. Immediately add eight drops of test solution #2 to the same test tube.

4. Tightly cap the test tube with the provided cap and shake vigorously for 5 seconds. Do not cap the tube with your finger since that might interfere with test results.

5. Set the timer for 5 minutes and wait for the color to develop.

6. After 5 minutes, read the test results by comparing the test solution color to the saltwater ammonia color chart provided in the test kit. View the tube color against a white background in a well-lit area. Determine the closest color match between the test solution and the color chart and record the associated NH_3/NH_4^+ values. Record the value on the "Water Quality Monitoring" datasheet.

7. Pour 5 mL of solution into the appropriate disposal vessel.

8. After using the API kit container, clean it with mild soap and water and store until next use.

Note that samples may also be collected at the end of each test for possible additional ammonia analyses. Log these samples on the "Analytical Sample Inventory Bench Sheet" and archive the samples at -20°C.

A. Testing Protocol 1: Red Drum and Speckled Seatrout Acute Toxicity Procedure

A.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Prepare WAFs with the appropriate treatment oil (see test-specific TCTs) according to the *Protocols for Preparing Water Accommodated Fractions* in the QAPP. Prepare HEWAFs the evening of the embryo or larval collection. If a single test requires multiple WAF preparations, prepare all WAF solutions at the same time, composite the solutions in a decontaminated stainless steel or glass container, and thoroughly mix before making test treatment dilutions.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary chain-of-custody (COC) documentation, as described in the QAPP.

A.2 Test Media Preparation

Perform static-exposure acute toxicity tests in 250- or 600-mL glass beakers with a total test solution volume of 200 and 400 mL, respectively (see test-specific TCTs). For the preparation of WAF dilutions, measure the appropriate source water and stock WAF volumes using a glass graduated cylinder or pipettes. Be sure to create enough volume for all treatment replicates and required ALS Environmental samples. Add both source water and WAF volumes to a 2,000-mL Erlenmeyer flask and swirl to mix. For larger volume dilutions, use a 1-gal glass bottle or similar glass container. Pour the solution into sample vessels. After filling the sample vessels, collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental as described in the QAPP. Fill each sample jar, provided by ALS Environmental, to capacity. Fill all exposure vessels and sample jars and discard the unused WAF into a lined 55-gal drum for off-site disposal.

A.3 Embryo or Larval Exposure

1. Set up the water bath with an aquarium heater/chiller to achieve the appropriate temperature, indicated in the test-specific TCT. Place labeled test beakers with treatment solutions into the water bath to acclimate the test solutions to the correct temperature. See

test-specific TCTs for beaker and exposure water volumes, treatment levels, and other test-specific testing parameters. Use the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet to track tank IDs, respective dilutions, and start/stop dates and times.

2. Before test initiation, measure water-quality parameters (temperature, conductivity/ salinity, DO, ammonia, and pH) as described in the *Stratus Consulting General Laboratory Procedures and Practices – Red Drum, Speckled Seatrout, and Pacific White Shrimp* (Stratus Consulting GLPP) document. Record the water-quality results on standard "Water Quality Monitoring" data entry bench sheets.

3. ***For embryo exposures*** (see test-specific TCTs): Collect embryos from the egg collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the egg collector. Take care not to collect too many embryos in one beaker; the layer of floating embryos in the collection beaker should not exceed 1.5 cm.

   For larval exposures (see test-specific TCTs): Collect the desired aged larvae from incubation tanks. Collect larvae and water by dipping a decontaminated glass beaker into stocked incubation tanks. Please note that hatchery staff will stock incubation tanks with embryos. Embryos hatch into larvae in the early evening approximately 17 to 20 hours post-fertilization.

4. ***For embryo exposures (***see test-specific TCTs): If loading embryos using a compound microscope, use a pipette to transfer the embryos from the collection beaker onto a microscope slide. Use the low-magnification microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Transfer the embryos into test beakers in random order by gently washing them from the surface of the microscope slide. Once the complete transfer is verified, dispose of the slide by inserting it into a hard-sided sharps receptacle. Record start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain embryos. See the test-specific TCT for the number of organisms required per replicate, as well as the number of replicates required per treatment.

   If loading embryos using a stereo microscope, use a pipette to collect the embryos from the collection beaker into a new Petri dish with source water. Use the stereo microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Gently expel the viable embryos into the test beakers. Inspect the pipette to verify complete transfer of the embryos into the test beakers. Record start time for each

replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain embryos. See test-specific TCTs for the number of organisms required per replicate, as well as the number of replicates required per treatment.

For larval exposures (see test-specific TCTs): Transfer larvae from the collection beaker to exposure vessels using a pipette. Before dispensing them into exposure vessels, count the larvae by looking through a pipette cylinder that is backlit with a desk lamp. After dispensing, reexamine the pipette to ensure all larvae were successfully transferred. Record the start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain larvae. See test-specific TCTs for the number of organisms required per replicate, as well as the number of replicates required per treatment.

5. Keep the beakers in the water bath for the duration of the test, except when you are inspecting them. The light cycle may vary during test maintenance because of routine hatchery operations.

6. After 24 hours of exposure, count and record the number of live and dead organisms in each exposure chamber. Count organisms by gross observation under a desk lamp against a dark background. Record the organism as dead if you can fully identify intact carcasses or dead eggs. Do not count pieces of decomposed carcass remnants as dead organisms. Remove all whole dead organisms and carcass or embryo remnants from the exposure chamber into a freezer-safe sample container using a treatment-dedicated pipette and archive at -20°C according to the QAPP.

7. Repeat step 6 for each 24-hour time period prior to test termination.

8. At the end of the study, count and record the number of live and dead organisms remaining in each exposure chamber using the gross observation methods described in step 6. Pipettes may also be used to inspect and count larvae, as described above. Note that immobile or morbid organisms may be removed from the exposure chamber by using a pipette and then inspected under a microscope at magnification. Morbid organisms are defined as alive but immobile. The number of morbid organisms may be enumerated using both a microscope and gross observations. If performing microscope assessments, only perform them at the end of test. Test organisms may be fixed for potential future analyses. To preserve samples, collect live and morbid organisms and fix them in 10% buffered formalin. Archive all remaining live and dead organisms that are not fixed in formalin at -20°C according to the QAPP. Record test end times on the "Tank ID,

Dilution, or Stock Code Definitions" data entry bench sheet. See test-specific TCTs for exposure duration and test length.

9. If needed, collected end-of-test water chemistry samples and discard the remaining test solutions into a lined 55-gal drum for off-site disposal.

B. Testing Protocol 2: Red Drum and Speckled Seatrout Acute Differential Exposure Toxicity Testing

B.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Prepare WAFs with the appropriate treatment oil (see test-specific TCTs) according to the *Protocols for Preparing Water Accommodated Fractions* in the QAPP. Prepare HEWAFs the evening of the embryo or larval collection.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

B.2 Test Media Preparation

Perform acute differential static-exposure toxicity tests in 250- or 600-mL glass beakers with a total test solution volume of 200 and 400 mL, respectively (see test-specific TCTs). For the preparation of WAF dilutions, measure the appropriate source water and stock WAF volumes using a glass graduated cylinder or pipettes. Be sure to create enough volume for all treatment replicates and required ALS Environmental samples. Add source water and WAF volumes to a 2,000-mL Erlenmeyer flask and swirl to mix. For larger-volume dilutions, use a 1-gal glass bottle or similar glass container. Pour the solution into sample vessels. After filling sample vessels, collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental, as described in the QAPP. Fill each sample jar, provided by ALS Environmental, to capacity. After all exposure vessels and sample jars are filled, discard unused WAF into a lined 55-gal drum for off-site disposal.

B.3 Embryo Exposure

1. Set up the water bath with an aquarium heater/chiller to achieve the appropriate temperature that is indicated in the test-specific TCT. Place labeled test beakers with treatment solutions into the water bath to acclimate the test solutions to the correct

temperature. See test-specific TCTs for beaker and exposure water volumes, treatment levels, and other test-specific testing parameters. Use the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet to track tank IDs, respective dilutions, and start/stop times and dates.

2. Before test initiation, measure water quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH) as described in the Stratus Consulting GLPP document. Record water quality results on standard "Water Quality Monitoring" data entry bench sheets.

3. Collect embryos from the egg-collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the egg collector. Take care not to collect too many embryos in one beaker; the layer of floating embryos in the collection beaker should not exceed 1.5 cm.

4. Use a pipette to transfer the embryos from the collection beaker onto a microscope slide. Use a low-magnification compound microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Transfer the embryos into test beakers in random order by gently washing them from the surface of the microscope slide. Once the complete transfer is verified, dispose of the slide by inserting it into a hard-sided sharps receptacle. Record the start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain embryos. See the test-specific TCT for the number of organisms required per replicate, as well as the number of replicates required per treatment.

5. Keep the beakers in the water bath for the duration of the test, except when you are inspecting them. The light cycle may vary during test maintenance because of routine hatchery operations.

6. After the appropriate exposure duration time, transfer embryos/larvae from the treatment solutions into clean source water. Perform all of the transfers using pipettes. Use a new pipette for each treatment to avoid cross-contamination. See test-specific TCTs for exposure durations and test lengths.

7. During transfer and at 24 hours, count and record the number of live and dead organisms in each exposure chamber. Count organisms by gross observation under a desk lamp against a dark background. Record an organism as dead if you can fully identify intact carcasses or dead eggs. As such, do not count pieces of decomposed carcass remnants as dead organisms. Remove all whole, dead organisms, and remnants into a freezer-safe

sample container using a treatment-dedicated pipette and archive at -20°C, according to the QAPP.

8. Repeat step 7 for each 24-hour time period prior to test termination.

9. At the end of the study, count and record the number of live and dead organisms remaining in each exposure chamber using the gross observation methods described in step 7. Pipettes may also be used to inspect and count larvae, as described above. Note that immobile or morbid organisms may be removed from the exposure chamber by using a pipette and then inspected under a microscope at magnification. Morbid organisms are defined as alive but immobile. The number of morbid organisms may be enumerated using both a microscope and gross observations. If performing microscope assessments, only perform them at the end of test. Test organisms may be fixed for potential future analyses. To preserve samples, collect live and morbid organisms and fix them in 10% buffered formalin. Archive all the remaining live and dead organisms that are not fixed in formalin at -20°C. Collect and archive them according to the QAPP. Record test end times on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet. See test-specific TCTs for exposure duration and test length.

10. Discard the remaining test solutions into a lined 55-gal drum for off-site disposal.

C. Testing Protocol 3: Morphological Assessment of Oil Cardiotoxicity in Red Drum Embryos

These tests were conducted in in collaboration with NWFSC.

C.1 General Considerations

Decontaminate all glassware, spatulas, and materials according to the *Decontamination SOP*, described in the QAPP.

Prepare WAFs with the appropriate treatment oil (see TCTs) according to *Protocols for Preparing Water Accommodated Fractions* in the QAPP, Appendix A. Prepare HEWAFs the evening of the embryo or larval collection. If a single test requires multiple WAF preparations, all WAF solutions will be prepared at the same time, composited in a decontaminated stainless steel or glass container, and thoroughly mixed before making test treatment dilutions.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless noted otherwise; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

C.2 Test Media Preparation

Perform acute differential static exposures toxicity tests in 600-mL glass beakers with a total test solution volume of 400 mL. For the preparation of WAF dilutions, measure the appropriate source water and stock WAF volumes using a glass graduated cylinder or pipettes. Be sure to create enough volume for all treatment replicates and required ALS Environmental samples. Add both source water and WAF volumes to a 2,000-mL Erlenmeyer flask and swirl to mix. For larger-volume dilutions, use a 1-gal glass bottle or similar glass container. Pour the solution into replicate/sample vessels. After filling the sample vessels, collect 250 mL of each bulk solution and ship it overnight on ice to ALS Environmental, as described in the QAPP. Each sample jar, provided by ALS Environmental, should be filled to capacity. After all exposure vessels and sample jars are filled, discard unused WAF into a lined 55-gal drum for off-site disposal.

C.3 Embryo Exposure

1. Set up the water bath with an aquarium heater/chiller to achieve the appropriate temperature, as indicated in the test-specific TCT. Place labeled test beakers with treatment solutions into the water bath to acclimate the test solutions to the correct temperature. See test-specific TCTs for beaker and exposure water volumes, treatment levels, and other test-specific testing parameters. Use the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet to track tank IDs, respective dilutions, and start/stop dates and times.

2. Before starting the test, measure the water-quality parameters (temperature, conductivity/salinity, DO, ammonia, and pH), as described in the Stratus Consulting GLPP document. Record water-quality results on standard "Water Quality Monitoring" data entry bench sheets.

3. Collect embryos from the egg collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the egg collector. Take care not to collect too many embryos in one beaker; the layer of floating embryos in the collection beaker should not exceed 1.5 cm.

4. Use a pipette to collect the embryos from the collection beaker. Use the stereo microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Gently expel the embryos into the test beakers. Load approximately 200 organisms per replicate.

5. Keep the beakers in the water bath, except when being inspected. The light cycle may vary during test maintenance because of routine hatchery operations.

6. At roughly 24 hours post-fertilization, harvest 100 mL of test solution containing the newly hatched larvae. Pour the solution through a small straining basket to collect the larvae, placing approximately 20 larvae into a labeled microcentrifuge tube containing RNAlater for possible genetic analyses. Immediately place the tube in liquid nitrogen. Repeat for each replicate.

7. At approximately 12 hours post-hatch or approximately 36 hours post-fertilization, observe and record mortality. Count organisms by gross observation. Enumerate dead organisms only if you can fully identify intact carcasses or dead eggs. Decomposed carcass remnants may not be counted as dead organisms. Remove all whole dead organisms and remnants from the exposure chamber using a treatment-dedicated pipette and archive them at -20°C, according to the QAPP.

8. Also at approximately 12 hours post-hatch or approximately 36 hours post-fertilization, harvest 100 mL of test solution containing fish. Pour the solution through a small straining basket to collect the fish. Place approximately 20 individuals into a labeled microcentrifuge tube containing RNAlater for possible genetic analyses. Immediately place the tube into liquid nitrogen. Repeat for each replicate.

9. Divide the remaining 200 mL of test solution with larvae into two equal 100-mL volumes. Try to divide the samples so that an equal number of larvae remain in each aliquot. Set one 100-mL aliquot aside for harvesting at the end of imaging. Repeat for each replicate.

10. Use the remaining 100 mL aliquot from each replicate for imaging. To capture images of individuals, follow the protocols described in Section C.3 (Digital Videomicroscopy of Hatched Larvae). Repeat for each replicate.

11. Collect any remaining live organisms and fix them in paraformaldehyde.

12. Archive all of the imaged larvae, together with the dead larvae collected in step 7, at -20°C according to the QAPP.

13. After imaging is complete, harvest larvae from the 100-mL aliquot that was set aside in step 9. To harvest fish, pour the solution through a small straining basket and place approximately 20 individuals into a labeled microcentrifuge tube containing RNAlater for possible genetic analyses. Immediately place the tube into liquid nitrogen. Repeat for each replicate.

14. Discard the remaining test solutions into a lined 55-gal drum for off-site disposal.

C.4 Digital Videomicroscopy of Hatched Larvae

1. Randomly select a replicate from one of the treatment groups.

2. Capture two or three larvae at a time using a wide-bore glass pipette.

3. Mount larvae in 2% methyl cellulose in source water.

4. Image all the larvae in sets of two or three, keeping the larvae in methyl cellulose for less than 10 minutes.

5. Visually inspect and photo-document all larvae using a Nikon SMZ800 stereo microscope fitted with a phototube and Unibrain Fire-i400 1394 camera, connected via

firewire to a laptop with BTV Pro. Keep the magnifications for imaging identical for all samples collected. Position larvae with the anterior to the left and dorsal to the top of the frame.

a. Video capture. Take a 10-second video at the highest magnification (6.3x). Focus on the cardiac/pericardial region for each larvae. Align eyeballs and neuromasts on top of each other.

b. Image capture. Capture composite images of the entire larvae for three fish per beaker at 5x magnification.

6. After imaging, use a wide-bore glass pipette to transfer larvae to an appropriate container for archiving. Repeat the process of capturing and mounting two or three larvae at a time until 20 larvae from the treatment vessel have been imaged, then move on to the next beaker. Refresh the methyl cellulose as needed.

7. Save and copy files to two back-up hard drives.

8. Repeat steps 1–7, imaging all of the exposure vessels.

9. Process images according to the *Viewing Video Files for Cardiac Endpoint Measurements* SOP in the NWFSC GLPP.

D. Testing Protocol 4: Red Drum Oil Slick Exposures

D.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

D.2 Preparation of Polyvinyl Chloride Coupling

To prepare the polyvinyl chloride (PVC) coupling for slick exposures, gather the required number of 2-inch PVC couplings. Use one coupling for each replicate (see test-protocol TCTs). Drill a hole through both sides of the top half of the PVC coupling so that a small wooden dowel can slide through the holes. Thoroughly wash and rinse the PVC coupling with Simple Green and tap water, followed by an RO rinse. Soak the PVC coupling in RO water for a minimum of two hours. Dry before using.

D.3 Slick Preparation

1. Fill the labeled exposure chamber with 450 mL of source water. Set up the water bath with an aquarium heater/chiller to achieve the appropriate temperature, as indicated in the test-specific TCT. Place labeled test beakers in the water bath to acclimate the test solutions to the correct temperature. See test-specific TCTs for beaker and exposure water volumes, treatment levels, and other test-specific testing parameters. Use the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet to track tank IDs, respective dilutions, and start/stop dates and times.

2. Weigh 2 g of oil in a clean aluminum weigh boat.

4. Tare the PVC coupling on the top loading balance.

5. Carefully use a metal spatula to smear as much of the pre-weighed 2 g of oil as possible around the inside of the bottom half of the PVC coupling. Keep the oil ring approximately 1 cm away from the edge of the PVC coupling.

6. Weigh the PVC coupling with the oil to determine the total amount of oil that was added to the PVC coupling. Record the results.

7. Place a dowel through the holes drilled in the top portion of the PVC coupling.

8. Repeat steps 1–7 for each replicate.

9. Rest the ends of the wooden dowel on the top of the exposure beaker so that the oil from the PVC coupling is in contact with source water (Figure D.1). Repeat for each replicate.

10. If a PVC coupling control is required (see test-specific TCTs), set up one treatment that includes the PVC coupling but excludes the oil.

11. Place the PVC coupling in the water and allow the PVC coupling to soak in the exposure beakers for approximately 5.5 hours while in the water bath.

Figure D.1. Top view of the slick exposure set-up. Photo: Abt Associates.

D.4 Embryo Exposure

1. Before starting the test, measure water-quality parameters in the control beakers without PVC couplers (temperature, conductivity/salinity, DO, ammonia, and pH), as described in the Stratus Consulting GLPP document. Record water quality results on standard "Water Quality Monitoring" data entry bench sheets.

2. Collect embryos from the egg collection chambers located on the side of each brood-stock tank. Collect embryos and water by dipping a decontaminated glass beaker into the

egg collector. Take care not to collect too many embryos in one beaker; the layer of floating embryos in the collection beaker should not exceed 1.5 cm. Collect the embryos from the collection beaker into a new Petri dish with source water.

3. Use a pipette to collect embryos from the Petri dish. Use the stereo microscope to assess embryo viability. Discard any embryos that look unfertilized or malformed. Collect a total of 20 embryos.

4. To avoid disturbing the oil slick, gently place the pipette into the exposure water between the PVC coupling and the edge of the beaker.

5. Simultaneously, expel the embryos while carefully removing the PVC coupling from the beaker. Inspect the pipette to verify complete transfer of embryos into test beakers. Record the start time for each replicate on the "Tank ID, Dilution, or Stock Code Definitions" data entry bench sheet and replace the exposure vessel back into the water bath. Mark the lip of the loaded beakers with tape and repeat the above procedure until all beakers contain embryos.

6. During testing, keep the beakers in the water bath except when being inspected. The light cycle may vary during test maintenance because of routine hatchery operations.

7. After 24 hours of exposure, count and record the number of live and dead organisms in each exposure chamber. Count organisms by gross observation under a desk lamp against a dark background. Record the organism as dead if you can fully identify intact carcasses or dead eggs. Do not count pieces of decomposed carcass remnants as dead organisms. Remove all whole dead organisms and remnants from the exposure chamber using a treatment-dedicated pipette; archive them at -20°C, according to the QAPP.

8. Repeat step 7 for each 24-hour time period prior to test termination.

9. At the end of the study, count and record the number of live and dead organisms remaining in each exposure chamber using the gross observation methods described in step 6. Immobile or morbid organisms may be removed from the exposure chamber using a pipette and inspected under a microscope at magnification. Morbid organisms are defined as alive but immobile. Enumerate the number of morbid organisms using both a microscope and gross observations. If performing microscope assessments, only perform them at the end of the test. Collect live and morbid organisms and fix them in 10% buffered formalin for potential analysis, and archive all remaining live and dead organisms that are not fixed in formalin at -20°C, according to the QAPP. See test-specific TCTs for exposure duration and test length.

10. Use a siphon to composite the water from the four replicates of each treatment into a clean beaker. Avoid collecting the top 50 mL of water.

11. Swirl the composite water beaker and pour 250 mL of the solution into the analytical chemistry bottle. Ship it on ice overnight to ALS Environmental for analysis.

12. Discard the remaining test solutions into a lined 55-gal drum for off-site disposal.

E. Testing Protocol 5: Red Drum Chronic Spiked Sediment Exposures

E.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

Place any unused prepared sediment into a Ziploc bag and store in the dark at 4°C (short-term) or in a freezer at -20°C (long-term). Store large quantities (> 5 kg) of prepared sediment in metal pans, covered with aluminum foil and stored in the dark at 4°C.

E.2 Test Organisms

Obtain red drum juveniles at approximately three weeks post-hatch, from one of the one-acre outdoor ponds located at the TPWD hatchery in Lake Jackson, Texas. Capture juvenile red drum from the deepest section of the pond, near the weir, using a long-handled dip net. Transfer fish from the net to buckets filled with pond water aerated with oxygen. Immediately transport fish to the TPWD hatchery incubation room for acclimation and testing.

Acclimate juvenile fish to 100% dilution/culture (source) water and hold for 2 days before test initiation to fully acclimate fish to testing water characteristics. Keep fish within 2°C of the test temperature for the 48 hours immediately before initiation of the study (see test-specific TCTs). Feed fish commercial fish food at least twice daily during the holding and test periods. See Source water and Fish culturing sections of the *Stratus Consulting General Laboratory Procedures and Practices – Red Drum, Speckled Seatrout, and Pacific White Shrimp* for additional information. Only healthy looking, vigorous juveniles should be used to stock test exposure tanks.

E.3 Test Media Preparation

Mix oil into uncontaminated sediments (ALAJ46-C1127-SB701B) to prepare test media; use a KitchenAid stand mixer using the following procedure:

1. For each treatment, weigh and thaw 4 kg of sediments overnight. Details regarding loading rates (grams of oil/kg of sediment) used for each treatment can be found in test-specific TCTs.

2. Starting with the control treatment, weigh out approximately 4 kg of sediment, dispense into a KitchenAid mixing bowl, turn mixer on for 30 minutes at moderate speed (4.5 on mixer), and scrape the sides of the bowl with a metal spatula every 2–4 minutes as needed.

3. Transfer control sediment to a clean, decontaminated mixing bowl and cover with aluminum foil.

4. Working in order from low to high concentration treatments, measure out required mass of slick oil in a pre-cleaned aluminum weigh boat or glass beaker. Tare a weigh boat or beaker and 2–3 Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat.

5. Weigh approximately 4 kg of sediment, dispense into the KitchenAid mixing bowl, and transfer the oil from the measuring vessel into the mixing bowl, placing it in several areas around the bowl. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass of oil transferred.

6. Mix the oil into the sediment for 30 minutes at moderate speed (4.5 on mixer) and scrape the sides of the bowl with a metal spatula every 2–4 minutes as needed.

7. Once the mixing is complete, transfer sediment oil mixture to a clean, decontaminated stainless steel mixing bowl and cover with aluminum foil. Be sure to thoroughly scrape the mixer bowl and paddle with a stainless steel putty knife or spoon to remove all of the excess oiled sediment.

8. Repeat steps 4 through 7 until all of the sediments in each treatment are prepared.

9. For each treatment, load four pre-cleaned exposure tanks and an 8-oz glass analytical chemistry sample sediment jar with prepared sediment. To load, add scoops of approximately 200 g sediment to each tank followed by a smaller scoop of approximately 10 g sediment to the sample jar. Repeat 5 times so that all sediment is used and each tank contains approximately 1–1.2 kg of wet sediment with the sample jar containing approximately 50 g of sediment (i.e., 3/4 full). To ensure sediment is equally divided among four replicate tanks, weigh each tank with sediment, and adjust volumes as needed.

10. Randomly place tanks with sediment back onto the rack system and replace tank lids, airlines, waterlines, and effluent lines.

11. Store labeled samples at 4°C until shipment to ALS Environmental using guidelines described in the project QAPP.

12. After all tanks are loaded with their appropriate sediment mixtures, open up the water inflow values and adjust to a slow and steady flow until each tank is filled to just under the overflow.

13. Turn off the water inflow and airlines to all tanks and allow the sediment to settle in the tank overnight under static conditions.

14. Several hours before beginning the test, turn on the flow-through water and aeration. Do this by opening up water inflow values and use ball valves to adjust inflow to a slow and steady flow. Flow rates should be set by observing the stream of water flowing into each tank. A slow flow rate is set at the point where the incoming water is a series of individual drops that are just less than a steady solid stream. This steady series of drops equates to approximately 3 L per hour flowing into each exposure tank. The air lines should then be turned on and adjusted to a slow inflow at one bubble per second.

15. After a few hours of running clean source water through the exposure tanks, take water quality measurements near the sediment/water interface. If the water quality is similar to the source water, then it is acceptable to start loading test organisms into exposure tanks. If the water quality is not similar to the source water, check water quality at the water surface. If the water surface water quality is better than at the sediment/water interface, increase the air and allow more time to equilibrate. Recheck the water quality at the sediment-water interface and near the surface, as needed, until it is similar to the source water.

E.4 Test Initiation

Tests will be initiated when juvenile red drum are transferred to each exposure tank following the procedure described below.

1. Set up a digital balance, a fish measuring board with millimeter increments, and a few 1-L plastic pour beakers for transferring fish. Also obtain enough *Tank ID Dilution or Stock Code Definitions* bench sheets to document start times for each treatment and tank and enough *Organism Length and Weight* bench sheets to record individual fish lengths and weights for each treatment and tank.

2. Obtain a random grab of juvenile red drum from the holding/incubation tank and transfer them into a clean tub of source water. Aerate the staging tub with an air stone while working on fish.

3. Randomly capture one fish from the staging tub by hand and inspect for gross abnormalities. Body condition should also be assessed. Abnormal or emaciated individuals will not be used for testing. Do not use MS-222 or any other method to anesthetize fish.

4. Place normal-looking juvenile fish on a damp measuring board and determine standard length to the whole millimeter. Standard length is measured from the tip of the upper jaw to the end of the hypural plate where the vertebral elements support the rays of the caudal fin.

5. After measuring standard length, quickly and carefully transfer the fish to a small weigh boat with water on a tared digital balance. Record the weight of the fish to the nearest hundredth of a gram.

6. Pour the fish from the weigh boat to a 1-L plastic pour beaker filled with fresh source water.

7. Repeat steps 2 to 6 until 10 individual red drum are measured and weighed.

8. Transfer all 10 fish to a randomly selected treatment exposure tank by lifting the tank lid and slowly pouring fish and water to avoid disturbing sediments. Record the time when transferred on the *Tank ID Dilution or Stock Code Definitions* data entry bench sheet.

9. Repeat steps 2 through 8 until all tanks have been stocked with 10 measured and weighed fish. Note that after every 4 to 5 tanks, return the batch of fish in the staging tub to the holding tank, and net a new random grab of fish and transfer them into the staging tub filled with fresh source water.

10. Collect a composite Day 0, unfiltered water sample from the control and each oil treatment for polycyclic aromatic hydrocarbon (PAH) analysis. Each 250-mL amber glass bottle will be filled using approximately 60 mL of water from each of the four respective treatment tanks. Transfer the 60-mL aliquot from each tank into each bottle using a new, glass 25-mL serological pipette. Carefully collect water samples from under the water surface so sediments are not disturbed.

E.5 Test Maintenance

Tests will be monitored and maintenance performed at least twice daily for the entire test duration. Monitoring and maintenance will include assessing test performance, checking water quality, feeding test organisms, and verifying that the exposure system is working as designed by following the procedure described below.

1. Each morning, before doing any maintenance procedures that would potentially disturb the fish and cloud the water with suspended sediment, slowly and methodically search each tank for dead fish.

2. If a dead fish is observed, remove it using long-handled forceps or your gloved hand, and take and record standard length and weight measurements on a new *Organism Length and Weight* bench sheet. If the dead fish is too decomposed to be handled, forgo this step. Archive each dead fish or remnants thereof according to the project QAPP. If the tank is too cloudy to be thoroughly inspected, note it on the *Test Performance Monitoring* bench sheet.

3. After inspecting tanks and removing any dead fish, take water quality measurements. At a minimum, water quality measurements (temperature, pH, DO, conductivity/salinity, and total ammonia) will be taken from one tank from each treatment per day, so that after four days all tanks will be sampled. If warranted (i.e., aeration system stops working, all fish from a tank die suddenly), take water quality measurements from more tanks.

4. After recording water quality measurements, assess pH, DO, and total ammonia water quality results to determine if the tank inflow and aeration are sufficient for maintaining good water quality. Target water quality parameters are: pH > 7.50, DO > 4 mg/L, and total ammonia < 1 mg/L. If tanks are not meeting target water quality parameters, increase the inflow and/or increase aeration.

5. After taking water quality measurements and adjusting inflows as needed, feed each tank with the standard ration of prepared fed (Salmon Starter #2, Brand). The standard ration is 0.25 g of feed per feeding, with two feedings per day.

6. If *Artemia* are available, squirt approximately 1 mL of concentrated nauplii into each tank using a graduated, disposable pipette.

7. On day 1, 2, 4, 7, or 10, take water chemistry samples. Collect one unfiltered composite water sample from the control and each oil treatment for PAH analysis. Each 250-mL amber glass bottle will be filled using approximately 60 mL of water from each of the four respective treatment tanks. Transfer the 60-mL aliquot from each tank into each

bottle using a new, glass 25-mL serological pipette. Carefully collect water samples from under the water surface so sediments are not disturbed. Store labeled samples at 4°C until shipment to ALS Environmental, using guidelines described in the project QAPP.

8. Next, inspect the exposure system and, if necessary, replace the filter cartridges. Cartridges will be replaced at least once daily, but may need to be replaced more often when water is backing up in the sump while the pump is running, the pump housing is hot to the touch, or the pump is constantly running with water above the sump float switch. Also check that the water supply in the head tank is maintaining a consistent head, water lines are flowing, airlines are bubbling, and drain lines inserted into the drain pipe manifold are not clogged. Tank overflow bulkhead screens may also need to be periodically cleaned. Do this by having a spare, clean screen assembly on hand; remove the old screen and immediately replace with the clean screen. Do not leave the overflow bulkhead unscreened for more than a second or two while it is replaced with a clean screen.

9. Return in the late afternoon/early evening to feed fish. Perform the second daily feeding by adding 0.25 gm of feed into each tank.

10. Repeat steps 1 through 9 each day of the test. On the day the test is terminated, assess each tank for dead fish, conduct water quality measurements, and feed fish. In addition, collect end-of-test water chemistry samples. Water chemistry samples will be taken as described in step 7 above. Additional test termination procedures are described in the next section.

E.6 Test Termination

Upon test termination, testing staff should have all or most of the sample containers pre-labeled and water quality, first feeding, and water chemistry sampling described in Section E.5 above completed. All assessment and tissue sampling equipment and reagents should also be prepared and ready for use before the following test termination protocols are followed:

1. Pull the first randomly selected exposure tank from the system. Disconnect and turn off the inflow and airline tubing. The outflow tubing line may also need to be disconnected from the outside hose barb on the exposure tank overflow bulkhead.

2. Net all of the fish from the tank and transfer into a 2-L plastic pour beaker with fresh source water, aerated with an air stone. You may have to carefully pour the tank overlying water through a net over a bucket to capture all the fish in the tank and ensure that no fish remain in the tank. Decant as much overlying water as possible into a bucket.

Using a clean decontaminated stainless steel scoop, thoroughly mix and scoop out approximately 2 ounces of sediment into a treatment-specific 8-oz composite sediment chemistry sampling jar. This process will be repeated with each tank until each jar is filled with sediment from all remaining replicates for that treatment. Keep jars capped and cool between tanks. Dump the remaining sediment into a waste bucket for offsite disposal. Note that most of step 2 can be done while other testing staff are working on fish from the previous tank; however, to limit the length of time fish are held in the 2-L pour beaker, do not break down and net fish out of more than one tank while fish from the previous tank are being worked on.

3. Label the 2-L pour beaker containing the fish and bring into the wet laboratory for weight/length measurements and dissection.

4. Once fish are delivered to the wet laboratory, testing staff will immediately start to work on the fish.

5. First, randomly grab one to two fish from the 2-L pour beaker and place into a weight boat containing a 400-mg/L solution of MS-222 in source water. Wait approximately 30 seconds. until all opercular movement has stopped.

6. Next, transfer one of the fish from the MS-222 bath to the measuring board and record standard length to the nearest millimeter. Record results on the *Organism Length and Weight* bench sheet.

7. Next, transfer the fish from the measuring board to a new, clean dry weigh boat on a tared balance and weigh to the nearest hundredth of a gram. Record results on the *Organism Length and Weight* bench sheet.

8. After recording weight, transfer the weigh boat and fish to the microbiome sampling station.

9. Using clean, ethanol dipped, and flamed micro scissors, carefully cut and peel back the right gill operculum to expose the gills. Detach one to three gill arches by cutting the dorsal and ventral ends of the gill arches. Using the same micro scissors, transfer the gill tissue to a 1.5-mL certified DNA/RNA free micro centrifuge tube. Fill micro centrifuge tube with at least 1 mL of RNAlater RNA Stabilization Reagent (Qiagen, Duesseldorf, Germany) to fully submerse the tissue.

10. Using a second set of clean, ethanol dipped, and flamed micro scissors, carefully slice open the abdominal cavity from the anus to the head and tease out the gastrointestinal tract (GI). If possible, pull back the GI to expose its attachment to the stomach. Try not to disturb any other organs or tissue. Detach the GI from the stomach and anus so that the

entire GI is sampled. Using the same micro scissors, transfer the GI tissue to a 1.5-mL certified DNA/RNA-free micro centrifuge tube. Fill the micro centrifuge tube with 1 mL of RNAlater RNA Stabilization Reagent (Qiagen, Duesseldorf, Germany) to fully submerse the tissue.

11. Between each use, clean dissection tools with soap and hot water, dip into 90% ethanol, flame off remaining ethanol, and let air dry on a new, clean paper towel before using to collect microbiome samples.

12. Place fish and remaining tissues into a 50-mL conical Falcon tube with 10% buffered formalin. All post-dissection fish from one exposure tank/replicate will be stored in the same conical Falcon tube.

13. Repeat steps 5 through 12 until the first four fish in each tank are sampled for microbiome and histological analyses. If needed, top off the 50-mL Falcon tube with more 10% buffered formalin and store microbiome samples at 4°C for the first 24 hours, then transfer to -20°C.

14. Measure and weigh each remaining fish as described in steps 5 through 7. After weighing each fish, use a scalpel or micro scissors to carefully cut open the abdominal cavity laterally from the anus to the isthmus and place into a 50-mL Falcon tube with 10% buffered formalin. All remaining fish from one exposure tank/replicate not sampled for potential microbiome analysis will be stored in the same conical Falcon tube.

15. If needed, top off the 50-mL Falcon tube with more 10% buffered formalin and store at room temperature.

16. Repeat steps 1 through 14 until all exposure tanks have been taken down and fish and sediments have been sampled.

17. Clean exposure tanks and system. All remaining sediments from a single test should fit into one 5-gallon bucket. Oil-contaminated overlying water generated during tank breakdown and cleaning should be decanted into the sump of the exposure system with the filtration system running. Any solids and oily water remaining in the sump after disconnecting the filter system will be collected in 5-gallon buckets. Buckets containing oily water and sediments will be capped and transported to the nearest offsite oil waste recycling center for disposal. Any oil water generated during testing activates will also be containerized and either dropped off or be picked up by an offsite oil waste recycling company.

F. Testing Protocol 6: Pacific White Shrimp Chronic Spiked Sediment Exposures

F.1 General Considerations

Decontaminate all glassware, spatulas, and other materials according to the *Decontamination SOP*, described in the QAPP.

Follow the *Analytical Sample Shipping and COC SOP* found in the QAPP for sample collection, labeling, and handling. Store all analytical samples at 4°C unless otherwise noted; record all necessary sample numbers and prepare necessary COC documentation, as described in the QAPP.

Place any unused prepared sediment into a Ziploc bag and store in the dark at 4°C (short-term) or in a freezer at -20°C (long-term). Store large quantities (> 5 kg) of prepared sediment in large metal pans, covered with aluminum foil, and stored in the dark at 4°C.

F.2 Test Organisms

Obtain PL Pacific white shrimp, 8 to 10 mm in total length, from SIS. Transfer all of the shrimp and the water they were shipped in to an empty, clean incubation tank. Turn on the inflow so that a small trickle of source water begins to fill the incubation tank. After approximately 12 hours trickling water into the incubation tank, measure incubation tank water quality and compare to raw source water quality. If incubation water quality is similar to source water quality, open the incubation tank inflow to a steady stream. Inflow should be set at a rate that is sufficient to cause some circular current around the tank, but not overwhelm the incubation tank overflow screens or rack system sump and filter. Refer to the *Stratus Consulting General Laboratory Procedures and Practices – Red Drum, Speckled Seatrout, and Pacific White Shrimp* for a detailed description of the shrimp incubation and rack system. Hold PL shrimp in 100% dilution/culture (source) water for 2 days before test initiation to fully acclimate fish to testing water characteristics. Keep shrimp within 2°C of the test temperature for the 48 hours immediately before initiation of the study (see test-specific TCTs).

F.3 Test Media Preparation

Mix oil into uncontaminated sediments (ALAJ46-C1127-SB701B) using a KitchenAid stand mixer, following the procedure described below.

1. For each treatment, weigh and thaw 4 kg of sediments overnight. Details regarding loading rates (grams of oil/kg of sediment) used for each treatment can be found in test-specific TCTs.

2. Starting with the control treatment, weigh out approximately 4 kg of sediment, dispense into a KitchenAid mixing bowl, turn mixer on for 30 minutes at moderate speed (4.5 on mixer), and scrape the sides of the bowl with a metal spatula every 2–4 minutes as needed.

3. Transfer control sediment to a clean, decontaminated mixing bowl and cover with aluminum foil.

4. Working in order from low to high concentration treatments, measure out required mass of slick oil in a pre-cleaned aluminum weigh boat or glass beaker. Tare a weigh boat or beaker and 2–3 Kimwipes on the top loading balance. Using a stainless steel spatula, add slightly more than the desired mass of oil onto the weigh boat.

5. Weigh out approximately 4 kg of sediment, dispense into a KitchenAid mixing bowl, and transfer the oil from the measuring vessel into the mixing bowl, placing it in several areas around the bowl. Wipe off any oil remaining on the spatula with the tared Kimwipes. Reweigh the weigh boat and Kimwipes to calculate and record the actual mass of oil transferred.

6. Mix oil into the sediment for 30 minutes at moderate speed (4.5 on mixer) and scrape the sides of the bowl with a metal spatula every 2–4 minutes as needed.

7. Once the mixing is complete, transfer the sediment oil mixture to a clean, decontaminated stainless steel mixing bowl and cover with aluminum foil. Be sure to thoroughly scrape the mixer bowl and paddle with a stainless steel putty knife or spoon to remove all of the excess oiled sediment.

8. Repeat steps 4 through 7 until all of the sediments in each treatment are prepared.

9. For each treatment, load four pre-cleaned exposure tanks and an 8-oz glass, analytical chemistry sample sediment jar with prepared sediment. To load, add scoops of approximately 200 g sediment to each tank followed by a smaller scoop of approximately 10 g sediment to the sample jar. Repeat 5 times so that all sediment is used and each tank contains approximately 1–1.2 kg of wet sediment with the sample jar containing approximately 50 g of sediment (i.e., 3/4 full). To ensure the sediment is equally divided among four replicate tanks, weigh each tank with sediment, and adjust volumes as needed.

10. Randomly place sediment-loaded tanks onto the rack system and replace tank lids, airlines, waterlines, and effluent lines.

11. Store labeled samples at 4°C until shipment to ALS Environmental using guidelines described in the project QAPP.

12. After all tanks are loaded with sediment mixtures, open up water inflow values and adjust to a slow and steady flow until each tank is filled to just under the overflow.

13. Turn off the water inflow and airlines to all tanks and allow the sediment to settle in the tank overnight under static conditions.

14. Several hours before beginning the test, turn on the flow-through water and aeration. Do this by opening up water inflow values and use ball valves to adjust inflow to a slow and steady flow. Flow rates should be set by observing the stream of water flowing into each tank. A slow flow rate is set at the point where the incoming water is a series of individual drops, which is less than a steady solid stream. This steady series of drops equates to approximately 3 L per hour flowing into each exposure tank. Airlines should then be turned on and adjusted to a slow inflow at one bubble per second.

15. After a few hours of running clean source water through sediment loaded and settled exposure tanks, take water quality measurements near the sediment water interface. If the water quality is similar to source water, then it is acceptable to start loading test organisms into exposure tanks. If the water quality is not similar to the source water, check the water quality at the water surface. If the water surface water quality is better than at the sediment/water interface, increase the air and allow more time to equilibrate. Recheck the water quality at the sediment/water interface and near the surface, and increase the air and/or water inflow as needed until the exposure tank water quality is similar to the source water.

F.4 Test Initiation

Tests will be initiated when PL shrimp are transferred to each exposure tank following the procedure described below.

1. Obtain a few 2-L plastic pour beakers and enough *Tank ID Dilution or Stock Code Definitions* bench sheets to document start times for each treatment and tank.

2. Dip the plastic pour beaker into the shrimp incubation/holding tank to obtain a random grab of shrimp. Obtain a new random grab of shrimp for the incubation/holding tank after stocking approximately every four to five exposure tanks.

3. Using a disposable pipette with the tip cut off, aspirate a random grab of a few shrimp and dispense into a plastic Petri dish. Inspect each shrimp for gross abnormalities and presence of food in gut (PL shrimp are transparent). Dispose of any abnormal-looking shrimp and shrimp that do not contain food in their digestive system.

4. Repeat steps 2 and 3 until 20 individual, healthy-looking shrimp are in the plastic Petri dish.

5. Randomly select an exposure tank and gently pour shrimp from the Petri dish into the tank. Thoroughly inspect the Petri dish to ensure that all 20 shrimp were transferred. If needed, gently wash any remaining shrimp from the Petri dish into the exposure tank using a disposable pipette and source water. Record start time on the *Tank ID Dilution or Stock Code Definitions* bench sheet.

6. Repeat steps 2 through 5 until all exposure tanks have been stocked.

7. Collect a composite Day 0, unfiltered water chemistry sample from the control and each oil treatment for PAH analysis. Each 250-mL amber glass bottle will be filled using approximately 60 mL of water from each of the four respective treatment tanks. Transfer the 60-mL aliquot from each tank into each bottle using a new, glass 25-mL serological pipette. Carefully collect water samples from under the water surface while not disturbing the sediment. Confirm that shrimp are not aspirated into the pipette each time it is used to collect exposure tank water by thoroughly inspecting pipette contents behind a dark-colored background.

F.5 Initial Shrimp Length Measurements

Measure total shrimp length to the nearest millimeter for 40 individual shrimp and calculate an average length for the shrimp at the start of the test. Given that shrimp length measurements can cause damage to the shrimp, do not use any of these shrimp in test exposures. Follow the steps below to measure total shrimp length.

1. Using a 2-L plastic pour beaker, collect a sub-sample of shrimp from the stock shrimp tank.

2. Pour a sample with shrimp through a nylon screen to concentrate shrimp on the screen.

3. Using clean, metal forceps, carefully transfer an individual shrimp from the screen to a black, plastic metric ruler.

4. Align shrimp so that its rostrum is facing left and the body of the shrimp is fully stretched out.

5. Measure to the nearest millimeter the length of the shrimp starting from the rostrum to the tip of the uropod (i.e., tail).

6. Archive all in shrimp in a 15-mL conical tube filled with 10% neutral buffered formalin.

F.6 Test Maintenance

Tests will be monitored and maintained at least twice daily for the entire duration of the test. Monitoring and maintenance will include assessing test performance, checking water quality, feeding test organisms, and verifying that the exposure system is working as designed by following the procedure described below.

1. Take water quality measurements. At a minimum, water quality measurements (temperature, pH, DO, conductivity/salinity, and total ammonia) will be taken from one tank from each treatment per day, so that after four days all tanks will be sampled. If warranted, take water quality measurements from more tanks.

2. After recording water quality measurements, assess pH, DO, and total ammonia water quality results to determine if tank inflow and aeration are sufficient in maintaining good water quality. Target water quality parameters are: pH > 7.50, DO > 4 mg/L, and total ammonia < 1 mg/L. If the tanks are not meeting target water quality, turn up the inflow and/or increase aeration.

3. After taking water quality measurements and adjusting inflows as needed, feed each tank with the standard ration of *Artemia* nauplii. Fed each tank by aspirating 200 µL of nauplii feeding stock solution into a micro pipette and dispense into each tank. Keep the nauplii feeding stock solution well mixed when drawing standard rations. Refer to the next procedure in Section F.7 for a description of how to prepare the nauplii feeding stock solution.

4. After all shrimp exposure tanks have received food, pour remaining nauplii feeding stock into the incubation/holding tank.

5. On test Day 4, take water chemistry samples. Collect one unfiltered composite water sample from the control and each oil treatment for PAH analysis. Each 250-mL amber glass bottle will be filled using approximately 60 mL of water from each of the four respective treatment tanks. Transfer the 60-mL aliquot from each tank into each bottle using a new, glass 25-mL serological pipette. Carefully collect water samples from under

the water surface while not disturbing the sediment or aspirating shrimp. Store labeled samples at 4°C until shipment to ALS Environmental using the guidelines described in the project QAPP.

6. Next, inspect the exposure system and, if necessary, replace the filter cartridges. Cartridges will be replaced at least once daily, but may need to be replaced more often when water is backing up in the sump while the pump is running, the pump housing is hot to the touch, or the pump is constantly running with water above the sump float switch. Also check that the water supply in the head tank is maintaining a consistent head, water lines are flowing, airlines are bubbling, and drain lines inserted into the drain pipe manifold are not clogged. Tank overflow bulkhead screens may also need to be periodically cleaned. Do this by having a spare, clean screen assembly on hand; remove the old screen and immediately replace with a clean screen. Do not leave the overflow bulkhead unscreened for more than a second or two while it is replaced with a clean screen.

7. Return in the late afternoon or early evening to conduct the second daily feeding. Perform the second daily feeding by adding 200 µL of nauplii feeding stock solution into each tank using a micro pipette. Pour all remaining nauplii feeding stock solution into the incubation/holding tank after all exposure tanks have received food.

8. Repeat steps 1 through 7 each day of the test. On the day the test is terminated, conduct water quality measurements and collect end-of-test water chemistry samples; however, do not feed the shrimp. Collect water chemistry samples following procedures described in step 5 above. Additional test termination procedures are described in the Test Termination (Section F.8) procedures section.

F.7 Preparation of Nauplii Feeding Stock Solution

Nauplii feeding stock solution is used to feed experimental shrimp and maintain shrimp held in the incubation/holding tank throughout testing activities. Two batches of 24-hours-old *Artemia* nauplii cultures will be cultured, but staggered in age by approximately 12 hours so that each culture is used to make a fresh feeding stock during each feeding. Stocks are prepared by testing staff daily following the procedure described below.

1. Thoroughly clean one 10-L *Artemia* hatching cone using clean source water and a soft bristled brush.

2. Set hatching cone into the cone rack and ensure that the bottom petcock is closed.

3. Fill hatching cone with 10 L of clean, UV-sterilized, 5-µm, filtered TPWD hatchery source water. The UV sterilized filtered source water can be obtained from the source water hose attached to the filter system located at the back of the incubation room, near the shrimp holding tank. Ensure that the 250-gal holding tank that is attached to the filter system does not run dry. Fill the holding tank with raw source water as needed.

4. Insert a hard plastic air line into the hatching cone and adjust airflow so that water becomes turbulent, but does not bubble over the top.

5. Check the salinity of hatching cone water and add commercial sea salt mix or RO water to adjust salinity to approximately 32 ppt.

6. Obtain *Artemia* cysts from the refrigerator, measure out 40 grams of cysts, and pour into one hatching cone.

7. After approximately 12 hours, turn off or remove airline and let settle for approximately 1 hour. Turn on the spotlight and aim it at the bottom of the hatching cone to aid settlement. Settlement is complete when empty and unhatched cysts are floating, the center section is clear, and the bottom contains a thick layer of reddish-orange, live nauplii.

8. Harvest all of the bottom layer of nauplii by opening up the bottom petcock over an empty, clean 2-L plastic pour beaker. Close the petcock when almost all of the bottom layer has drained.

9. Add clean source water to the 2-L plastic pour beaker to bring the total volume of nauplii stock to 1 L.

10. Use stock solution to feed shrimp exposure tanks and holding tank. Stock solution should contain approximately 3,000 nauplii per millimeter.

11. Repeat steps 1 through 10 after each feeding.

F.8 Test Termination

Upon test termination, testing staff should have all or most of the sample containers pre-labeled and water quality, first feeding, and water chemistry sampling (described in Section E.6) completed. All equipment and reagents should also be prepared and ready for use before the following test termination protocols are performed:

1. Pull the first randomly selected exposure tank from the system. Disconnect and turn off the inflow and airline tubing. The outflow tubing line may also need to be disconnected from the outside hose barb on the exposure tank overflow bulkhead.

2. Carefully pour the tank overlying water and shrimp through a 200-µm Nitex screen positioned over a bucket to capture water as it is poured through the screen. Decant as much overlying water as possible, without disturbing sediment, through the screen. Visually inspect to ensure no shrimp are still in the tank.

3. Pour clean source water through the screen to rinse as much sediment off the screen as possible, and place the screen with shrimp into the tray. Rinse shrimp from the screen into a staging tub filled with clean source water. Ensure that no shrimp remain on the screen. Thoroughly clean or replace the screen between tanks.

4. Transfer shrimp from the screen to an aluminum weigh boat using metal forceps. Blot dry, count, and weigh. Record counts on the *Test Performance Monitoring Bench Sheet* and weights on the *Weight and Length Bench Sheet.*

5. Transfer all shrimp from the weigh boat to a 15-mL conical Falcon tube containing 10% neutral buffered formalin. It may be necessary to decant most of the formalin and refill with fresh formalin after all shrimp have been added.

6. Repeat steps 1 through 5 until all exposure tanks have been taken down and shrimp and sediments have been sampled.

7. Using a clean decontaminated stainless steel scoop, thoroughly mix and scoop out approximately 2 oz of sediment into a treatment-specific 8-oz composite sediment chemistry sampling jar. This process will be repeated with each tank until each jar is filled with sediment from each replicate tank for each treatment. Cap jars and store at 4°C until shipped to ALS for analysis. Discard remaining sediment into a waste bucket for offsite disposal.

8. Clean exposure tanks and system. All remaining sediments from a single test should fit into one 5-gal bucket. Oil-contaminated overlying water generated during tank breakdown and cleaning should be decanted into the sump of the exposure system with the filtration system running. Any solids and oily water remaining in the sump after disconnecting the filter system will be collected in 5-gal buckets. Buckets containing oily water and sediments will be capped and transported to the nearest offsite oil waste recycling center for disposal. Any oil water generated during testing activates will also be containerized and either dropped off or picked up by an offsite oil waste recycling company.

9. The following day, transfer shrimp from the 15-mL sample tube with 10% neutral buffered formalin to a small holding vessel (e.g., weigh boat). Pull off excess formalin with a transfer pipette and return to the 15-mL tube.

10. Measure final shrimp length for each individual in the sample according to steps 3–5 in Section F.5. Once the length has been measured, place individual shrimp back in the appropriate sample tube.

11. Repeat steps 9 and 10 for each shrimp sample so that the total length of all individual shrimp collected during test termination has been measured.

15. Marin Biologic Laboratories, Inc. General Laboratory Procedures and Practices

15.1 Introduction

Marin Biologic Laboratories, Inc. (Marin), conducted human adrenal cell line toxicity tests to identify toxicological effects of the 2010 *Deepwater Horizon* (DWH) oil spill. This chapter describes general laboratory practices and procedures used at Marin. Marin provided Stratus Consulting with work plans and data reports describing the procedures used to conduct each test. Stratus Consulting used information from work plans and reports to prepare this General Laboratory Procedures and Practices (GLPP) chapter.

15.2 Testing Methods

Marin tested the effects of DWH oil exposure on cortisol, aldosterone, and progesterone production, as well as steroidogenesis genes in human adrenal cells. Additional adrenal cell line exposures were conducted to characterize adrenocorticotropic hormone (ACTH) and foskolin activity in stimulating cortisol and aldosterone production.

15.2.1 Test organisms

Experiments conducted by Marin used the human adrenal cell line H295R obtained from the American Type Culture Collection (ATCC) and grown in culture according to the ATCC standard protocol (available at http://www.atcc.org/products/all/CRL-10296.aspx#culturemethod).

The base-growth medium for this cell line is ATCC-formulated DMEM:F12 Medium (ATCC Catalog No. 30-2006). The base-growth medium was amended to include the following components; 0.00625 mg/mL insulin, 0.00625 mg/mL transferrin, 6.25 ng/mL selenium, 1.25 mg/mL bovine serum albumin, and 0.00535 mg/mL linoleic acid. After these additions, the amended base-growth medium was adjust to a final concentration of 2.5% Nu-Serum IV (Corning #355104) and 1% penicillin/streptomycin antibiotic (Penn Strep). Additives [insulin, transferrin, selenium, bovine serum albumin (BSA), and linoleic acid] were obtained from ITS + Premix (Corning #354352).

Adherent adrenal cells were grown at 37°C in a 5% CO_2-air incubator. Cells were sub-cultured by removing the culture medium and briefly rinsing with TryplExpress (trypsin-EDTA solution). After 5 to 15 minutes, the adherent cell layer dispersed. The cells were then diluted with the appropriate volume of fresh growth medium. The target density when sub-culturing cells was 4-8 x 10^4 cells/cm^2.

A cell culture was established by conducting an initial freeze of six cell vials in liquid nitrogen. To determine the proper conditions of maintenance for the cell line and for initiating a cell assay, a seven-day grow-out study was performed and growth curve established.

15.2.2 Exposure media preparation

Human adrenal cells were exposed to different concentrations of high-energy water accommodated fractions (HEWAFs) and low-energy water accommodated fractions (LEWAFs) that were prepared according to the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. Standard water accommodated fraction (WAF) preparation bench sheets used to document the analyst, method steps, volumes/weights, and times when preparing HEWAFs and LEWAFs.

Slick A (DWH4748co or DWH4748cp), Slick B (DWH7616), and artificially weathered source oil (DWH7977) HEWAFs and Slick A (DWH4748co or DWH4748cp) LEWAFs were tested. WAFs were prepared using cell-line growth medium without fetal bovine serum or other protein supplements, to avoid their inactivation during sonication. These protein supplements were added at the appropriate concentrations for testing.

All WAF preparations were filtered using a single 0.7-µm glass fiber filter as described in Appendix G of the QAPP.

Aliquots (250 mL) of each undiluted HEWAF or LEWAF were sampled and sent for chemical analysis to ALS Environmental as described in the project QAPP.

15.3 Reporting and Testing Documentation

A workbook was created to document the analyst, method steps, volumes/weights, and times used. This workbook was used for all assays. In addition, data reports were prepared for each experiment. Raw data files were also submitted to Stratus Consulting.

A. Testing Protocol 1: Assessing HEWAFs for Potential Microbial Contamination

HEWAFs prepared with Slick A, Slick B, and artificially weathered source oil were tested for microbial contamination before initiation of definitive tests.

A.1 Test Design

For microbial contamination assessment, each test included four different HEWAF concentrations prepared in the cell culture media prepared as described in the Marin GLPP, but with the penicillin/streptomycin antibiotic added. Tests were carried out in 96-well flat bottom plates and 5-mL T-25 culture flasks. Cultures were maintained at 37°C in a humidified incubator for up to seven days and monitored microscopically for microbial outgrowth. All treatments were run in triplicate.

B. Testing Protocol 2: Assessing Cell Growth Following Exposure to HEWAF

B.1 Test Design

To establish sub-lethal effect concentrations for subsequent HEWAF tests, three dilutions of HEWAF were assessed for their effect on H295R cell line growth; these were the same three HEWAF concentrations tested in Testing Protocol 1. Cell growth was assessed daily for seven days by fluorescence using Alamar blue dye. At each time point, 20 µL of 0.6 M Alamar blue was mixed with 200 µL of cell cultures in a white, 96-well plate. After 1–4 hours of incubation, staff recorded the fluorescence resulting from the intracellular reducing conditions of live cells. Cell growth in each treatment was reported as growth relative to a control cell culture. All treatments were run in triplicate.

C. Testing Protocol 3: Assessing Effects on Steroid Production and Gene Expression of Adrenal Cells Following Exposure to HEWAF

HEWAF made from two oil samples was tested for effects on cortisol, aldosterone, and progesterone production; and STARD1, CYP11A1, and HSD3B2 gene expression in the human adrenal H295R cell line.

Each test included five HEWAF concentrations, prepared using 1:2 serial dilutions starting at 1:10 stock HEWAF to growth medium, and two sets of control treatments. Additionally, 20 µL of 10^{-6} M ACTH was added to one set of controls to serve as a positive control. All treatments were run in triplicate.

C.1 Cell Culture Exposure

1. Plate 2 mL H295R cells in wells of a 12-well plate at 4 x 10^4 cells/cm^2 and allow adherence for 24 hours

2. Remove the supernatant and add 2 mL of appropriate WAF treatment, or ACTH agonist

3. Incubate cultures at 37°C in a humidified CO_2 incubator for 48 hours.

C.2 Steroid Assays (Cortisol, Aldosterone, and Progesterone)

1. After 48 hours of exposure, collect culture medium from 96-well plate and freeze the sample at -80°C until analysis.

2. For progesterone and aldosterone, thaw samples on ice and assayed directly, using Cayman Enzyme Immunoassay (EIA) kit #582601 at a 1:20 dilution of samples, or using IBL International kit #RE52301 with undiluted samples, respectively.

3. For cortisol, extract samples before analysis. To extract, thaw medium on ice and acidify 500-µL samples to ~ pH 2 with three drops of 3M HCl.

4. Extract two times with 2–5 mL diethyl ether.

5. Evaporate under a gentle stream of nitrogen to remove all solvent.

6. Reconstitute the remaining residue in 500 µL enzyme-linked immunosorbent assay (ELISA) buffer (Cayman Chemical, Ann Arbor, MI, USA) and vortex several times to mix. Freeze extract and store at -80°C until further analysis.

7. Measure steroids by competitive ELISA, following the manufacturer's procedures for cortisol kit #500360, aldosterone kit #10004377, progesterone kit #582601 (Cayman Chemical, Ann Arbor, MI).

C.3 Quantitative PCR Assay for Gene Expression

For this procedure, gene expression was measured by quantitative polymerase chain reaction (qPCR) and expressed relative to a housekeeping gene. In addition, transcription of three steroidogenic genes, STARD1, CYP11A1, and HSD3B2, was quantified in H295R cells.

1. Expose cells according to procedures in Section C.1.

2. After 48 hours, extract and purify RNA from cells using RNeasy Mini Spin Columns (Qiagen #74104), QIA Shredder (Qiagen #79656), and RNase-Free DNase Set (Qiagen #79254).

3. Prepare complementary DNA (cDNA) from each RNA sample. For reverse transcription, use the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA).

4. Perform real-time qPCR on the resulting cDNA by mixing 10 µL TaqMan Gene Expression Master Mix, 1 µL TaqMan gene expression assay, 5 µL RNase-free water, and 4 µL cDNA template. Set the thermal cycle profile to the following: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles at 95°C for 15 s; and 60°C for 1 minute.

5. Quantify expression of mRNA using the Ct method. Ct is the PCR cycle at which the signal crosses the amplification threshold value set in the linear phase of the reaction. Normalize the Ct values for each gene of interest to the "housekeeping" gene β-actin. Use normalized values to calculate the degree of induction or inhibition and express as a "fold difference," compared to normalized control values. Report all data as the statistical difference in "fold induction" between the exposed and control cultures.

D. Testing Protocol 4: Assessing Steroidogenesis Effects on Adrenal Cells Following Exposure to HEWAF and LEWAF

Adrenal cells were exposed to HEWAF and LEWAF made with Slick A oil to determine their effect on cortisol and aldosterone production to the supernatant by the human adrenal cell line H295R after 2 and 7 days of incubation and to determine their CYP11A1 and CYP11B2 gene expression**.** All treatments were run in triplicate.

D.1 Cell Culture Exposure

Adrenal cells were assessed for steroidogensis effects following two and seven days of exposure. For each test, a matrix of six WAF concentrations (including control) and up to three agonist conditions (ACTH induction, forskolin induction, or no agonist) were assessed. All treatments were run in triplicate. Different initial cell concentrations are necessary to achieve approximately 50–90% confluence at the Day 2 and Day 7 time points.

1. For the two-day exposures, plate 2 mL H295R cells at 4 x 10^4 cells/cm^2 in a 12-well plate and allow adherence for 24 hours. For the seven day exposures, plate 2 mL of H295R cells at 1 x 10^4 cells/cm^2 in a 12-well plate and allow adherence for 24 hours.

2. After adherence, aspirate off the cell culture medium and replace with 2 mL of the appropriate treatment medium. For the two-day exposures, also add appropriate agonist to wells: use a 1-µM solution for ACTH and a 5-µM solution for forskolin. For the seven-day exposures, add agonist to wells on the fifth day.

3. Incubate cultures at 37°C in a humidified CO_2 incubator for 2 or 7 days based on the treatment (see test-specific TCT for exposure durations).

D.2 Viable Cell Measurement

After appropriate incubation time (2 or 7 days), quantify viable cells to normalize assay results.

1. To quantify viable cells, remove 1 mL of cell culture supernatant from each well and set aside for assays

2. Add 100 µL of Alamar blue to the remaining cell culture in each well and incubate for 2 to 4 hours

3. Measure fluorescence at 560 nm/590 nm.

D.3 Steroid Assays

1. For aldosterone quantification, pipette 100 µL of the cell culture supernatant that was set aside, above, into a 96-well plate from Cayman EIA kit #582601 or an IBL International kit #RE52301, and follow instructions. For cortisol, acidify 100 µL of cell culture supernatant to ~ pH 2 using three drops of 3M HCl.

2. Extract two times with 2–5 mL diethyl ether solvent.

3. Evaporate under a gentle stream of nitrogen to remove all solvent.

4. Reconstitute the remaining residue in 500-µL EIA buffer provided with the kit (Cayman Chemical, Ann Arbor, MI, USA, Kit #500360) and vortex several times to mix.

5. Dilute samples 1:10 in EIA buffer and then analyze according to instructions for Cayman EIA kit #500360.

6. If not analyzed immediately, freeze extract and store at -80°C until analysis.

D.4 Gene Expression

This protocol describes the quantification of CYP11A1 and CYP11B2 gene expressions measured by QPCR and expressed relative to a housekeeping gene.

1. On Day 2 and Day 7, collect cell culture supernatant from each well, and spin to create cell pellet.

2. Freeze and store at -80°C until extraction. Extract RNA from cell pellets and purify using RNeasy Mini Spin Columns (Qiagen #74104), QIA Shredder (Qiagen #79656), and RNase-Free DNase Set (Qiagen #79254).

3. Prepare cDNA from each RNA sample. For reverse transcription, use the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA).

4. Perform real-time qPCR on the resulting cDNA by mixing 10 µL TaqMan Gene Expression Master Mix, 1 µL TaqMan gene expression assay, 5 µL RNase-free water, and 4 µL cDNA template. Set the thermal cycle profile to the following: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles at 95°C for 15 s; and 60°C for 1 minute.

5. Analyze cDNA for expression of the genes CYP11A, CYP11B2, β-actin "housekeeping gene."

6. Quantify expression of mRNA using the Ct method. Ct is the PCR cycle at which the signal crosses the amplification threshold value set in the linear phase of the reaction. Normalize the Ct values for each gene of interest to the "housekeeping" gene β-actin. Use normalized values to calculate the degree of induction or inhibition and express as a "fold difference," compared to normalized control values. Report all data as the statistical difference in "fold induction" between the exposed and control cultures.

16. Louisiana Universities Marine Consortium General Laboratory Procedures and Practices

16.1 Introduction

The goal of this project is to determine the effects of *Deepwater Horizon* (DWH) oil on mortality rates, hatch rates, and the development of four resident fish species in the Gulf of Mexico. These endpoints were assessed in a series of toxicity tests conducted on Gulf menhaden (*Brevoortia tyrannus*) embryos, larvae, and juveniles; Bay anchovy (*Anchoa mitchilli*) embryos and larvae; red snapper (*Brevoortia patronus*) embryos; and sand seatrout (*Cynoscion arenarius*) embryos.

16.2 Methods

16.2.1 Test organisms

Menhaden and sand seatrout embryos: Wild Gulf menhaden and sand seatrout eggs were collected at sea approximately 25–30 miles offshore into the Gulf of Mexico from Cocodrie, LA. Eggs were collected with a neuston net (0.8-mm mesh) that was towed at the water surface. Unsorted net collections were transported back to the Louisiana Universities Marine Consortium (LUMCON) Marine Center in large coolers. At the laboratory, menhaden eggs were sorted from other plankton by allowing them to float and skimming them with 500-µm netting or a small beaker. The skimmed embryos were transferred into clean, filtered seawater with the same temperature and salinity as the seawater at the collection site; the embryos were kept in this water until the test began. Collections occurred in February and March, as weather allowed.

Surface temperatures and salinity measurements were collected during egg collection to help determine an appropriate temperature during housing, exposures, and post-exposure incubations.

Menhaden juveniles: Wild Gulf menhaden juveniles were collected by cast net in the LUMCON boat basin near Cocodrie, LA and held in a recirculating seawater system overnight before oil exposures. A pilot test was conducted to monitor ammonia and oxygen and to establish test loading rates that did not stress the fish under the expected conditions of the planned test.

Bay anchovy: Bay anchovy embryos and larvae were obtained from broodstock maintained at LUMCON. Broodstock were established by collecting juvenile anchovy off the LUMCON dock (29°15’14.42”N, 90°39’49.64”W) using neuston net (0.8-mm mesh), fitted with a solid glass

codend to reduce the chance of damage to the juveniles; laboratory staff reared the juveniles to adult stage. The adult anchovy population was maintained in a recirculating seawater tank (575-gallon, Red Ewald, Inc., Karnes City, TX, USA) with continuous aeration at 26–27°C, 25 ppt, and a 15:9 light:dark photoperiod. Fish were fed dry commercial fish feed (size 0.3, Cargill, Minneapolis, MN, USA) and a liquid mixture of live-hatched Artemia (Brine Shrimp Direct, Ogden, UT, USA), cultured rotifers, and *Nannochloropsis oculata* phytoplankton. Both were delivered by automatic feeders set to distribute feed at eight-minute intervals throughout daylight hours. A total of 4 L of liquid feed and 10–15 g dry feed were distributed daily to approximately 35 fish. Spawned embryos were caught in an egg collection device within the same system as adult fish and held overnight until testing began the following morning.

Red snapper: Mature red snapper (*Lutjanus campechanus*) were collected ~ 36 miles offshore of Louisiana in June and July 2015 by hook and line and transported back to LUMCON in a transport tank fitted with oxygen aeration. Amquel was added to reduce ammonia stress during transport (~ 1 g per 4 L). At the time of collection all mature fish (> 43 cm total length) were inspected on the boat to tentatively identify sex. Suspected males were immediately sacrificed by placing them under ice, while females were held in a live tank fitted with aeration. At the laboratory, all iced males were dissected to remove their gonad and the gonads cut into 2-cm sections. The sperm from the 2-cm sections were then stripped into a solution of cold Hank's balanced salt solution (202 mM) and stored at 4°C until use. All living female snapper were anesthetized using clove oil (3.3 mg:20 L clove oil:seawater) and sexed by catheterization with Teflon tubing attached to a 10 ml syringe. A small sample of gonad was removed from each female to confirm sex and to evaluate the size and condition of the oocytes for spawning. Suitable females were then injected with a priming dose (250 IU per kg) of Ovaprim (Western Chemical, Inc., Ferndale, WA, USA) on the day of collection and held in a recirculating seawater system overnight at 30 ppt and 27–28°C on a 14:10 light:dark photoperiod. The next morning they were given a resolving dose (500 IU per kg) of Ovaprim. That evening each female was inspected for properly developed, hydrated oocytes. Females with properly developed hydrated oocytes were stripped spawned and their oocytes placed into a glass bowl with sperm solution warmed to 27°C for fertilization. Fertilized embryos were water hardened and monitored for proper development. Embryos of females with properly developing embryos and a high fertilization rate were selected for use in oil exposures.

16.2.2 Source water

The source water used to maintain all fish embryos and larvae, and that was used in all oil exposures, was raw seawater collected regularly by boat from offshore Louisiana and stored in a holding system at LUMCON. Before use, the source water was UV sterilized and diluted down to appropriate salinity using 5-µm filters (Whirlpool, Benton Charter Township, MI, USA).

16.2.3 Exposure media preparations

Gulf menhaden toxicity tests were conducted using two different water accommodated fraction (WAF) preparations: high-energy and low-energy WAFs (HEWAFs and LEWAFs, respectively). Both HEWAFs and LEWAFs were prepared as described in the standard operating procedure (SOP) *Protocols for Preparing Water Accommodated Fractions* described in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (QAPP), located in Attachment 3. WAFs were prepared using Slick B (GU2888-A0719-OE701) or Slick A oil (CTC02404-02). See test-specific test condition tables (TCTs) for details.

Tests were also conducted using the slick preparation method. Generally, slick preparation consisted of placing a polyvinyl chloride (PVC) coupler with a 1-cm-thick strip of oil into the exposure chamber and allowing the PVC to soak for a given length of time before initiating the exposure. A more detailed discussion of this preparation can be found in test-specific procedures in the LUMCON General Laboratory Procedures and Practices (GLPP).

16.2.4 Water quality

Ammonia, dissolved oxygen, water temperature, and pH were monitored daily in all exposures and controls. For some tests, water temperature was also monitored continuously using a HOBO data logger (see test-specific TCTs). The exposure chambers were maintained at the appropriate temperature with the water batch connected to a heater/chiller. Salinity was measured in controls at the beginning and end of each experiment with a refractometer. See Section 16.4 for details on water quality protocols.

16.2.5 Analytical chemistry and archive sampling

To sample water for polycyclic aromatic hydrocarbons (PAHs), each 250-mL sample jar (provided by ALS Environmental) was filled to capacity. Sample collection, labeling, and handling were conducted as specified in the *Analytical Sample Shipping and COC SOP*, found in the QAPP. Sample storage and documentation [e.g., preparing required chain-of-custody (COC) forms] was conducted as described in the QAPP. Analytical chemistry samples were shipped on ice to ALS Environmental as soon as possible after collection

Analysis of PAHs in the water of each treatment was conducted by ALS Environmental Laboratories. Filtered and unfiltered water samples were collected. Methods for filtration can be found in the project QAPP, Appendix G. See test-specific TCTs for details on water sampling plans.

All unused or remaining WAF was disposed of according to LUMCON's waste disposal protocols.

16.3 Reporting and Testing Documentation

Data management, documentation, quality assurance/quality control (QA/QC), and reporting were handled as described in the QAPP. Photographs taken during planned tests were handled and documented as described in the QAPP.

16.4 General Testing Protocols

16.4.1 Water Quality SOPs

Temperature measurement SOP

1. Temperature measurement is performed using the Atkins AquaTuff 351 temperature probe.

2. Place thermometer in solution and wait for 1 minute. Record value.

3. Make sure to clean thermometer with absolute ethanol and distilled water between measurements.

Measurement of salinity SOP

1. Salinity is measured using a refractometer (Vital Sine SR-6 Refractometer). Using a glass Pasteur pipette, place 1–2 drops of test solution on the measurement window and close the lid. Point toward the light, making sure to keep the refractometer level.

2. Record value.

3. Decontaminate after each use by gently washing with warm soapy water, rinsing with deionized (DI) water, and carefully drying with Kimwipes.

Measurement of dissolved oxygen SOP

1. Dissolved oxygen is measured using a handheld digital YSI ProODO dissolved oxygen meter and probe (#626279).

2. The oxygen meter should be calibrated daily and the calibration logs should be filled out.

3. Calibration:

    a. *Salinity*: Because the solubility of oxygen in water decreases as salinity increases, it is important to input the test salinity into the meter before calibration and use. To input the salinity value into the meter, push the probe button and select *salinity* from the menu. Enter the appropriate value. Input the mean value of the measured salinity for the test replicates; salinity should not vary much between replicates. Record the calibration salinity used on the calibration log.

    b. *Temperature*: This meter does not need temperature calibration, although the meter reading should be recorded on the calibration log.

    c. *One-point calibration in water-saturated air*: This procedure is as described in the manufacturer manual. Moisten the calibration sponge with a small amount of water and ensure no water droplets are present on the sensor/temperature caps; do not immerse the sensors in water. Place sensors in calibration sleeve and allow to equilibrate for 5 minutes. Press *calibration* button, highlight *DO %* and press *enter*. Highlight the barometer value and press *enter*. Although the value can be changed, the value measured by the meter should be used. If the barometer value is off, then a barometric pressure calibration should be performed. Record the barometric pressure value on the calibration log. Wait for temperature and DO % values to stabilize and highlight *accept calibration* and press *enter*. No calibration efficiency rating is provided, but the user should check off on the calibration log that the meter was calibrated.

    d. *Barometric Pressure*: This value does not need to be routinely calibrated. If the value appears to be inaccurate, press the calibrate button and select *barometer*. Simply input an acceptable value and select *calibration*.

4. To test the dissolved oxygen in the test replicates, first set the meter to auto-stabilize by pressing probe and selecting *auto-stable*; the sensitivity should be set to the middle value, which allows a data variance of 1.275%. Place probe in the sample and briefly move to release air bubbles from the sensor. Continued movement is not necessary because of the optical luminescent measurement. Allow the value to stabilize and record it on the data sheet.

5. Between replicates, the probe should cleaned using Simple Green Cleaning Solution followed by distilled water.

Total ammonia (NH_3/NH_4^+) – API ammonia test kit SOP

1. Collect 5 mL of test solution and place it into the glass test tube provided in the API test kit. The test tube has a white 5-mL graduation line.

2. Add eight drops of test solution #1 to the test tube.

3. Immediately add eight drops of test solution #2 to the same test tube.

4. Tightly cap the test tube with the provided cap and shake vigorously for 5 seconds. Do not cap the tube with your finger because that might interfere with the test results.

5. Set the timer for 5 minutes and wait for the color to develop.

6. After 5 minutes, read the test results by comparing the test solution color to the saltwater ammonia color chart provided in the test kit. View the tube color against a white background in a well-lit area. Determine the closest color match between the test solution and the color chart and record the associated NH_3/NH_4^+ values. Record the value on the "Water Quality Monitoring" datasheet.

7. Pour the 5-mL solution into the appropriate disposal vessel.

8. After using the API kit container, clean it with mild soap and water and store until next use.

Measurement and maintenance of copper in broodstock tanks SOP

1. Measure copper levels 2 to 3 times per week in broodstock tanks using the HACH DR/890 Portable Colorimeter (Free Copper kit) Method 8506, briefly discussed below.

2. Fill one sample vial provided by HACH with 10 mL of DI water (the blank).

3. Select the appropriate program (Program 20) and press *enter* on the HACH portable colorimeter.

4. Place the blank into the colorimeter and cover with the instrument cap and press *zero*; the display should show 0.00 mg/L.

5. Fill another sample vial with 10 mL of the water sample and add the contents of one CuVer 1 Copper reagent powder pillow to the sample. Swirl to mix.

6. Press *timer* and *enter* and begin the 2-minute reaction period.

7. After the timer beeps, place the prepared sample into the colorimeter and cover with the instrument cap. Press the *read* button.

8. Record the value in the laboratory notebook.

9. Add copper to tanks where measured values are below 0.25 mg/L.

A. Testing Protocol 1: Gulf Menhaden/Sand Seatrout Embryo and Larval Acute Toxicity WAF Exposures

A.1 Test Solutions

HEWAF preparations followed the SOP provided in the QAPP. Stocks of HEWAF were prepared within 24 hours of test initiation in a decontaminated (acetone/hexane/DCM rinse) stainless steel commercial Waring CB15 blender (Torrington, CT, USA) at an oil:seawater loading rate of 1 g:1 L. WAF was transferred to a decontaminated separatory funnel (ACE Glass, Inc., Vineland, NJ, USA) and allowed to separate for 1 hour before using it in the exposure.

The appropriate amount of 100% stock WAF was added to each test beaker and two dummy beakers for each treatment (treatment-dependent), followed by clean seawater to bring the volume up to 1 L for embryo exposures and 2 L for larval exposures. Dummy beakers were used for water chemistry sampling only and did not contain test organisms.

A.2 Exposures

1. Embryo and larval exposures were conducted in 1-L and 2-L glass beakers, respectively, with beakers covered in black plastic to alleviate the problem of larvae swimming into the sides of the beakers and injuring themselves.

2. Test beakers were placed in a temperature-controlled water table equipped with hanging fluorescent lamps to adjust and maintain all exposure water at the desired temperature.

3. Embryos were loaded into each test beaker, once the exposure solution reached desired temperature; exposures were allowed to run without renewals for 48–96 hours. All test beakers were covered with food-grade plastic wrap for the duration of the exposure and were maintained at 18 ± 1°C on a 8 L:16 D light cycle. See test-specific TCTs for number of treatments, number of replicates per treatment, number of organisms per replicate, and number of hours per test. See LUMCON GLPP for source and maintenance of test organisms.

4. Dissolved oxygen, temperature, salinity, ammonia, and pH were measured starting at T_0, and then at 24-hour intervals in exposures and controls.

5. At the end of the test, the numbers of live, dead, and moribund larvae, and alive and dead unhatched embryos, were assessed. Fish were considered dead if they did not respond to gentle prodding by pipette, and moribund if alive but sedentary after gentle prodding. For some tests, live larvae were transferred to beakers with 2 L of clean water; latent mortality was assessed for an additional five days (see test-specific TCT).

6. All embryos and larvae from exposures were preserved in 10% neutral buffered formalin for long-term archive storage at LUMCON.

A.3 Water Sample Analysis

1. Unfiltered water samples (250 mL) were collected from replicate "dummy" beakers prepared for each treatment at test initiation and from one test beaker per treatment at test termination.

2. Samples were analyzed for PAH analytes by gas chromatography/mass spectrometry (GC/MS) in single-ion monitoring mode following the U.S. Environmental Protection Agency Method 8270D. All water samples were analyzed by ALS Environmental (Kelso, WA).

B. Testing Protocol 2: Bay Anchovy Embryo Acute Toxicity WAF Exposures

B.1 Bay Anchovy Embryos

1. Bay anchovy are evening spawners; spawned embryos were caught in an egg collection device within the same system as adult fish and held there overnight until use in exposure testing the following morning. See LUMCON GLPP for the sourcing and maintenance of test organisms.

B.2 Source of Crude Oil and Test Solutions

1. HEWAF and LEWAF preparations followed the SOPs provided in the QAPP. Stocks of HEWAF were prepared within 24 hours of test initiation in a decontaminated [acetone/hexane/dichloromethane (DCM) rinse] stainless steel commercial Waring CB15 blender (Torrington, CT, USA) at an oil:seawater loading rate of 1 g:1 L. WAF was then transferred to a decontaminated separatory funnel (ACE Glass, Inc., Vineland, NJ, USA) and allowed to separate for 1 hour before the WAF was used in exposure. Stocks of LEWAF were prepared one day before exposure in a decontaminated aspirator bottle (Kimax, Elmsford, NY, USA) at an oil:seawater loading rate of 1 g:1 L. LEWAF was stirred on a stir plate for 18–24 hours before use in exposure. For both oil preparation methods, the appropriate amount of 100% stock WAF was added to each test beaker and one dummy beaker for each treatment (treatment-dependent), followed by clean seawater to bring the volume up to 1 L. Dummy beakers were used for water chemistry sampling only and did not contain test organisms.

B.3 Exposures

1. Embryo and larval exposures were conducted in 1-L and 2-L glass beakers, respectively, with beakers covered in black plastic to alleviate the problem of larvae swimming into the sides of the beakers and injuring themselves.

2. Test beakers were placed in a temperature-controlled water table equipped with hanging fluorescent lamps to adjust and maintain all exposure water at the desired temperature.

3. Embryos (n = 25) were then loaded into each test beaker and exposures allowed to run without renewals for 48 hours or 72 hours (see test-specific TCT).

4. Tests consisted of 4–6 oil treatments, plus a control using clean source water, and 3–4 replicates, depending on the availability of embryos (see test-specific TCT).

5. All test beakers were covered with food-grade plastic wrap for the duration of the exposure, and were maintained at 26–27°C on a 14 L:10 D light cycle.

6. Dissolved oxygen, temperature, salinity, ammonia, and pH were measured starting at T_0, and then at 24-hour intervals in exposures and controls.

7. At test conclusion, the numbers of live, dead, and moribund larvae, and alive and dead unhatched embryos were assessed. Fish were considered dead if they did not respond to gentle prodding by pipette, and moribund if alive but sedentary after gentle prodding.

8. All unhatched embryos and larvae from exposures were preserved in 10% neutral buffered formalin for long-term storage at LUMCON.

B.4 Water Sample Analysis

1. 250 mL were collected from filtered samples (HEWAF exposures only) and unfiltered samples from replicate dummy beakers prepared for each treatment at test initiation, and from one test beaker per treatment at test termination.

2. The samples were analyzed for PAH analytes by GC/MS in single-ion monitoring mode following the U.S. Environmental Protection Agency Method 8270D. All water samples were analyzed by ALS Environmental (Kelso, WA).

C. Testing Protocol 3: Bay Anchovy Embryo Acute Toxicity Slick Exposures

C.1 Bay Anchovy Embryo

1. Bay anchovy are evening spawners; spawned embryos were caught in an egg collection device within the same system as adult fish and held there overnight until they were used in exposure testing the following morning. See LUMCON GLPP for source and maintenance of test organisms.

C.2 Test Solutions

1. Surface slicks were prepared by applying an approximately 1-cm-thick strip of oil (2 g) to the inner side of a 3-inch PVC coupler fitting. The PVC fitting was then placed into a 1-L beaker filled with seawater and suspended by a wooden dowel such that the oil ring aligned with the surface of the water. The oiled fitting was allowed to soak for 4–6 hours immediately before exposure so the oil could migrate off the PVC and onto the water's surface. Two dummy beakers for each treatment were prepared using the same methods for test beakers. Dummy beakers were used for water chemistry sampling only and did not contain test organisms.

C.3 Exposures

1. Embryo and larval exposures were conducted in 1-L and 2-L glass beakers, respectively, with beakers covered in black plastic to alleviate the problem of larvae swimming into the sides of the beakers and injuring themselves.

2. During the surface slick soak, test beakers were placed in a temperature-controlled water table equipped with hanging fluorescent lamps to adjust and maintain all exposure water at the desired temperature.

3. At the conclusion of the slick preparation steps, embryos (n = 25) were carefully loaded beneath the slick by pipetting them between the PVC ring and the edge of the beaker at the same time the PVC ring was removed. Exposures were allowed to run without renewals for 48 hours.

4. Tests consisted of 4–6 oil treatments, plus a control using clean source water, and 3–4 replicates, depending on the availability of embryos (see test-specific TCT).

5. All test beakers were covered with food-grade plastic wrap for the duration of the exposure, and were maintained at 26–27°C on a 14 L:10 D light cycle.

6. Dissolved oxygen, temperature, salinity, ammonia, and pH were measured starting at T_0, and then at 24-hour intervals in dummy beakers to avoid disturbing the surface slick, and in actual test beakers for the control treatment.

7. At the end of the test, the numbers of live, dead, and moribund larvae, and alive and dead unhatched embryos, were assessed. Fish were considered dead if they did not respond to gentle prodding by pipette, and moribund if alive but sedentary after gentle prodding.

8. All unhatched embryos and larvae from exposures were preserved in 10% neutral buffered formalin for long-term storage at LUMCON.

C.4 Water Sample Analysis

1. Unfiltered water samples (250 mL) were collected from replicate dummy beakers prepared for each treatment at test initiation and termination. To capture PAHs on the surface slick, a decontaminated 10-cm-diameter absorbent pad was placed on the slick for 3 minutes; upon removal, the pad was transferred into a wide-mouth jar for chemical analysis.

2. Water and pad samples were analyzed for PAH analytes by GC/MS in single-ion monitoring mode following the U.S. Environmental Protection Agency Method 8270D. All water samples were analyzed by ALS Environmental (Kelso, WA).

D. Testing Protocol 4: Red Snapper Embryo Acute Toxicity WAF Testing

D.1 Fish Collection and Spawning

1. Mature red snapper (*Lutjanus campechianus*) were collected from offshore Louisiana by hook and line and transported back to LUMCON in a live tank fitted with aeration. See LUMCON GLPP for source and spawning procedures for red snapper test organisms.

D.2 Test Solutions

1. HEWAF preparations followed the SOP provided in the QAPP. Stocks of HEWAF were prepared within 24 hours of test initiation in a decontaminated (acetone/hexane/DCM rinse) stainless steel commercial Waring CB15 blender (Torrington, CT, USA) at an oil:seawater loading rate of 1 g:1 L. The WAF was then transferred to a decontaminated separatory funnel (ACE Glass, Inc., Vineland, NJ, USA) and allowed to separate for 1 hour before use in exposure testing. The appropriate amount of 100% stock WAF was added to each test beaker (treatment-dependent), followed by clean seawater to bring the volume up to 1 L.

D.3 Exposures

1. Embryo and larval exposures were conducted in 1-L and 2-L glass beakers, respectively, with beakers covered in black plastic to alleviate the problem of larvae swimming into the sides of the beakers and injuring themselves.

2. Beakers were placed in a temperature-controlled water table equipped with hanging fluorescent lamps to adjust and maintain all exposure water at desired temperature.

3. Embryos (n = 25) were then loaded into each test beaker and exposures allowed to run without renewals for 48 hours.

4. Tests consisted of five oil treatments, plus a control that used clean source water, and relied on four replicates per treatment.

5. All test beakers were covered with food-grade plastic wrap for the duration of the exposure, and were maintained at 27–28°C on a 14 L:10 D light cycle.

6. Dissolved oxygen, temperature, salinity, ammonia, and pH were measured starting at T_0, and then at 24-hour intervals in exposures and controls.

7. At the end of the test, the numbers of live, dead, and moribund larvae, and alive and dead unhatched embryos, were assessed. Fish were considered dead if they did not respond to gentle prodding by pipette, and moribund if alive but sedentary after gentle prodding.

8. All unhatched embryos and larvae from exposures were preserved in 10% neutral buffered formalin for long-term storage at LUMCON.

D.4 Water Sample Analyses

1. Unfiltered water samples (250 mL) were collected from replicate dummy beakers prepared for each treatment at test initiation and from one test beaker per treatment at test termination.

2. Samples were analyzed for PAH analytes by GC/MS in single-ion monitoring mode following the U.S. Environmental Protection Agency Method 8270D. All water samples were analyzed by ALS Environmental (Kelso, WA).

17. Exposure Characterization and Chemistry Tests General Laboratory Procedures and Practices

17.1 Introduction

Exposure characterization and chemistry (ECC) tests were conducted by several toxicity testing laboratories. The goal of these tests was to characterize the chemistry and exposure conditions for toxicity tests conducted as part of the *Deepwater Horizon* Natural Resource Damage Assessment (DWH NRDA) aquatic toxicity testing program. To characterize the expected exposure for a toxicity test, the methods of a toxicity test (or set of tests) were repeated without the addition of test organisms. There are two types of ECC tests described in this chapter: (1) tests that replicated a specific test or set of tests and therefore followed the corresponding toxicity test's protocols, and (2) tests that more generally characterized different exposure types [e.g., static, water accommodated fraction (WAF) exposures, slick exposures] but did not follow specific toxicity testing protocols. The endpoints for ECC tests included analytical chemistry, fluorescence, droplet size and frequency, and oil slick/sheen thickness [see test-specific test conditions tables (TCTs)]. This chapter describes the General Laboratory Procedures and Practices (GLPP) used to conduct these ECC studies.

17.2 Methods

17.2.1 Water

For ECC tests that replicated specific toxicity tests, see the corresponding individual laboratory GLPP chapters for a description of the water source used in that ECC test (corresponding toxicity tests are listed in the test-specific protocols in the ECC GLPP). In cases where this information is not available or the ECC test does not correspond to a particular set of toxicity tests described in the individual laboratory GLPP chapters, the water source is described in the test-specific ECC testing protocols.

17.2.2 Exposure media preparations

Three different WAF preparations were used to prepare aqueous treatments for the aquatic toxicity testing program: high energy (HEWAF), chemically enhanced (CEWAF), and low energy (LEWAF). The testing program also included four different oil types: Source, Artificially Weathered Source, Slick A, and Slick B. For the standard WAF preparation methods used in the aquatic toxicity testing program, see the protocols described in the *Quality Assurance Project*

Plan: Deepwater Horizon Laboratory Toxicity Testing (QAPP). In some cases, we performed ECC tests that assessed WAF preparations with slight deviations to the three methods used by the aquatic toxicity testing program. For those tests, the modifications are described in the test-specific ECC testing protocols.

For sediment exposures, field-contaminated sediments were used as well as clean, reference sediments collected from the field and spiked with either Slick A or Slick B oil. For additional information regarding the collection of all field-collected sediments, see the *Field Samples* table in the DWH Trustee toxicity database generated in support of the DWH NRDA [available through the National Oceanic and Atmospheric Administration (NOAA) Data Integration, Visualization, Exploration, and Reporting (DIVER) data repository (DIVER, 2015)]. For the spiked-sediment preparation methods, see the individual toxicity testing protocols for the corresponding spiked sediment tests.

A number of laboratories tested the effects of thin oil sheens on different aquatic organisms; these tests are identified as "Slick" tests. While the general protocol for creating thin sheens was the same across laboratories, due to differences in exposure vessel size and shape, each laboratory had slight modifications to the slick formation protocol. Thus, slick formation protocols can be found in the individual toxicity testing protocols for the corresponding slick tests.

17.2.3 Water quality monitoring

For many ECC tests, water quality was generally not monitored. For cases where water quality was measured, see the laboratory-specific work plans for their water quality standard operating procedures (SOPs).

17.2.4 Sample filtration

All sample filtration was conducted according to the standard filtration protocol described in Appendix G of the QAPP. For the pilot ECC tests (described in Testing Protocol 15, Appendix O, of this GLPP chapter), the sample filtration was conducted using one 0.7-µm glass fiber filter (or GF/F filter).

17.2.5 Analytical chemistry sampling

Analytical sampling and analysis of all water and sediments were carried out following the procedures outlined in Appendix G of the QAPP.

17.3 Reporting and Testing Documentation

Data management, documentation, quality assurance/quality control (QA/QC), and reporting were handled as described in the QAPP.

17.4 Standard Operating Procedures

17.4.1 Particle size and frequency/concentration analysis by Coulter counter

Startup procedures

1. Ensure that the waste jar is empty.
2. Attach the correct aperture tube. The size is specified in the test-specific testing protocols.
3. Turn on the Coulter counter.

Calibration

1. Prepare a fresh dilution of calibration beads according to manufacturer's instructions. For samples analyzed with the 70-µm aperture, use the Standard, L10 latex particles with a nominal size of 10 µm from Beckman Coulter. For samples analyzed with the 140-µm aperture, use the Beckman Coulter Standard L20 latex particles with a nominal size of 20 µm.

2. Place the beaker of fresh calibration standard on the sample platform and raise the platform until the aperture tube, electrode, and stirrer are immersed.

3. Turn on the stirrer.

4. Press Set-up and select the size units (µm), aperture size, and metered volume (typically set to 1 mL).

5. Press CAL and enter the calibrator size. Ensure the Measure Aperture field says Yes.

6. Press Start to begin the calibration.

7. Once the calibration is complete, store new K_d.

8. Perform the calibration daily before running analyses.

Sample analysis

1. Before the sample analysis, analyze a blank seawater sample to determine the background counts. These counts will be subtracted from every sample to correct for background noise.

2. Prepare the sample. For some WAF samples, the droplet concentration will be too high to be quantified directly. For these samples, dilute the WAF before analysis using the same water that was used to prepare the WAF. The dilution factor required for the various WAF samples will need to be determined by the operator on a case-specific basis. Once the sample droplet concentrations are within the range that can be quantified, proceed to step 3.

3. Transfer the sample to a 25-mL sampling cuvette.

4. Place the sample in the cuvette slope and raise the platform until the aperture tube and electrode are immersed. Ensure that the sample is well-mixed before starting the sample analysis.

5. Press Start to initiate an analysis. At the end of the analysis, save the output for post-processing using custom Matlab scripts.

6. In some cases, the aperture can become obstructed. This will be evident when looking through the aperture-viewing screen. In most cases, pressing Stop will be sufficient to unblock the aperture, and then the sample will need to be recounted by pressing Start. If the aperture remains blocked, there are two options: clean the orifice by using a small brush, or flush the aperture.

7. After the sample analysis, flush the Coulter counter with clean water.

17.4.2 Fluorescence measurements by Cyclops handheld fluorometer

1. Turn on the Cyclops-7 and check that the instrument is functioning properly. See the Cyclops User Manual for more information (Turner Designs, 2016).

2. Ensure the Cyclops-7 is clean. If the instrument needs to be cleaned, see the Cyclops User Manual for cleaning instructions (Turner Designs, 2016).

3. Transfer a 250–500 mL water sample (depending on available volume) into a clean, 500-mL glass beaker and place the beaker on a non-reflective black surface.

4. Submerge the optical end of the Cyclops-7 into the center of the beaker. Ensure that the sensor is more than 3 inches above the bottom of the container and that the depth to the bottom is kept consistent across samples. Keep the sensor in the middle of the beaker so that there are more than 2 inches between the sensor and the walls of the beaker.

5. Depending on the concentration, activate the X1, X10, or X100 gain setting. If the sample signal is higher than ~ 3 V, a lower gain setting is needed. Conversely, if the gain setting is lower than ~ 0.3 V, a higher gain setting is needed. See the Cyclops User Manual for additional details on how to activate different gain settings (Turner Designs, 2016).

6. Once the appropriate gain setting has been activated, record the output for the sample.

7. Rinse the sensor with deionized water before measuring the next sample.

17.4.3 Sampling of oil films using sorbent pads

1. Using clean scissors, cut 3M T-151 sorbent pads to fit the surface area of the exposure vessels being sampled.

2. Decontaminate the pads by rinsing (or soaking) pads in dichloromethane. Remove the pads and allow them to dry in a fume hood for several hours or overnight. If not using immediately, store in a clean, Ziploc bag once dry.

3. Using solvent-rinsed forceps, carefully place a pre-cut, decontaminated pad on the surface of the water with the oil sheen to be sampled. Gently press the top of the pad with forceps to ensure complete contact between the pad and the water's surface.

4. Allow the pad to sit for 30 seconds to 1 minute so that the oil can sorb to the pad.

5. Using the solvent-rinsed forceps, collect the pad from the surface. Check to ensure that there is no oil sheen still visible on the water. If there is still a visible sheen on the surface, re-apply the pad to the surface and allow the oil to sorb to the pad for another 30 seconds.

6. If sampling multiple exposure vessels with the same pad, after the sheen from the first exposure vessel is fully sorbed onto the pad, transfer the pad to the next exposure vessel and repeat steps 4 and 5. Continue until the desired number of exposure vessels have been sampled. If needed, both sides of the sorbent pad can be used to collect the oil sheens.

7. Once complete, shake any excess water from the pad, and place it into a pre-cleaned sample jar (8–16 oz depending on size of pads). Make a note of how many exposure

vessels were sampled by each pad, and what the surface area of the vessel was for each sample so that a total sampled surface area can be calculated.

8. Store the pad at -20°C until it is shipped to ALS Environmental for analysis.

17.4.4 Preparing a calibration curve for determination of slick thickness using light transmission

1. Produce oil films with different thicknesses by pressing 0.005, 0.01, 0.015, or 0.02 g of oil between two glass coverslips.

2. For each weight, prepare four replicate coverslips.

3. Use a microscope to determine the resulting thickness of the oil by measuring the gap between the two coverslips [Figure 17.1 (A)]. Measure at least two sides of each set of coverslips to capture the potential variation in thickness across the oil film within the coverslips

4. Next, place each set of coverslips on an optical measurement stage between an optical fiber emitting green laser light and a power meter [Figure 17.1 (B)] and measure the power transmission of the green laser through the oil film samples. For each measurement, take an average of the transmitted power measurements collected over a 60-second interval to account for minor fluctuations in the laser. Also, take five measurements at different locations across each oil film sample.

5. Before each sample measurement, take an average blank reading by taking triplicate measures of the light transmission between two coverslips without oil to determine the background light attenuation.

6. To measure the relative power of each sample, divide the average sample transmission by an average blank reading (i.e., the transmission of green light through two coverslips without oil).

7. Generate a calibration curve by plotting relative power versus oil film thickness (as determined with a microscope).

8. Fit the data using the linear regression of log-transformed data to obtain an estimated absorption coefficient for the oil (i.e., the slope of the calibration curve).

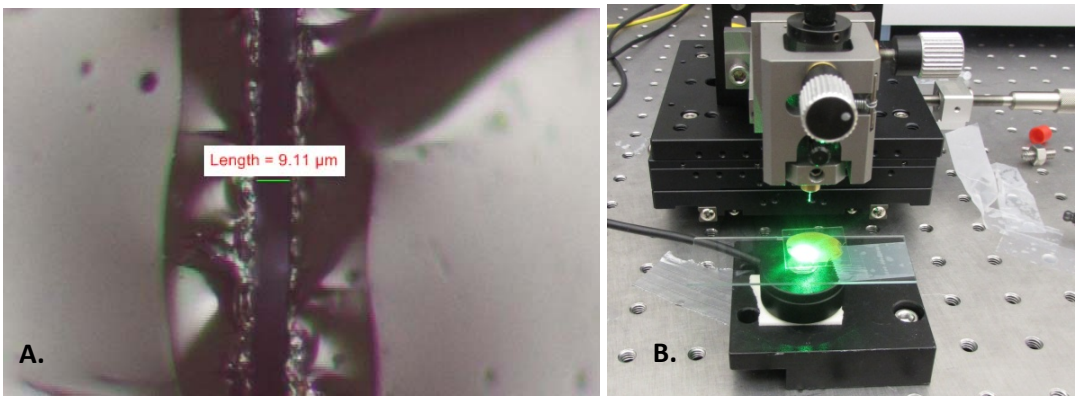

Figure 17.1. Measurement of oil thickness between two glass coverslips (A) and setup used to measure the light transmission through the oil film between two coverslips (B).

17.4.5 Slick thickness measurement using light transmission

1. Position the fiber optic approximately 2 cm above the surface of the sample, pointed directly down on the measurement area.

2. Position the detector beneath the measurement stage holding the sample dishes, centered directly below the fiber optic. This position of the fiber optic minimizes the distance between the sample's surface and the optical probe, while still allowing the exchange of test dishes and control dishes on the measurement stage (see Figure 17.2).

3. Do not change the distance between sample surface, fiber optic, and detector between measurements of both samples and controls.

4. Before starting the measurements, let the laser warm up for 1–2 hours to stabilize.

5. Before each sample, take a set of triplicate blank transmission measurements from a crystalizing dish containing 200 mL of clean seawater (i.e., no oil).

6. For each sample dish, collect a set of five transmission measurements. Take the five measurements from the center of the dish and from four points approximately half a centimeter from the edge of the dish at positions that match the 3-, 6-, 9-, and 12-hour markers on a clock in relation to the center point. Ensure the sample surface is stable and the fluid is not moving when the measurements are taken. For each measurement, take an average of the transmitted power measured over a 60-second interval.

7. Calculate the relative power for each set of five sample transmission measurements by dividing the sample measurement by the average of the three control measurements taken directly before the sample measurements.

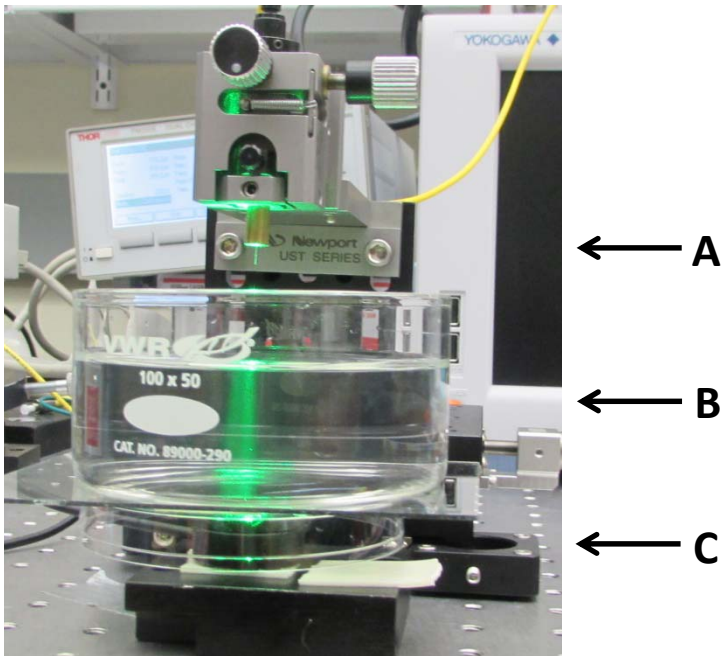

Figure 17.2. Experimental setup for the slick thickness measurements: (A) optical fiber, (B) sample dish with seawater, and (C) detector.

References

DIVER. 2015. Data Integration, Visualization, Exploration and Reporting Application. Web Application. *Deepwater Horizon* Natural Resource Assessment Data. National Oceanic and Atmospheric Administration. Available: https://dwhdiver.orr.noaa.gov/.

Turner Designs. 2016. Cyclops Submersible Sensors: User's Manual. June 9. Turner Designs, San Jose, CA. Last accessed 7/22/2016. Available: http://www.turnerdesigns.com/t2/doc/manuals/998-2100.pdf.

A. Testing Protocol 1: 96-Hour Exposure Characterization (CHEM96HR)

The primary objective of these 96-hour ECC tests was to characterize the chemistry of WAF exposures over the course of a typical 96-hour acute toxicity test. Generally, these tests included analytical samples collected from one or more WAF dilutions immediately after completion of the WAF preparation, and at different time points thereafter up to 96 hours.

A.1 96-Hour Chemistry Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* in Appendix A, Section A.3, of the QAPP.

2. Prepare all WAFs according to the protocols described in Appendix A of the QAPP.

3. For exposure solutions, use the preparation and handling methods described in the corresponding definitive tests except do not add test organisms. This includes, but is not limited to, using the same methods to prepare WAFs and dilutions, using the same type and frequency of aeration (if applicable), and holding exposure vessels under the same environmental conditions (e.g., temperature, photoperiod, light intensity). For each 96-hour ECC test, refer to the corresponding toxicity testing protocols and TCTs for additional information on WAF dilution methods, source water, and preparation of exposure vessels (Table A.1).

4. For the static tests, collect water samples from the middle of the exposure vessel using a syringe or siphon. For the static recirculating tests, collect water samples from the outflow of the Imhoff cone exposure vessel or from the reservoir beaker.

5. Only sample each exposure vessel once. When more than one water sample is required, set-up replicate exposure vessels for each time point sampled. If the volume of a replicate exposure vessel is smaller than the sample volume required for analysis, prepare additional replicates for each time point so that a sufficient volume of water can be collected.

6. Send all samples to ALS Environmental for analysis.

7. For additional information, including renewal regime, oil type, WAF preparation method, and exposure vessel size, refer to the test-specific TCTs.

Table A.1. List of static and static recirculating 96-hour ECC tests and their corresponding toxicity testing protocols.

Test_ID	Lab	Renewal regime	Oil	WAF prep	Corresponding toxicity test(s)[a]	Corresponding toxicity test GLPP chapter	Corresponding toxicity testing protocol(s)
C01	RSMAS	Static	A	CEWAF	288, 316	7	5, 6
C02	RSMAS	Static	A	HEWAF	278, 279	7	5, 6
C03	RSMAS	Static	S	CEWAF	285, 515	7	5, 6
C04	RSMAS	Static	S	HEWAF	299, 320, 512	7	5, 6
C05	RSMAS	Static	WS	CEWAF	291, 294	7	5, 6
C06	RSMAS	Static recirculating	A	HEWAF	234, 235, 292, 341, 359, 362, 373	7	10, 11
C07	RSMAS	Static recirculating	WS	HEWAF	273	7	12
C08	GCRL	Static	A	CEWAF	137, 142, 148, 155, 167, 184, 193, 227, 238, 253, 255, 325	3	1
C09	GCRL	Static	A	HEWAF	125, 127, 154, 173, 185, 192, 194, 225, 236, 248, 254, 524, 614, 632	3	1
C10	GCRL	Static	B	CEWAF	138, 144	3	1
C11	FGCU	Static	A	CEWAF	103, 106, 124, 628	2	1
C12	FGCU	Static	A	HEWAF	506, 507, 508	2	1
C13	FGCU	Static	WS	CEWAF	102, 105, 519	2	1
C17	RSMAS	Static	WS	HEWAF	290, 298, 339	7	5, 6
C18	RSMAS	Static recirculating	A	HEWAF	234, 235, 292, 341, 359, 362, 373	7	10, 11
C25	RSMAS	Static	A	HEWAF	278, 279, 314	7	5, 6
C26	RSMAS	Static recirculating	A	HEWAF	234, 235, 292, 341, 359, 362, 373	7	10, 11

A = Slick A oil; B = Slick B oil; S = Source oil; WS = Artificially Weathered Source oil; FGCU = Florida Gulf Coast University; GCRL = University of Southern Mississippi Gulf Coast Research Laboratory; RSMAS = University of Miami Rosenstiel School of Marine and Atmospheric Science.

a. In some cases a static ECC test can be used to characterize the concentration change expected to occur for a static-renewal test in-between the renewals.

B. Testing Protocol 2: Replicate Stock WAF Chemistry (STOCKCHM)

To better characterize the variation in starting concentration for different WAFs prepared with the same method and oil type, laboratories prepared and analyzed paired filtered and unfiltered water samples from replicate stock WAFs. For additional information regarding source water, refer to the GLPP corresponding to the laboratory that conducted the specific ECC test.

B.1 Stock WAF Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* in Appendix A, Section A.3, of the QAPP.

2. Prepare a sufficient volume of WAF to collect paired filtered and unfiltered stock WAF samples. Ensure that each pair of samples (filtered and non-filtered) are collected from the same WAF preparation or from a well-mixed composite of more than one WAF preparation. Collect no more than one set of samples (one filtered and one non-filtered) from a single or composite WAF preparation.

3. Prepare all WAFs according to the protocols described in Appendix A of the QAPP.

4. Immediately after WAF preparation, collect one filtered and one unfiltered stock WAF sample as described in Appendix G of the QAPP.

5. Send all samples to ALS Environmental for analysis.

6. Prepare three replicate WAFs for each test.

C. Testing Protocol 3: 96-Hour WAF Characterization Studies (DROPLET1)

The primary objective of these ECC tests was to characterize both the dissolved and droplet fractions of the 12 different WAFs used during the DWH aquatic toxicity testing and the changes to those fractions across a typical static, 96-hour test. For these tests, filtered and unfiltered WAF samples from 1%, 10%, and 100% WAF dilutions of a 1-g/L stock WAF were collected at 0-, 8-, 24-, 48-, and 96-hour time points. At each time point, samples were analyzed for droplet size and frequency by Coulter counter, total polycyclic aromatic hydrocarbon (PAH) concentration by fluorescence using an Aqualog benchtop spectrofluorometer, and total PAH concentration by fluorescence using a Cyclops handheld fluorometer. In addition, samples were collected and sent to ALS Environmental for chemical analysis. Note that LEWAF tests only included analytical chemistry samples.

These tests were conducted at the University of Maryland. All water had 35 ppt salinity prepared using Instant Ocean sea salts and 0.2-µm filtered, sterile water.

C.1 96-Hour Droplet Characterization Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* in Appendix A, Section A.3, of the QAPP.

2. Prepare all WAFs according to the protocols described in Appendix A of the QAPP. Due to the large volumes required, prepare several stock WAFs and composite prior to performing dilutions.

3. Prepare 1%, 10%, and 100% dilutions of stock WAF and transfer to 2-L or 4-L glass aspirator bottles (see test-specific TCTs for size).

4. Do not prepare control treatments (solutions that do not contain oil or dispersant) for these ECC tests.

5. At each time point, collect the WAF sample from one replicate aspirator bottle per treatment. To collect the sample, allow 20–50 mL of WAF to drain from the aspirator bottle into a waste container. Then collect the WAF into a glass holding vessel, stopping before the water surface drops below the drain of the aspirator bottle.

6. From each sample, filter approximately half of the collected volume according to the protocol in Appendix G of the QAPP, leaving the other half unfiltered

7. Collect a set of sub-samples from both the filtered and unfiltered WAF samples for size analysis, fluorescence analysis by Aqualog, fluorescence analysis by Cyclops, and chemical analysis at ALS Environmental. See Table C.1 for volume, sampling vessel description, storage, and hold time requirements for each sub-sample.

Table C.1. Sampling parameters for DROPLET1 tests

Analysis	Sample volume (mL)	Sample vessel	Storage	Hold time
PAH chemical analysis	250	Certified, pre-cleaned 250-mL amber glass bottle	4°C until shipped	7 days
Fluorescence by Aqualog	10 plus 10 mL ethanol	20-mL scintillation vial	4°C until analyzed	4 days
Fluorescence by Cyclops	250–500	500-mL glass beaker	Room temperature	Run immediately after sampling
Coulter counter	40	Glass transfer vessel	Room temperature	Run immediately after sampling

8. Perform droplet size and frequency/concentration analyses according to SOP 17.4.1 in the ECC GLPP (*Particle size and frequency/concentration analysis by Coulter counter).* Use 70-µm aperture size for these analyses.

9. Immediately following the collection of the sample, perform fluorescence analyses using the handheld Cyclops according to SOP 17.4.2 in the ECC GLPP (*Fluorescence measurements by Cyclops handheld fluorometer*).

10. Perform Aqualog fluorescence analyses according to the methods described in Appendix F of the QAPP. Samples, diluted in ethanol per Table C.1, can be stored at 4°C for up to four days prior to analysis.

11. Store all analytical samples at 4°C until shipped to ALS Environmental for analysis.

12. For test specifics such as renewal regime, oil type, WAF preparation method, and exposure vessel size, see the test-specific TCTs.

D. Testing Protocol 4: Characterization of Metals Concentrations in Stock WAFs (DROPLET2)

The purpose of this study was to determine the concentrations of metals in the 12 WAFs used during the DWH aquatic toxicity testing program. These tests were conducted at the University of Maryland. All water had a salinity of 35 ppt, prepared using Instant Ocean sea salts and 0.2-µm filtered, sterile water.

D.1 WAF Metals Characterization Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* in Appendix A, Section A.3, of the QAPP.

2. Prepare a 1 g oil/L stock WAF according to the protocols described in Appendix A of the QAPP.

3. Filter at least 700 mL of stock WAF according to the filtration protocol described in Appendix G of the QAPP. Fill a 250-mL plastic sample bottle for metals analysis and a 250-mL amber glass bottle for PAH analysis with filtered WAF sample.

4. Fill a second 250-mL plastic metals sample bottle and a 250-mL amber glass PAH bottle with unfiltered WAF sample.

5. Store all samples at 4°C until shipped to ALS Environmental for analysis.

6. See the test-specific TCTs for information regarding the oil type and WAF preparation method that was used for each test.

E. Testing Protocol 5: Characterization of CEWAFs Prepared with Different Dispersant-to-Oil Ratios or Different Mixing Energies (DROPLET3)

The primary objective of this study was to characterize the effects that different WAF preparation parameters had on the final chemistry of a CEWAF. In particular, this matrix of tests evaluated the effects of using different dispersant-to-oil ratios to prepare CEWAFs as well as investigated the effects of slower mixing speeds on the final WAF chemistry. Both the dissolved and droplet fractions were evaluated. All WAFs were prepared using a loading of 1 g oil/L seawater. For some tests, WAF samples were collected before the settling period during WAF preparation and at 0, 8, and 24 hours; for all other tests, water samples were only collected before the settling period during WAF preparation and just after the settling period (i.e., at 0 hour). These samples were analyzed for droplet size and frequency/concentration by Coulter counter and chemistry. Additionally, a set of three HEWAFs were prepared and samples from both the pre-settled and 0-hour time points were analyzed by Coulter counter for comparison to the different CEWAFs.

The effect of mixing speed on the chemistry of a CEWAF was also evaluated. To investigate these effects, CEWAFs were prepared according to the CEWAF protocols described in Appendix A of the QAPP, except we used a mixing speed that resulted in no vortex instead of the typical 25% vortex mixing speed. We referred to these WAF preparations as low-energy CEWAFs or LECEWAFs in the TCTs.

These tests were conducted at the University of Maryland. All water had a salinity of 35 ppt, prepared using Instant Ocean sea salts and 0.2-µm filtered, sterile water.

E.1 CEWAF Characterization Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* in Appendix A, Section A.3, of the QAPP.

2. Prepare all WAFs according to the protocols described in Appendix A of the QAPP except for changes to the dispersant-to-oil ratio or mixing energy (see test-specific TCTs).

3. Just after mixing, but before the WAF is allowed to settle (i.e., the 1-hour settling period for HEWAFs and the 3–6-hour settling period for the CEWAFs), collect a 40-mL sample for droplet size analysis by Coulter counter.

4. Allow the remainder of the WAF volume to settle as described in the WAF preparation protocols in Appendix A of the QAPP.

5. Collect the WAF sample from one replicate aspirator bottle per treatment at each time point.

6. To collect the WAF sample, allow 20–50 mL of WAF to drain from the aspirator bottle into a waste container. Then collect the WAF into a glass holding vessel, stopping before the water surface drops below the drain of the aspirator bottle.

7. From each sample, filter approximately half of the collected volume, leaving the other half unfiltered. Filter all samples according to the filtration protocol described in Appendix G of the QAPP.

8. Collect a set of sub-samples from both the filtered and unfiltered WAF samples for size and chemical analysis. See Table E.1 for volume, sampling vessel, storage, and hold time requirements for each analysis.

Table E.1. Sampling parameters for DROPLET3 tests

Analysis	Sample volume (mL)	Sample vessel	Storage	Hold time
PAH chemical	250	Certified, pre-cleaned 250 mL amber glass bottle	4°C until shipped	7 days
Coulter counter	40	Glass transfer vessel	Room temperature	Run immediately after sampling

9. Perform droplet size and frequency/concentration analyses according to SOP 17.4.1 provided in the ECC GLPP (*Particle size and frequency/concentration analysis by Coulter counter*). Analyze the samples as soon as possible after sample preparation. For all pre-settled WAF samples, use an aperture size of 140 µm to analyze samples. For all other time points, use an aperture size of 70 µm.

10. Store all analytical chemistry samples at 4°C until shipped to ALS Environmental for analysis.

11. For test specifics such as renewal regime, oil type, WAF preparation method, and exposure vessel size, see the test-specific TCTs.

F. Testing Protocol 6: Characterization of Static Renewal Tests with Daily Ultraviolet Exposure (CHEMUV48)

The primary objective of this study was to characterize the changes in exposure chemistry that occurred over a typical 48-hour, static renewal, ultraviolet (UV) exposure test. Samples were collected at the beginning of the test from the initial WAF dilutions, stored in the dark, and collected again 12 hours later. At 12 hours, dishes were renewed with fresh WAF dilutions, and then the dishes were exposed to different UV levels for 4–6 hours. Samples of all WAF dilutions were collected for chemical analysis. This process was repeated for a second day. A final set of samples was collected 12 hours after a second round of UV exposure.

This test was conducted at the University of North Texas (UNT). For additional information on source water, see the UNT GLPP.

F.1 UV Chemistry Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* in Appendix A, Section A.3, of the QAPP.

2. Prepare all WAFs according to the protocols described in Appendix A of the QAPP.

3. Use the same preparation and handling methods used during a typical UV definitive toxicity test, except do not add test organisms. This includes, but is not limited to, using the same methods to prepare WAFs and dilutions; using the same 250-mL, glass crystallizing dishes to hold exposure media; and holding exposure vessels under the same environmental conditions (temperature and photoperiod). However, light intensity is expected to change due to day-to-day variations in ambient solar light.

4. Do not prepare control treatments (solutions not containing oil or dispersant) for ECC tests.

5. To collect enough water at each time point for a sample, combine water from all three 250-mL glass crystallizing dishes. Use a siphon to collect sample water from the middle of the exposure vessel, leaving the top 20–30 mL in the exposure vessel to avoid contamination from oil at the surface of the water.

6. Renew WAF every 12 hours and place the exposure vessels under 10%, 50%, and 100% ambient solar UV light for 4–6 hours during the day. At night, keep all of the exposure vessels at room temperature in the dark. Include a set of dark control exposure vessels (vessels in the dark during the entire study).

7. Send all water samples to ALS Environmental for analysis.

8. For test specifics such as renewal regime, oil type, WAF preparation method, and exposure vessel size, see the test-specific TCTs.

G. Testing Protocol 7: Characterization of Static WAF Exposure by Fluorescence Spectroscopy (FLUORCHM)

The primary objective of these ECC tests was to quantify total PAH concentrations in the water column of WAF exposures over the course of a typical 96-hour acute toxicity test using fluorescence spectroscopy. All of these tests were conducted by the researchers at RSMAS. The testing procedures generally followed the protocols and procedures used by RSMAS for static, acute toxicity tests, except for the addition of organisms. For additional information on source water, see the RSMAS GLPP.

G.1 96-Hour Chemistry by Fluorescence Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. Follow the *Decontamination SOP* in Appendix A, Section A.3, of the QAPP.

2. Prepare desired WAFs in advance according to the *Protocols for Preparing WAFs* SOP in Appendix A, Section A.1, of the QAPP.

3. Dilute the WAF to treatment concentrations in bulk, with enough volume for all treatment replicates and sample analysis (see test-specific TCTs). Add both water and WAF volumes to 5-L aspirator bottle(s) equipped with a closed-valve Tygon tubing outflow. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels either through the tubing outflow or by decanting. Allow at least 50 mL of the WAF to drain from the outflow tubing before sampling.

4. Collect an initial 5-mL sample from each WAF dilution for PAH quantification by fluorescence spectroscopy, as described in the RSMAS GLPP.

5. Fill 1-L glass exposure beakers with a total test solution volume of 1 L. Because oil/PAHs can adhere to plastic, use only glass-graduated cylinders or Hamilton syringes to add the WAF.

6. Take initial measurements of water temperature, pH, DO, and salinity, within each test chamber, as outlined in the *Temperature measurement SOP*, *pH measurement SOP*, *Measurement of DO SOP***,** and *Measurement of salinity SOP*, respectively, after setting up replicate beakers. All SOPs can be found in the RSMAS GLPP.

7. Place the replicate beakers in a temperature-controlled room. See test-specific TCT for additional details.

8. Every 24 hours, collect a 5-mL sample from each treatment and replicate. Use a pipette to collect the sample, and ensure that the sample is collected from the middle of the water column.

9. Prepare and store the samples for final PAH quantification by fluorescence spectroscopy, as described in the RSMAS GLPP.

10. Discard any remaining test solutions as outlined in the *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

H. Testing Protocol 8: Study to Evaluate the Use of Sorbent Pads to Reduce PAH Concentration in Water Exposures (CHEMPAD1)

The purpose of this test was to evaluate the potential for using submerged 3M T151 sorbent pads to reduce the PAH concentration in the water column. All of these tests were conducted by researchers at the University of Miami RSMAS. The testing procedures generally followed the protocols and procedures used by RSMAS for static, acute toxicity tests, except for the addition of organisms. For additional information on source water, see the RSMAS GLPP.

H.1 Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* in Appendix A, Section A.3, of the QAPP.

2. Prepare the LEWAF in advance according to the *Protocols for Preparing Water Accommodated Fractions* SOP in Appendix A of the QAPP.

3. Dilute WAF to treatment concentrations in bulk, with enough volume for all treatment replicates and sample analysis (see test-specific TCTs). Add both water and WAF volumes to 5-L aspirator bottle(s) equipped with a closed-valve Tygon tubing outflow. Spin the solution for 5 minutes on a stir plate with a 50% vortex. Separate the solution into replicate/sample vessels either through the tubing outflow or by decanting. Drain at least 50 mL of the volume from the outflow tubing before sampling. Measure seawater volume using a graduated cylinder. Because oil/PAHs can adhere to plastic, use only glass-graduated cylinders or Hamilton syringes to add WAF.

4. Collect an initial 5-mL sample from each WAF dilution for PAH quantification by fluorescence spectroscopy, described in the RSMAS GLPP.

5. Collect 250 mL of each bulk solution and ship overnight on ice to ALS Environmental, as described in the QAPP.

6. Add 1 L of the appropriate WAF dilution to replicate 1-L glass beakers. For samples, prepare one replicate per time point. See test-specific TCT for details.

7. In each replicate beaker, add a 2 x 2 in square piece of sorbent padding (3M T-151) fastened to a stir bar (to hold the pad down under the water).

8. After all of the replicates are set up and initial samples have been collected, cover the exposure chambers with glass panes to limit evaporation. Maintain the replicates in the environmental control chamber at 27°C with a 16:8 light/dark photoperiod.

9. At each time point, collect a WAF sample from one replicate beaker per treatment. Collect the sample from the middle of the exposure vessel by syringe or siphon, so that the sample better represents the water column exposure concentrations that the organisms experienced during a test.

10. After the samples are collected, discard any remaining test solution and the piece of sorbent padding from the replicate beaker as outlined in *WAF and toxicity test water disposal procedures SOP* found in the RSMAS GLPP.

I. Testing Protocol 9: Development of a Crude Oil Artificial Weathering Method (OILWEATH)

The purpose of this study was to develop a crude oil artificial weathering method. The weathering process was conducted outdoors to capture the effects of cloud cover, wind, and natural sunlight. Aquarium wave generators mimicked wave mechanics. A UV transparent acrylic pane was placed over the framework to prevent wildlife and rain water from disturbing the weathering process. Over the course of the 36-day experiment, oil and water samples were collected to evaluate the weathering process.

This test was conducted at GCRL. For additional information on source water, see the GCRL GLPP.

I.1 Artificial Weathering Tank Construction

1. Place replicate 20-gallon tanks on a platform that receives direct sunlight during most of the day.

2. Secure ACRYLITE UV transmitting plastic with hinges to a frame 6 inches from the top of tanks (see Figure I.1). The hinges will allow the plastic to be lifted during sampling.

3. Fill each tank with 35 L of artificial seawater at 15 ppt salinity.

4. Place a Maxspect Gyre-Generator pump into each tank submerged 1.5 cm below the water surface, attached to the side of the tank.

Figure I.1. Artificial weathering apparatus.

I.2 Weathering Protocol

1. Add 227 mL of source oil directly in the center of the tank prior to the Gyre-Generator being turned on.

2. Turn on the Gyre-Generator, and set the pulse rate to 3 seconds. Run continuously throughout the experiment.

3. At test initiation, collect an initial oil sample by scooping 1–2 g oil from the surface and transfer to a small glass vial.

4. Monitor water level, temperature, and salinity daily. Adjust water level and salinity, if needed.

5. Exchange approximately 80% of the underlying water in each replicate tank according to the following schedule: during the first week exchange water every other day, then exchange water every four days for the next three weeks, and then exchange the water every five days for the remainder of the test. To exchange water, use a pump placed at the bottom center of the tank to avoid disturbing the slick.

6. At each water change, collect a sub-sample of the water removed during the water exchange and one or two 1–2 g sample(s) of weathered slick oil by scooping with a decontaminated spatula. Place water samples in 250-mL amber glass sample jars and the oil samples into a 10-mL Wheaton amber glass vial. Store all samples at 4°C.

7. Send a representative selection of samples to ALS Environmental for analysis. Store the remaining oil samples at 4°C for potential future toxicity testing.

8. Continue the experiment for 36 days.

J. Testing Protocol 10: Characterization of Thin Sheen Chemistry (SLICKCHM)

The primary objective of these tests was to chemically characterize thin sheen exposures that were used during DWH aquatic toxicity testing. These tests typically included the analysis of underlying water samples and samples of the oil sheen itself.

J.1 Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination procedures, follow the *Decontamination SOP* as described in Appendix A, Section A.3, of the QAPP.

2. Create thin sheens using the slick preparation protocols described in the corresponding toxicity test GLPP Chapter and Testing Protocol. The corresponding toxicity tests are listed in Table J.1.

Table J.1. List of static, 96-hour ECC tests and their corresponding toxicity testing protocols.

Test_ID	Lab	Renewal regime	Oil	Corresponding toxicity test(s)	Corresponding toxicity test GLPP chapter	Corresponding toxicity testing protocol(s)
K03	LSU	Static	A	377, 594, 617	11	5, 6
K04	LSU	Static	B	378, 595, 618	11	5, 6
L06	LUMCON	Static	A	937, 944, 945,949, 950	16	3
L07	LUMCON	Static	B	938, L10	16	3
L08	LUMCON	Static	A	937, 944, 945,949, 950	16	3
L09	LUMCON	Static	B	938, L10	16	3
L11	LUMCON	Static	A	937, 944, 945,949, 950	16	3
L12	LUMCON	Static	B	938, L10	16	3
X20	Stratus	Static	A	X19	14	4
X21	Stratus	Static	B	X18	14	4
X22	Stratus	static	A	643	8	13
X23	Stratus	static	B	644	8	13

LSU: Louisiana State University; LUMCON: Louisiana Universities Marine Consortium; Stratus: Stratus Consulting.

3. After sheen preparation, sample the oil sheen according to the Pad Sampling SOP described in the ECC GLPP. Store pad samples at -20°C until shipped to ALS Environmental for analysis.

4. Once the sheen has been sampled, collect a 250-mL water sample from the middle of the water column by siphon or syringe. Composite the water samples across several replicates if needed. Store water samples at 4°C until they are shipped to ALS Environmental for analysis.

5. For test specifics such as test duration, oil type, loading rate, and exposure vessel size, see the test-specific TCTs.

K. Testing Protocol 11: Characterization of Slick Thickness by Light Transmission (SLICKUSF)

The primary objective of these tests was to characterize the thickness of the thin oil sheens generated and used as a part of the DWH aquatic toxicity testing program. We measured the slick thickness of thin sheens prepared using the same oils and methods as those used by the researchers conducting slick toxicity tests. To measure the thickness of the oil sheen, we used the Beer-Lambert law relating light attenuation (i.e., light absorption) to slick thickness using an absorption coefficient that we determined from a calibration curve. The Beer-Lambert law relates relative light transmission through a liquid film (T) to the film's thickness, as follows:

$$T = e^{-\alpha L},$$

where α is the absorption coefficient of the substance of interest and L is the path length of the transmitted light or the thickness of the film. For additional details on these tests, see Forth et al. (2016).

These tests were conducted at the University of South Florida. For all tests, artificial seawater was prepared by mixing Instant Ocean sea salts with deionized water.

K.1 Slick Preparation Protocol

1. Fill 250-mL glass crystalizing dishes (100 mm in diameter) with 200 mL of 32 ppt salinity seawater, prepared by mixing deionized water and Instant Ocean salts.

2. Prepare oil slicks on water according to UNT GLPP Chapter 8, Testing Protocol 13 (Figure K.1).

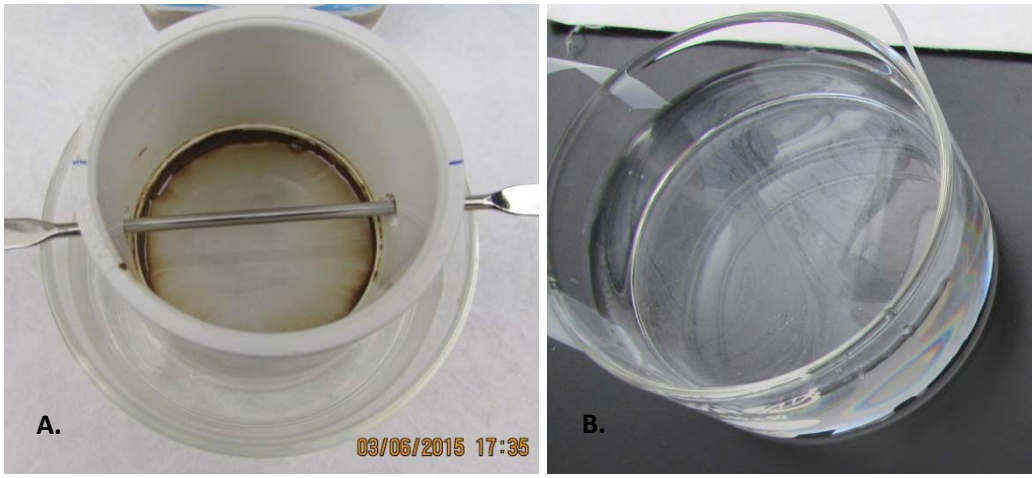

Figure K.1. Preparation method for generating oil slicks (A) and an example oil slick (B) using this method.

K.2 Slick Characterization

1. After preparation of the sheen, measure slick thickness according to SOP 17.4.5 (*Slick thickness measurement using light transmission*) in the ECC GLPP.

2. For tests where pad sampling was conducted (see test-specific TCTs), sample the oil sheen according to the SOP 17.4.3 (*Sampling of oil films using sorbent pads*) described in the ECC GLPP.

3. Once the sampling is complete, place the pad in a pre-cleaned sample jar, 8–16 oz depending on pad size, and store at -20°C until it is shipped to ALS Environmental for analysis.

4. To determine slick thickness from transmission measurements, use the calibration curve developed for the corresponding oil type using SOP 17.4.4 (*Preparing a calibration curve for determination of slick thickness using light transmission*) described in the ECC GLPP.

5. For test specifics such as oil type, loading rate, and exposure vessel size, see the test-specific TCTs.

L. Testing Protocol 12: Preparation and Characterization of Oil Slicks with Different Thicknesses (SLICKALT)

The primary objective of these pilot studies was to develop an alternate slick preparation method that would allow us to create slicks with different slick thicknesses. This alternate method is in contrast to the standard thin sheen preparation method described in Testing Protocol 11, which was the primary method for creating slick exposures for the DWH aquatic toxicity testing program. We characterized the resulting slick thicknesses by chemical analysis of the oil slick collected using sorbent pads and by light transmission measurements. Below we describe this alternate slick preparation method, and the protocols used to characterize those slicks.

These tests were conducted at the University of South Florida. For all tests, artificial seawater was prepared by mixing Instant Ocean sea salts with deionized water.

L.1 Alternate Slick Preparation Protocol

1. Add the appropriate volume of clean seawater to a decontaminated exposure chamber (see test-specific TCT).

2. For the less viscous Source and Artificially Weathered Source oils, transfer oil by pipette or syringe. For the thicker, more viscous Slick A and Slick B oils, weigh the appropriate mass of oil in an aluminum weigh boat (different amounts of oil will generate different slick thicknesses; see test-specific TCTs).

3. Transfer oil from the weigh boat to the water surface and re-weigh the weigh boat to get an accurate mass of oil that was transferred to the exposure vessel.

4. Repeat steps 2 and 3 for each exposure vessel.

5. If preparing slicks with Slick A or Slick B oils, place the exposure vessels outdoors and expose to sunlight for 2–3 hours. This will enable the thick oil to spread across the surface of the water.

L.2 Slick Characterization

1. After preparation of the sheen, measure slick thickness according to SOP 17.4.5 (*Slick thickness measurement using light transmission*) in the ECC GLPP.

2. Once the transmission measurements have been taken, sample the oil sheen according to the SOP 17.4.3 (*Sampling of oil films using sorbent pads*) described in the ECC GLPP.

3. Once sampling is complete, place the pad in a pre-cleaned sample jar, 8–16 oz depending on pad size, and store at -20°C until it is shipped to ALS Environmental for analysis.

4. To determine the slick thickness from transmission measurements, use the calibration curve developed for the corresponding oil type using SOP 17.4.4 (*Preparing a calibration curve for determination of slick thickness using light transmission*) described in the ECC GLPP.

5. For test specifics such as test duration, oil type, loading rate, and exposure vessel size, see the test-specific TCTs.

M. Testing Protocol 13: Preliminary Characterization of Oil Spiked and Field-Contaminated Sediments (SEDCHEM1)

The primary objective of these tests was to characterize spiked and field-contaminated sediments. These tests were conducted during method development for spiking sediments with oil, or for assessing oil concentrations and other potential contaminant concentrations in field-collected sediments before they were used in toxicity testing. Information on spiking methods can be found in the GLPP Chapter 3, Toxicity Testing Protocol 1, and information on all the field-collected sediments can be found in the Field Sediment Table of the NOAA DIVER data repository.

N. Testing Protocol 14: Characterization of Gulf Killifish Sediment Toxicity Test Exposure (SEDCHEM2)

The purpose of this study was to characterize the water column PAH exposure for the Gulf killifish sediment toxicity test conducted at LSU. Therefore, test chambers were prepared according to the same protocols as the toxicity test (Test 195), except for the addition of embryos. Throughout the study, water samples were collected to analyze for PAH concentration, total suspended sediment load (mass/volume), and sediment particle size. For additional details on this study, see the LSU GLPP.

N.1 Preparation of Exposure Chambers

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* as described in Appendix A, Section A.3, of the QAPP.

2. Spike reference sediments with Slick B oil according to the *Protocols for Preparation of Oil-spiked Sediments* provided in the LSU GLPP.

3. Load sediments with oil at different concentrations (see test-specific TCTs). Include a control treatment with no oil.

4. For each replicate, add 280 g (~ 150 mL) of sediment to a 950 mL Pyrex container.

5. Place nine glass marbles (diameter of approximately 1.5 cm) inside each Pyrex container. Place the marbles toward the glass wall where they align with the rim of the filter basket.

6. Place the polytetrafluoroethylene (PTFE) basket in the Pyrex container so that it rests on the glass marbles.

7. Slowly add 200 mL of 12-ppt artificial seawater into each test chamber. Allow suspended particles to settle before collecting the first water samples.

N.2 20-Day Test Duration

1. After preparation, place the test chambers on an orbital shaker at 20 RPM in an environmental chamber kept at room temperature on a natural light cycle (similar to the associated toxicity Test 195).

2. Every other day, remove 65 mL of water from the test chambers and replace it with 65 mL of new, clean 12-ppt artificial seawater. Retain the water that is removed from each test chamber for PAH analysis (see the water sampling protocol in Section N.3 below).

3. Continue test for 20 days.

N.3 Water and Sediment Sampling

1. At the test initiation, collect one sediment sample (8-oz sediment jar) per treatment and send to ALS Environmental for analysis.

2. On days 2, 8, 14, and 20, collect an unfiltered and filtered water sample from the 65 mL sample that is removed during the water renewal for PAH analysis. Composite water from all replicates to obtain the required sample volume (see Table N.1).

Table N.1. Sampling parameters for SEDCHEM2 test

Analysis	Sample volume (mL)	Sample vessel	Storage	Hold time
Unfiltered PAH chemical	250	Certified, pre-cleaned 250-mL amber glass bottle	4°C until shipped	7 days
Filtered PAH chemical	275	Certified, pre-cleaned 250-mL amber glass bottle	4°C until shipped	7 days
Sediment mass	NA	2.6-µm and 2 x 0.3-µm filters from filtered PAH sample	Dry in oven	Run immediately
Coulter counter	50	Glass transfer beaker	4°C until shipped	Run immediately

NA = not applicable.

3. For filtered samples, dry filters for 4–8 hours the day before sampling. Pre-weigh a dried 2.6-micron filter and two dried 0.3-micron filters. Use these dried and pre-weighed filters to filter a 275-mL sub-sample of the composited water sample from each treatment by first filtering through the 2.6 micron filter, and then filtering that filtrate through two,

stacked 0.3 micron filters. Transfer the final filtrate to a 250-mL amber glass sampling bottle and store at 4°C until shipped to ALS Environmental for PAH analysis.

4. Collect all three filters and dry in an oven overnight. Once dry, re-weigh to determine total sediment mass in the 275-mL sample.

5. Collect a 50-mL sub-sample from each composited sample and store at 4°C until shipped to the University of Maryland for sediment size analysis by a Coulter counter. See SOP 17.4.1 (*Particle size and frequency/concentration analysis by Coulter counter*) in the ECC GLPP for details on the analysis.

O. Testing Protocol 15: Early Pilot WAF Characterization Studies (EARLYCHM)

These ECC tests are early pilot studies that provided initial characterization of the WAF preparations used in the DWH NRDA aquatic toxicity testing program. Tests were conducted at Mote Marine Laboratory and the University of Maryland.

The first set of tests (i.e., J tests) was conducted at Mote Marine Laboratory. For these tests, WAFs were generated with five different loading ratios, 1:100, 1:500, 1:1000, 1:10000, and 1:100000, oil:water. All CEWAFs were mixed using a 1:10 dispersant:oil loading ratio with Corexit 9500. A subsample of each prepared WAF was then filtered, using a 0.7-µm glass fiber filter (or GF/F filter). Both unfiltered and filtered WAF solutions were analyzed for PAH components, and WAF solutions made with source oil were also analyzed for benzene, toluene, ethylbenzene, and xylene (BTEX). Alternatively, a series of WAF treatment concentrations were also prepared by diluting a single, high-concentration WAF with clean seawater. A loading ratio of 1:100 (weight-to-volume) oil to water was used to prepare the high-concentration WAF, and a subsample of each prepared WAF was then filtered, using a 0.7-µm GF/F filter. Both unfiltered and filtered WAF solutions were then diluted to 10%, 25%, 50%, 75%, and 90% WAF. As with the differential loading WAF preparations, both the unfiltered and filtered WAF dilution series solutions were analyzed for PAH and BTEX (source oil only).

The second set of tests was conducted at the University of Maryland. For these studies, the purpose was to provide an initial characterization of stock WAFs and WAF dilutions that were prepared using a 1-g oil/L loading. All filtered samples were filtered using a 0.7-µm GF/F filter and all CEWAFs were mixed using a 1:10 dispersant:oil loading ratio. The WAFs were prepared with 35-ppt salinity water (Instant Ocean salts) and kept at room temperature during preparation. Because these tests were conducted as pilots outside the normal toxicity testing procedures, they do not have associated TCTs. See Table O.1 for the test conditions for these tests.

Table O.1. Test conditions for University of Maryland pilot ECC tests

Test ID number	Sample ID	Toxicant	Preparation method	Test duration	Loading rate	Temperature (°C)	Salinity (ppt)
002	um-c0412-up-002-002	WS	LEWAF	0	1 g/L	22–23	35
002	um-c0412-up-002-001	NA	NA	0	1 g/L	22–23	35
002	um-c0412-fp-002-012	DISP	COR	0	1 g/L	22–23	35
002	um-c0412-up-002-007	DISP	COR	0	1 g/L	22–23	35
002	um-c0412-fp-002-024	WS	HEWAF	0	1 g/L	22–23	35
002	um-c0412-up-002-014	WS	CEWAF	0	1 g/L	22–23	35

Table O.1. Test conditions for University of Maryland pilot ECC tests (cont.)

Test ID number	Sample ID	Toxicant	Preparation method	Test duration	Loading rate	Temperature (°C)	Salinity (ppt)
002	um-c0412-up-002-019	WS	HEWAF	0	1 g/L	22–23	35
003	um-c0413-up-003-026	A	LEWAF	0	1 g/L	22–23	35
004	um-c0419-up-004-048	S	LEWAF	0	1 g/L	22–23	35
004	um-c0419-fp-004-066	S	HEWAF	0	1 g/L	22–23	35
004	um-c0419-fp-004-058	S	CEWAF	0	1 g/L	22–23	35
004	um-c0419-up-004-053	CEW	CEWAF	0	1 g/L	22–23	35
004	um-c0419-up-004-061	S	HEWAF	0	1 g/L	22–23	35
005	um-c0420-up-005-069	B	LEWAF	0	1 g/L	22–23	35
005	um-c0420-fp-005-080	B	CEWAF	0	1 g/L	22–23	35
005	um-c0420-up-005-077	B	CEWAF	0	1 g/L	22–23	35
005	um-c0420-fp-005-075	B	HEWAF	0	1 g/L	22–23	35
005	um-c0420-up-005-071	B	HEWAF	0	1 g/L	22–23	35

A = Slick A oil; B = Slick B oil; S = Source oil; COR = Corexit 9500 dispersant only; DISP = dispersant; NA = not applicable; WS = Artificially Weathered Source oil.

O.1 Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* as described in Appendix A, Section A.3, of the QAPP.

2. Prepare all WAFs according to the protocols described in Appendix A of the QAPP.

3. Filter at least 700 mL of stock WAF using the 0.7-μm GF/F filter, according to the filtration protocol described in Appendix G of the QAPP.

4. Fill a 250-mL amber glass PAH bottle with filtered WAF sample.

5. Fill a second 250-mL amber glass PAH bottle with unfiltered WAF sample.

6. Store all samples at 4°C until shipped to ALS Environmental for analysis.

P. Testing Protocol 16: WAF Chemistry from Unreportable Bioassay Tests (CHMISTRY/LAWAFCHM)

When conducting tests with non-standard species and exposure methods, researchers often first conducted pilot studies to develop methods, refine techniques, and determine the appropriate concentration ranges to use in a definitive test. In general, the bioassay data from these tests are substandard, and are thus not reported. For many of these pilot studies, data from similar definitive tests were reported instead.

In addition, definitive toxicity tests would on occasion be terminated because of high control mortality, poor water quality due to tank or environmental chamber malfunctions, and other failures. For these tests, the bioassay data are not reliable, and thus the data were not reported.

For some tests, the WAF chemistry data were deemed reliable even if the bioassay data were not. These WAF data were used in WAF characterization analyses. We have provided the chemistry that was used and the necessary information to interpret and analyze this data including the test conditions and sample inventory information.

Finally, the State of Louisiana conducted a set of tests where both bioassay and chemistry data were collected, but only the chemistry data were relied upon in the Programmatic Damage Assessment and Restoration Plan (PDARP). Inquiries regarding additional data and/or protocols can be made to the State of Louisiana. To distinguish these tests from the pilot and terminated tests described above, we have given them the StudyID LAWAFCHM in the DWH Trustee toxicity database made available through the NOAA DIVER data repository (DIVER, 2015). All other tests under this protocol were assigned the StudyID CHMISTRY.

P.1 WAF Chemistry Sampling Protocol

1. Decontaminate all glassware, equipment, and sample collection materials thoroughly prior to use; or use new and certified, pre-cleaned materials and equipment. For decontamination, follow the *Decontamination SOP* as described in Appendix A, Section A.3, of the QAPP.

2. Prepare all WAFs according to the protocols described in Appendix A of the QAPP.

3. Immediately after WAF preparation and WAF treatment dilution, fill 250-mL amber glass PAH sample bottles and/or 3 x 15-mL falcon tubes for DOSS analysis. Store all samples at 4°C until they are shipped for analysis.

4. If filtered samples are collected, filter stock WAF and/or diluted WAF treatments using the filtration protocol described in Appendix G of the QAPP.

5. Immediately after filtration, fill 250-mL amber glass PAH sample bottles.

6. Send all samples to ALS Environmental for analysis.

Appendix References

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