Portland Harbor
Injury Assessment
Juvenile Chinook Salmon
Controlled Dietary Exposure and Endpoint Analysis

Quality Assurance Project Plan (QAPP) and Work Plan (WP)

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Corresponding author:

Jessica Lundin

Ecotoxicology Program
NOAA Northwest Fisheries Science Center

2725 Montlake Blvd East
Seattle, WA 98112
Phone: 206.860.3310

Email: Jessica.Lundin@noaa.gov

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Acronyms and Abbreviations

CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
COC	Chain of Custody
COC	Contaminants of concern
DIVER	Data Integration Visualization Exploration and Reporting
DDT	Dichlorodiphenyltrichloroethane
EPA	U. S. Environmental Protection Agency
LC	Lethal Concentration
LOQ	Limit of Quantification
NIST	National Institute of Standards and Technology
NOAA	National Oceanic and Atmospheric Administration
NRDA	Natural Resource Damage Assessment
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PH	Portland Harbor
RSD	Relative Standard Deviation
QA	Quality assurance
QC	Quality control
QAPP	Quality assurance project plan
SRM	Standard Reference Material
TBT	Tri-butyl tin

1 Abstract

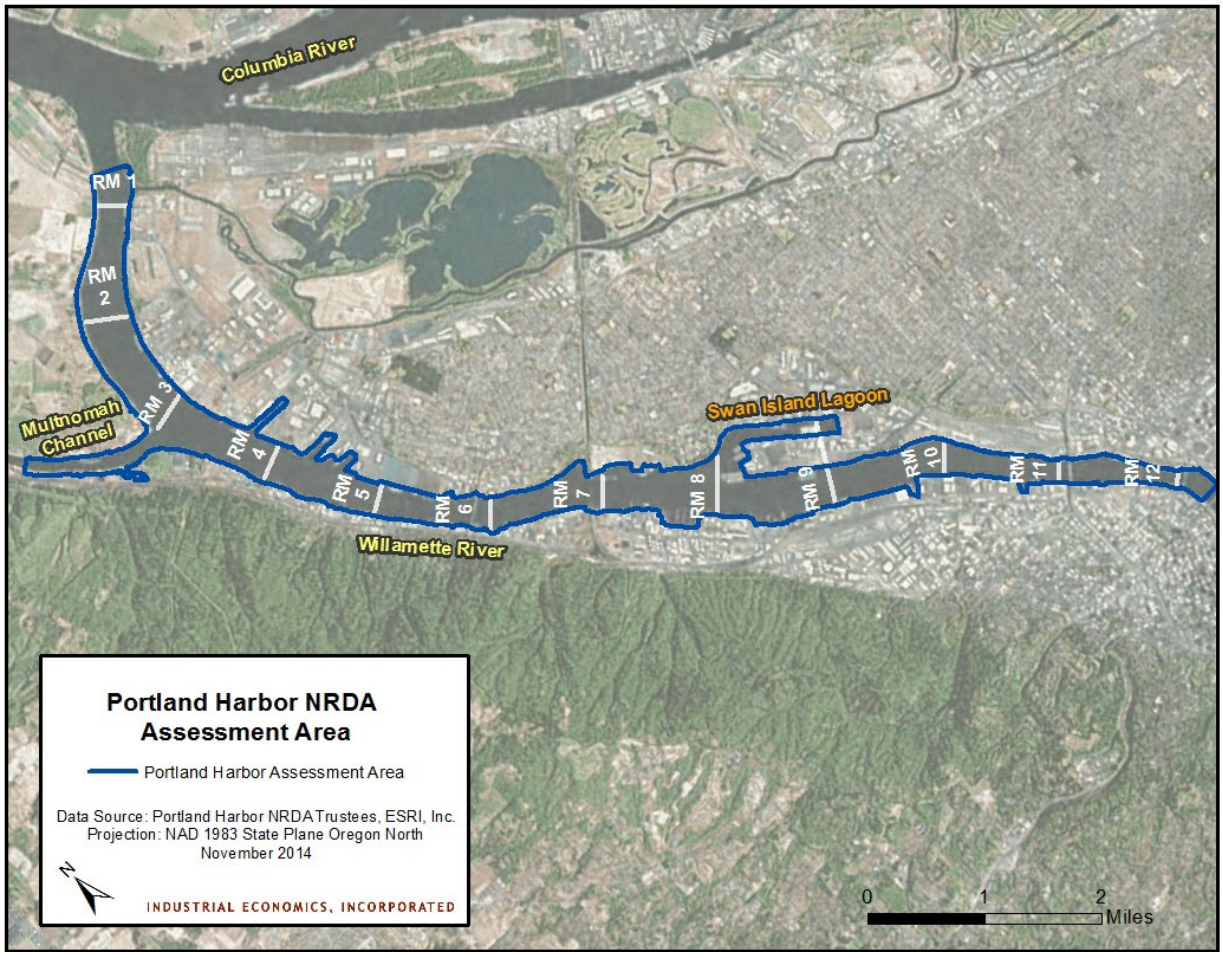
This study is intended to determine whether dietary exposure to contaminants of concern (COCs) at concentrations relevant to conditions in the Portland Harbor (PH) Superfund site is associated with growth and immune function impairment in juvenile Chinook salmon. In 2018, juvenile (sub-yearling) out-migrating Chinook salmon were collected from the Willamette River and genetically analyzed to confirm they were of Upper Willamette River origin. Otoliths from these fish were extracted and analyzed to determine growth rates, and tissues were analyzed for contaminant concentrations. Growth and contaminant concentrations at contaminated sites were compared to upstream reference locations. This laboratory study will assist in interpreting findings from the field study. The data from these and other studies will be used to quantify contaminant-related losses to salmon by using organism-based metrics that reflect an impact to the overall aquatic habitat complex (e.g., lost biomass).

2 Background: Rationale for generating the data

2.1 History of the Study Area

The Willamette River flows through the highly industrialized Portland Harbor prior to its confluence with the lower Columbia River. For more than a century, this harbor has functioned as a commercial shipping port and working waterfront. Over the decades, numerous industries have released toxic chemicals into the river. Common sources of pollution have included permitted and non-permitted end-of-pipe discharges, accidental spills during cargo transfers, and stormwater and groundwater transport from upland areas (Trustee Council 2007). Extensive legacy pollution in harbor sediments eventually led the U.S. Environmental Protection Agency (EPA) to add Portland Harbor to the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) National Priorities List (i.e., designated Superfund site) in December 2000. At present, the Superfund site extends from river mile 2 to 11 (Figure 1), inclusive of upland areas.

Figure 1. Map of Portland Harbor NRD Assessment Area



2.2 Contaminants of Concern

Priority contaminants of concern in this study include polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethanes (DDTs) and related metabolites, polycyclic aromatic hydrocarbons (PAHs). Antifouling agents such as butyltins (e.g., tributyltin (TBT)) are also of concern but are not included in this study due to financial and practical constraints.

2.3 Results from Previous Studies

In 2004, Meador et al (2006) dosed juvenile Chinook salmon with PAHs in food to evaluate growth and related physiological responses. The mixture of 21 PAHs was intended to mimic mixtures found in the stomachs of Chinook salmon from the Lower Duwamish and Hylebos waterways in Puget Sound. A significant reduction in mean fish dry weight was observed in the highest dose tested (1171 ug/g dry weight total PAHs). This concentration in food resulted in a whole body concentration of 0.67 ug/g wet weight total PAHs after 53 days of exposure to contaminated food. Most feeding treatments exhibited alterations in whole-body lipids and an increase in blood plasma indicators of “toxicant-induced”

starvation. Fish weighed 10-16 g (wet weight) at the start of the test, were fed commercially available fish pellets contaminated with PAHs dissolved in methylene chloride, and were held in full-strength sea water during the feeding trial.

Collier et al (2014) summarized the state of knowledge of the effects of PAHs on fish. In field and lab studies, juvenile fish (various species) exposed to PAH contaminated food, sediment, or water exhibited reduced growth and indicators of modified lipid metabolism. Effects were observed after exposures ranging from 24 hours to several weeks. Few studies have been done in fish to determine the effects on disease susceptibility of fish after exposure to PAHs. Several studies indicated increased susceptibility to disease after exposure to PAHs (e.g. Bravo et al 2011). Two studies suggest that fish exposed to PAHs did not exhibit reduced susceptibility to pathogens (Palm et al 2003; Prosser et al 2011).

Johnson et al (2014) summarized effects of PCBs, DDTs, and other persistent organic pollutants on fish. Changes in energy metabolism have been reported in fish exposed to PCBs in lab studies. Reductions in growth and body condition have been observed in field collected fish exposed to PCB contaminated sediment. Fish exposed to DDT in diet or through sediment have also exhibited reduced growth. In contrast, some studies have illustrated increases in fish growth after exposure to these contaminants. Studies examining the susceptibility of fish to pathogens after exposure to PCBs indicate reduced survival in brown bullhead, Arctic charr, rainbow trout, and Chinook salmon, with one study showing no effect on survival of Chinook salmon. No prior studies were described that evaluated survival of fish after exposure to DDT and pathogens.

In 2018, juvenile (sub-yearling) out-migrating Chinook salmon were collected from the Willamette River and genetically analyzed to confirm they were of Upper Willamette River origin. Otoliths from these fish were extracted and analyzed to determine growth rates, and tissues were analyzed for contaminant concentrations. Growth and contaminant concentrations at contaminated sites were compared to upstream reference locations; similarly, the association of modified growth and tissue contaminant concentrations was evaluated. A similar field study was conducted in the Lower Duwamish River in 2018. Six composite samples of stomach contents of out-migrating Upper Willamette River Chinook were chemically analyzed in 2018. Total PAH concentrations in stomachs ranged from 73.7 to 834 ppb wet weight (ww). Total DDT concentrations in stomachs ranged from 12.8 to 142 ppb ww. Total PCB concentrations in stomachs ranged from 25.7 to 58.0 ppb ww (DIVER, 2020).

Thirty-eight whole body (less livers and stomach contents) composite samples were analyzed from the Lower Willamette River in 2018. Total PAH concentrations in these samples ranged from 4.7 to 36 ppb ww. Total DDT concentrations in whole bodies ranged from 7.0 to 497 ppb ww. Total PCB concentrations ranged from 11.6 to 391 ppb ww. Lipids in whole bodies (less livers and stomach contents) ranged from 0.8-3.0% (DIVER, 2020).

Twelve composite samples of stomach contents of out-migrating hatchery-spawned Lower Duwamish River Chinook were chemically analyzed in 2018 (WDFW unpublished data). Total PAH concentrations in stomachs ranged from 11 to 8000 ppb wet weight (ww). Thirty eight whole body (less livers and stomach contents) composite samples from the same collection were analyzed. Total DDT concentrations in whole bodies ranged from 1.6 to 41 ppb ww. Total PCB concentrations ranged from 4.3 to 130 ppb ww. Lipids in whole bodies (less livers and stomach contents) ranged from 1.7-3.1%. Though stomach contents from these fish were not analyzed for DDTs and PCBs, stomach contents from composites of fish outmigrating from the Lower Duwamish River in 1989, 1990, and 2006 contained between 4 and 50 ppb ww of total DDT and between 92 and 400 ppb ww of total PCBs.

3 Project description

3.1 Project purpose and description

The purpose of this study is to determine whether dietary exposure to contaminants of concern (COCs) at concentrations relevant to conditions in Portland Harbor (PH) is associated with growth impairment. A secondary objective is to determine whether exposure to contaminant mixtures in food (at concentrations observed in fish from Portland Harbor) reduces survival of fish after challenge with a pathogenic bacteria (immunotoxicity). Though this lab study is primarily intended to support interpretation of Willamette River field observations in 2018, exposure conditions from both the Lower Willamette River and the Lower Duwamish River were evaluated in selecting mixture composition and doses for this work.

This study will expose juvenile Chinook to chemicals of concern [PCBs, DDTs, and PAHs] in diet to evaluate growth and immune response as injury endpoints associated with tissue concentrations (derived from dietary exposure). The study design for the growth evaluation includes 5 exposure doses based on a dilution series of a mixture of the above compounds, with a concentration range and proportion of chemicals present in the mixture intended to be representative of concentrations found in stomach contents of juvenile fish collected from the Portland Harbor area and the Lower Duwamish River. One solvent control group (no chemicals added to diet, but including the solvent used to create chemical mixtures) plus one clean control group (un-altered food) will also be tested. Growth metrics will include weight and length at the time of sacrifice (5-weeks of exposure to contaminated diet), and average growth rate across recent 7-, 14-, and 21-days of growth prior to sacrifice using otolith microstructural analysis. Whole body composite samples will be analyzed for COC concentrations to relate lab exposures to field conditions. Liver tissue composite sample may be analyzed for PAH metabolites if whole body concentrations are insufficient to make comparisons to field collected fish.

For the pathogen challenge, fish surviving the contaminant exposure will be exposed to a defined concentration of a gram negative bacterial pathogen, *Aeromonas salmonicida*. *A. salmonicida* has a large host range and is the etiological agent of the disease known as furunculosis. Furunculosis is a systemic disease of salmonid which can cause high mortality (Feckaninova et al. 2017). The same dietary treatment groups used in the growth study will be examined except for the clean control group. Each of the resulting 6 feeding treatment groups will be divided into two further disease treatments, either exposed to *A. salmonicida* or to bacterial media alone. Fish will be held 21-28 days after exposure to *A. salmonicida* and survival in each disease treatment tank will be tracked as the primary endpoint of this study component. Exposure of fish to *A. salmonicida* and resulting mortality will be examined through culture of kidney tissue on to bacterial agar. Brown pigment produced by the bacterial colonies is considered a presumptive identification for the presence of *A. salmonicida* in the fish.

3.2 Target fish

This study is intended to inform analysis of injury to Upper Willamette River Spring Chinook salmon juveniles that outmigrate through the Portland Harbor Superfund site as sub-yearlings. Criteria for identifying a hatchery to supply target fish for this study include (in order of importance): (1) Chinook salmon brood stock; (2) embryo availability by early January due to lab scheduling constraints; (3) Willamette River stock (vs Coastal stock); (4) Ocean-type (vs stream-type) brood stock to match field collected fish. The Little White Salmon National fish hatchery (operated by the US Fish and Wildlife Service) was identified as the best candidate to supply fish for this study.

3.3 Tasks required

Tasks involved in this study include:

- Obtain approximately 15,000 fertilized Spring Chinook salmon button-up fry from Little White Salmon National fish hatchery
- Acclimate fish to laboratory conditions in 3-5 tanks
- Begin feeding fry with uncontaminated commercial food, acclimate fish to pelletized food
- Mix target contaminants (standards) into commercial pelletized food to create target doses. Confirm target concentrations through chemical analysis (including control feeding treatments).
- Select fish within a target initial size range. Distribute fish randomly to feeding treatment tanks. Begin feeding contaminated food.
- After five weeks, end feeding trial, remove sub-samples of fish for chemical analysis and growth measurements
- Extract otoliths, stomach contents, livers from fish, composite whole bodies and livers
- Submit remaining whole body samples to the NOAA NWFSC lab for chemical analyses
- Submit otoliths to the NOAA NWFSC lab for microstructural analysis
- Redistribute remaining fish to 400L tanks for disease challenge
- Pilot study to calculate the lethal concentration (LC) response to the *A. salmonicida* pathogen using subset of control fish
- Exposure fish to desired LC of pathogen
- Track survival for 21-28 days. End pathogen trial, sacrifice fish for growth measurements.
- Complete QA/QC review of data
- Document activities and data related to sample collection and laboratory analyses, and results of data verification and validation activities through NOAA's Data Integration, Visualization, Exploration and Reporting (DIVER) tool (see Section 9)
- Analyze data, prepare report(s) and peer-reviewed publication(s)

3.4 Practical Constraints

The most pertinent practical constraint for this study is the availability of lab space and facilities required to maintain, expose to contaminants and grow fish, and the ability to treat contaminated effluent. Other constraints include availability of funding to support chemical analyses, and the amount of sample mass available for whole body and liver analyses from juvenile Chinook salmon.

The minimum required mass for PAH, PCB, and DDT chemistry analysis is 4 g. The mass required for each liver tissue composite is 50 mg. A minimum of 5 g of tissue would be required for TBT analysis, and therefore, TBT is not a priority for chemical analysis.

4 Organization and Schedule

4.1 Key individuals and their responsibilities

Table 1. Organization of project staff and responsibilities

Name	Title	Phone #	Email	Responsibilities
Mary Baker	Assessment Manager	206.526.6315	mary.baker@noaa.gov	Ensuring that the study will meet the broader requirements of NRDA
Jessica Lundin	Project Manager	206.860.3310	jessica.lundin@noaa.gov	Study planning and design, sample coordinator, lead author on reports and publications
Gina Ylitalo	Environmental Chemistry Program Manager	206.860.3325	gina.ylitalo@noaa.gov	Environmental Chemistry Program Manager, analytic chemistry lead
Jennie Bolton	Laboratory QA Officer	206.860.3359	jennie.bolton@noaa.gov	Provide quality assurance on chemistry sample data
Mary Arkoosh	Supervisory Research Microbiologist	541-867-0327	Mary.arkoosh@noaa.gov	Co-Lead author on reports and publications
Joe Dietrich	Research Ecologist	541-867-0264	Joseph.dietrich @noaa.gov	Co-Lead author on reports and publications, Manager of Fish Disease Laboratory
Ali Bahrami-Bayeh	NWFSC Health and Safety Officer		ali.bahrami-bayeh@noaa.gov	Provide project safety related guidance and oversight
Nicolas Eckhardt	Data Manager	206.526.4821	nicolas.eckhardt@noaa.gov	Oversight of data intake and management

4.2 Project schedule: Study timeframe

This study is planned to occur bewteen December, 2019 and October, 2020.

Table 2. Proposed schedule for completing laboratory work and analysis

Task	Dates	Lead staff (all NOAA)
Prepare diet formulations	December2019/January 2020	Gina Ylitalo
Acclimate fish	January 2020	Joe Dietrich
Feeding Trial-growth	February 2020-March 2020	Jessica Lundin Joe Dietrich
Pathogen challenge	March 2020-April 2020	Mary Arkoosh Joe Dietrich
Laboratory work - chemistry	June 2020-August 2020	Irvin Schultz Jessica Lundin
Laboratory work - otoliths	June 2020-August 2020	Paul Chittaro Jessica Lundin
Report draft	November 2020	Jessica Lundin Mary Arkoosh Others to be determined
Report final	February 2021	Same as above

5 Overall study design

This study will expose juvenile Chinook salmon to chemicals of concern [PCBs, DDTs, PAHs] in diet to evaluate growth and immune function (survival after pathogen challenge) as injury endpoints associated with tissue concentrations derived from dietary exposure.

15,000 Chinook salmon will be obtained from the White Salmon National fish hatchery as button-up fry (see section 3.2 for source considerations and section 5.3 for requirements for number of fish), and grown for about 1 month post-hatch on commercially available food not dosed with chemicals. Fry will be transferred to and raised at in the Fish Disease Laboratory (FDL) at NOAA's Newport Research Station at Hatfield Marine Science Center in Newport, Oregon. Fish will be held in indoor tanks with flowing dechlorinated city water. The flow-through system will provide dechlorinated water to each tank. The temperature of the water will be maintained between 8 and 11^{o}C and water in each tank will be aerated. Water quality parameters will be monitored throughout the study. The tanks will be distributed evenly throughout the space available and feeding treatment replicates will be randomly assigned to available tanks. The laboratory building in which the wet lab is located will be secured in the evenings and on weekends, with access limited to those with Federal Facility CAC keycards.

The first phase of the study design includes feeding with contaminated food with 5 exposure doses representing stomach contents from fish collected in the Lower Willamette River and the Lower Duwamish River (see section 5.1), plus one clean control group (un-altered food) and one solvent control group (no chemicals in diet, but including the solvent used to create chemical mixtures). All exposure groups will have 4 replicate tanks containing the target fish (at least 200 fish per tank), for a total of 28 exposure tanks. An additional tank of fish will be maintained to monitor growth to adjust the quantity of food offered over time. Based on prior experiments with the target species (Meador et al, 2006), survival is expected to be high during the feeding study, with limited variability in environmental conditions between tanks.

Feeding with contaminated diets will begin at about 1 month post hatch when all fish have acclimated to consuming pelletized food and they are large enough to consume pellets dosed with contaminants (approximately 1.5 g wet weight). Prior to allocating fish to feeding treatment tanks, fish in all tanks will be sorted to limit the size range at the beginning of the test using a floating adjustable fish grader which sorts fish through a combination of girth and length (target range to be determined). At this point, 30 fish will be removed and frozen for potential later analysis if a determination of pre-test contaminant concentrations is desired. The 1.0 m diameter, 400L fiberglass treatment tanks will contain a similar number of fish per tank of a similar size range. Standardizing the starting weight of fish would limit variation in growth at the end of the test within each treatment group given the available pool of fish (Meador et al., 2006). Sorting fish through a combination of girth and length is expected to limit the starting weight range.

Fish will be fed Otohime Fish Diet, reported to contain 11% lipid content. While they are acclimated to hatchery conditions they will be fed pellet sizes B1 and B2 (250-650 um).

Chemical diet treatments will be prepared at the NWFSC lab in Seattle. Nominal concentrations will be verified by analysis of a sample of food for each treatment. Otohime Fish Diet C1 (580-840 um) will be treated using a stock solution of a contaminant mixture in dichloromethane ($MeCl_2$) (50mg mL^{-1}). See section 5.1 for more information on target concentrations for each treatment. All fish food for a given treatment will be made in one batch. Fish pellets will be placed in a stainless steel bowl, a calculated amount of stock solution will be added to 4 L of MeCl2, and the entire amount will be added to the bowl covering all the pellets. The mixture will be stirred occasionally and allowed to air dry in a fume hood. Once dried, the pellets will be placed into glass jars previously fired at 450^{o} C, and wrapped in aluminum foil to prevent UV light exposure, with a tight-fitting lid, and kept at -20^{o}C until needed to feed fish.

Fish will be fed approximately 1.9% bw day^{-1} based on their average day 0 wet weight, increased each week to account for growth (determined with a growth equation that assumes a conversion efficiency of 20%, Meador et al, 2006). From a separate tank, at least 10 fish will be weighed frequently to determine their growth rate to keep the amount to be fed to all tanks at 1.9% bw day^{-1}. Feeding will occur at least twice per day for at least 6 days per week throughout the exposure period. 50 fish per tank will be collected after 5-weeks of dietary exposure. To collect 50 fish from each tank, the water level will be lowered and a dip net will be used to collect fish from the middle of the remaining water column. The length of time for dosing was selected by estimating residence time for juvenile salmonids from the Willamette River and other systems (Healy 1991; Thorpe 1994). Fish will be weighed, measured, and frozen for 'frozen' dissection after transport to the Northwest Fisheries Science Center in Seattle, Washington. Thirty (30) of these fish will be dissected to remove stomach contents, livers, otoliths and remaining tissues will be combined into three 10-fish composites for chemical analysis. The additional 20 fish will be remain frozen and will be archived in a locked, -80 deg freezer for later analysis if necessary. Replicate composites of whole body chemistry from each tank are intended to evaluate whether fish in the tank have achieved concentrations similar to those observed in field collected fish.

Fifty fish from each control feeding treatment tank will be collected, as described above. If control and solvent control fish are not different in weight and length (generalized linear model, $p<0.05$), the solvent control fish will be used for statistical comparisons of chemical concentrations, lipid content and class, and otolith microstructure comparisons to other treatments and the clean control fish will be archived. If lengths or weights are different between the two control treatments, otoliths and tissues will be analyzed from both control diet treatment types.

After collection, fish will be immediately frozen on dry ice, maintained in a frozen condition, and transferred to a -80 deg C freezer at the NOAA Northwest Fisheries Science Center. Frozen fish will later

be measured, weighed, and dissected over dry ice (section 6). Otoliths will be processed for microstructural analyses to determine growth rates. Livers will be removed for archival and potential chemistry analyses. The whole body (minus stomach contents, liver and otoliths) will be composited and undergo chemical contaminant analysis.

During the 5-week dietary exposure, a pilot study will be performed to generate a lethal concentration response curve using a subset of control fish and the pathogen *A. salmonicida* This pilot effort will determine the exposure concentration for the pathogen challenge component of the study. The second phase of the study (pathogen challenge) will begin immediately following the 5-weeks of dietary exposure. Fish will be offered clean food at a rate of up to 1% bw day^{-1}.

For the disease challenge phase, the clean control feeding treatment will be discontinued due to limitations in tank space. Fish from the clean control treatment will be weighed, measured, and archived at the end of the feeding trial phase. Fish from each replicate feeding treatment tank from all other groups will be divided into two new tanks, one replicate will not be exposed to *A. salmonicida* (nonpath), and the other will be treated with the target concentration of *A. salmonicida* (path) developed during the pilot effort. To divide fish into new tanks, the water level on the source tank will be lowered and a dip net will be used to collect fish from the middle of the remaining water column. Fish will be counted as they are collected and alternately placed in each of the two new tanks for the disease challenge phase. Each disease challenge tank will contain the same number of fish (ideally at least 50 fish per tank unless an insufficient number of surviving fish are available to reach this goal). Excess fish excluded from the test at this point will be archived. This will result in 48 400 L disease challenge tanks: the four replicates of each of six feeding treatments divided into 8 tanks, four exposed to the pathogen, and four non-path tanks. Fish from each of the 48 disease challenge tanks will be removed from their 400 liter circular tank, placed in to separate exposure vessels containing aerated fresh water and the target concentration of pathogen or bacterial media for 60 minutes. Following exposure to the pathogen or media control, the fish will be returned to their 400 liter tank, and observed daily for mortalities for 21-28 days (until three consecutive days are observed without additional mortality). Fish will be offered untreated Otohime Fish Diet during this period. Dead fish will be removed throughout the observation period and weight and length collected. Using sterile technique, kidney tissue from all dead fish will be streaked on to growth agar, and allowed to incubate for colony bacterial growth. Bacterial colonies will be checked visually for the presence of brown coloration which is presumptive identification for *A. salmonicida*. These fish will be collected, placed in individual labeled bags (labels inside and outside) and frozen for possible later dissection to archive otoliths. At the end of the observation period, all remaining fish from pathogen treatments will be sacrificed, weighed, and measured and 30 fish from each pathogen tank will be placed in individual labeled bags (labels inside and outside) and frozen for possible later dissection to archive otoliths. After measurement or dissection, all fish exposed to pathogens will be autoclaved due to biohazard concerns. Fish from non-path treatments will be weighed, measured, and archived for possible later analysis.

Proper handling and storage of all tissue samples will be maintained prior to and upon delivery to the receiving laboratory. Chain of custody will be initiated when fish are collected and transferred between facilities throughout the study (Section 6.3.4). Analytic methods and quality assurance protocols are described in Sections 7. Data evaluation and interpretation techniques are described in Section 8. Documentation and records management practices are described in Section 9.

5.1 Exposure Considerations/treatment groups

- Clean control fish will be fed unaltered Otohime Fish Diet. Solvent control fish will be fed pellets treated identically to those for other doses, including soaking with $MeCl_2$.
- Stomach content concentrations of PAHs (high and low molecular weight parent compounds and alkylated compounds), PCBs, and DDTs in composites of field collected fish from Portland Harbor and the Lower Duwamish River were used to select target dietary treatments intended to mimic stomach contents of field-collected fish both in magnitude and proportionality (Table 3). Median concentrations of total PCBs and total PAHs in stomach contents from both sites were used to select concentrations for dose 2. The highest stomach content value of DDT from Portland Harbor was excluded in selecting a median value for the total DDT concentration for dose 2.
- The list of compounds and relative proportions of each to be added to food are provided in Table 4.
- Dose 5's target total PAH concentration represents the highest concentration tested in Meador et al (2006) (converted from dry weight to wet weight using 9% moisture (Rangen, Inc. personal communication)). Target concentrations for other exposure groups were established by maintaining the relationship between each compound in dose 2 and creating log-based dilution concentrations.
- Whole body tissue concentrations of PCBs (lipid adjusted), DDTs (lipid adjusted), and PAHs in composites of field collected fish from Portland Harbor will be used to confirm that laboratory food exposures result in tissue concentrations similar to those found in the environment. The total lipid content in field collected fish is expected to be lower than in laboratory exposed fish based on Meador et al. (2006).
- Environmental conditions (temperature and salinity) will be representative of those in the Willamette River when fish were collected from the field.
- Two control feeding treatments will be included: untreated food, and solvent treated food (with no chemicals added, but including the solvent used to create chemical mixtures).
- Untreated food will be analyzed for contaminants of concern.
- Commercially available fish food (containing approximately 11% lipid) will be used for dosing with contaminants.

Table 3. Exposure groups-target concentrations in food

Dose name	**PCBs ng/g wet weight**	**DDTs ng/g wet weight**	**Total PAHs ng/g wet weight**
Control (CC)	None added (<1 ppb)	None added (<1 ppb)	None added (<1 ppb)
Solvent control (SC)	None added (<1 ppb)	None added (<1 ppb)	None added (<1 ppb)
T1	**9.1**	**3.7**	**120**
T2	**52**	**21**	**690**
T3	**300**	**120**	**3900**
T4	**1700**	**690**	**23000**
T5	**9900**	**4000**	**130000**

We used DDT, PCB, and PAH stomach content data from juvenile Chinook salmon collected from Portland Harbor in 2018 (for DDTs, PCBs, and PAHs), Duwamish River in 2006 (n = 1 for DDTs and PCBs only) and 2018 (n = 10 for PAHs only) to assist in selection of analytes to be added to the fish food as part of the feeding study. For selection of the DDT analytes, stomach content data from three 2018 Portland Harbor sites (Owens Corning, Swan Lagoon, and T4) (DIVER, 2020), and one 2006 Duwamish River site were pooled. For the PCB analyte selection, stomach content data from four 2018 Portland Harbor sites (Arkema, Owens Corning, Swan Lagoon, and T4) (DIVER, 2020), and one 2006 Duwamish River site were pooled. The PAH data from four 2018 Portland Harbor sites (Arkema, Owens Corning, Swan Lagoon, and T4) (DIVER, 2020) plus ten 2018 WDFW Duwamish River stomach content samples collected from six Duwamish River sites (Slip 4, opposite Slip 4, Turning Basin, Rhone Poulenc, Jack and Joe Block Park, Jack Perry Park) (WDFW unpublished data) were pooled for the PAH analyte selection.

The concentrations (on both mass and molar basis) of individual analytes within each contaminant class (DDTs, PCBs, PAHs) measured in the stomach content samples were summed (∑DDTs, ∑PCBs, ∑PAHs) and are presented in Table 4. The percent contribution of each individual analyte contributing to its corresponding summed contaminant class was determined using the following equation:

(Analyte concentration/summed concentrations of all analytes within a contaminant class) x 100

= Percent contribution of an analyte to the summed analytes within a contaminant class

Example sample A: pyrene concentration = 15 ng/g, ww, ∑PAHs = 200 ng/g, ww

% pyrene contributing to ∑PAHs in sample A = (15 ng/g, ww / 200 ng/g, ww) x 100 = 7.5%

This step was completed for all analytes within each of the three contaminant classes, thus providing percent contribution of each analyte to the summed value within a contaminant class on a mass and a molar basis.

For each of the six DDTs determined in the stomach content samples, analytes (*p,p'*-DDD, *p,p'*-DDE, *p,p'*-DDT) that contributed greater than or equal to 5% to the ∑DDTs (on either a mass or molar basis) were selected for inclusion in the feeding study.

For each of the 40 PCB congeners determined in the stomach content samples, one or two analytes from each of five homologue series (i.e., tri-, tetra-, penta-, hexa-, hepta-chlorinated PCBs) that were the primary contributors to the ∑PCBs (on either a mass or molar basis) were selected for inclusion in the feeding study. In most cases, these analytes contributed > 3% to the ∑PCBs except CBs 28, 52, 105, and 170 (percent contribution ranged from 2.3 to 2.9%). Individual octa-, nona-, and deca-chlorinated congeners were minor contributors to ∑PCBs (each analyte contributing < 1% to ∑PCBs), and thus were excluded from the feeding study.

For each of the 42 PAHs (including alkylated homologues) determined in the stomach content samples, analytes (2-methylnaphthalene, 2,6-dimethylnaphthalene, acenaphthene, fluorene, phenanthrene, 1-methylphenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene) that contributed greater than or equal to 2% to the ∑PAHs (on either a mass or molar basis) were selected for inclusion in the feeding study except benzo[*k*]fluoranthene (~ 2%) and benzo[*e*]pyrene. Although two analytes (naphthalene and benzo[*a*]pyrene) contributed < 2% to the ∑PAHs (based on either a mass or molar basis), they were both included in the feeding study due to either increased frequency of detection in juvenile Chinook salmon samples from the Pacific Northwest (naphthalene) or compound toxicity (benzo[*a*]pyrene).

Table 4. Compounds to be added to food

Analyte	% analyte to sum analytes (molar basis)	% analyte to sum analytes (mass basis)	Ratio abbreviation
p,p'-DDD	12.2	12.2	p,p'DDD/∑DDTs
p,p'-DDE	76.6	74.9	p,p'DDE/∑DDTs
p,p'-DDT	6.2	7.3	p,p'DDT/∑DDTs
Percent of sum DDTs[1]	*95.0*	*94.4*	
Naphthalene (NPH)	0.41	1.48	NPH/∑PAHs
2-methylnaphthalene (MN1)	0.6	0.68	MN1/∑PAHs
2,6-dimethylnaphthalene (DMN)	0.74	5.33	DMN/∑PAHs
acenaphthene (ACE)	3.24	2.78	ACE/∑PAHs
fluorene (FLU)	4.5	1.49	FLU/∑PAHs
phenanthrene (PHN)	24.9	11.4	PHN/∑PAHs
1-methylphenanthrene (MP1)	1.33	3.92	MP1/∑PAHs
fluoranthene (FLA)	24.5	13.4	FLA/∑PAHs
pyrene (PYR)	15.7	9.9	PYR/∑PAHs
benz[a]anthracene (BAA)	4.52	2.38	BAA/∑PAHs
chrysene (CHR)	4.31	5.59	CHR/∑PAHs
benzo[a]pyrene (BAP)	1.54	1.04	BAP/∑PAHs
benzo[b]fluoranthene (BBF)	2.86	2.30	BBF/∑PAHs
Percent of sum PAHs[2]	*89.2*	*61.69*	
PCB28	3.47	2.78	PCB28/∑PCBs
PCB52	3.43	2.99	PCB52/∑PCBs
PCB101	5.35	5.11	PC101/∑PCBs
PCB105	2.27	2.29	PCB105/∑PCBs
PCB118	5.53	5.44	PCB118/∑PCBs
PCB138	12	12.6	PCB138/∑PCBs
PCB153	12.7	13.2	PCB153/∑PCBs
PCB170	2.03	2.29	PCB170/∑PCBs
PCB180	5.21	5.71	PCB180/∑PCBs
PCB187	3.48	4.14	PCB187/∑PCBs
Percent of sum PCBs[3]	*55.47*	*56.55*	

[1] Two other DDT analytes were also detected in juvenile Chinook salmon stomach contents and together they contributed 5% and 5.6% to sum DDTs (∑DDTs) on a molar basis and mass basis, respectively. Individually, each of the two DDTs contributed < 5% to the ∑DDTs on either a molar or mass basis.

[2] Twenty nine parent PAHs and alkylated PAH homologs were also detected in juvenile Chinook salmon stomach contents and their combined contributions were 10.8% and 38.31% to sum PAHs (∑PAHs) on a molar basis and mass basis, respectively. Individually, each of the 29 parent PAHs or alkylated PAH homologs contributed < 2% to the ∑PAHs on either a molar or mass basis.
[3] Nineteen other PCB congeners were also detected in juvenile Chinook salmon stomach contents and their combined contributions were 44.53% and 43.5% to sum PCBs (∑PCBs) on a molar basis and mass basis, respectively. Individually, each of the 19 PCB congeners contributed < 2% to the ∑PCBs on either a molar or mass basis.

5.2 Developing a Lethal Concentration Curve for Disease Challenge

Prior to conducting a disease challenge experiment with a new group of fish it is necessary to conduct a lethal concentration curve study to determine the bacterial concentration that will result in the target percent cumulative mortality (e.g., LC 30 or LC 50) during the challenge experiment. The steps required to develop this curve as described below:

- Prepare an *A. salmonicida* stock culture and grow to an optical density reading that corresponds to the peak of the exponential growth phase
- Use the stock culture to expose groups of fish to bacterial dilutions ranging from $1x10^{-1}$ to $1x10^{-6}$ colony forming units (cfu)/ml.
- Place the required amount of water in the exposure vessels, add an air stone, and transfer the fish to the exposure vessels
- Add the appropriate amount of stock bacterial culture to each tank to arrive at the required bacterial dilution in the exposure bath (for example $1x10^{-1}$, $1x10^{-2}$, $1x10^{-3}$, $2x10^{-4}$, $1x10^{-4}$, $1x10^{-5}$, $1x10^{-6}$)
- Expose the fish in the exposure vessels to the dilutions of *A. salmonicida* and carefully transfer the fish back to the flow through 400 L tank
- Collect and verify mortalities daily
- Prepare an LC-response curve of *A. salmonicida* Exposure Dilution vs. Percent Cumulative Mortality.

5.3 Parameters to be determined

Parameters to be determined/Data to be collected in this study include:

- Lab environmental monitoring
    - Room air temperature (daily)
    - Water temperature system wide (continuous)
    - Dissolved oxygen in each tank (weekly or more frequently)
    - pH and chlorine system wide (weekly)
- Biological metrics at the end of 5-week dietary exposures (50 individual fish per tank)
    - Fish total length (mm)
    - Fish body mass (g)
    - Liver whole weight (mg) (proxy for nutritional status)
    - Body condition (e.g., Fulton's condition factor)
- Biological metrics four weeks after pathogen challenge (following five-week dietary exposure)
    - Survival

    - Fish total length (mm)
    - Fish body mass (g)
- Tissue chemistry
    - Composite samples of whole bodies (less stomach contents, otoliths, and liver) tissue analyses for PCBs, DDTs, PAHs, percent lipids, and lipid class (wax ester and sterol esters, triglycerides, free fatty acids, cholesterol, phospholipids and other polar lipids) – analysis after 5-week dietary exposures (3 composite analyses per feeding treatment tank)
    - (Optional) PAH metabolites in liver tissue after dietary exposure
    - Composite samples of whole bodies (less stomach contents, otoliths, and liver) tissue analysis for PCBs, DDTs, PAHs, percent lipids, and lipid class (wax ester and sterol esters, triglycerides, free fatty acids, cholesterol, phospholipids and other polar lipids) – four weeks after pathogen challenge (fish from non-path tanks only due to biohazard concerns)
- Growth
    - Otoliths (microstructure analysis) at the end of 5-week dietary exposures (30 individual fish per tank), average daily growth in most recent 7-, 14-, and 21-days
- Verification of infection
    - Presumptive identification of *A. salmonicida* in kidney tissues after pathogen challenge (mortalities only)

5.4 Number of fish required

Based on the analyses to be performed in Table 5, approximately 15,000 fish are required to complete the study. For each of the 28 tanks for the feeding phase, 100 surviving fish are needed to start the pathogen challenge phase; 50 fish will be removed after five weeks of feeding (150 fish per tank should survive the five week study for a total of 4200 fish of similar size to start the feeding study). Additional fish will be required to standardize starting fish size (4200), conduct range-finding for the pathogen challenge (900), verify tissue concentrations before the test begins (30), and to monitor growth rates (150).

Table 5. Analyses to be performed

Analysis			**# Fish**	**Samples for analysis**	**Notes**
Chemistry	Diet formulation	PCBs, DDTs, PAHs, % lipids	diet	4	5 doses, 1 control, 1 solvent control (in duplicate)
Chemistry	Whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class	720	72	6 exposure groups x 4 replicate tanks x 3 composites per replicate (10 fish per composite) **after 5 weeks of exposure**
Chemistry	Whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class	480	archived	6 exposure groups x 4 replicate tanks x 20 additional fish after 5 weeks of exposure

Chemistry (archived)	Whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class	120	archived	Clean control group x 4 replicate tanks x 50 fish
Chemistry (archived)	Whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class	30	archived	These fish represent contaminant and lipid concentrations prior to dosing with contaminated food.
Chemistry (archived)	Whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class	720	archived	Fish surviving after phase 2 disease challenge, non-mortalities, three replicates of 10 non-pathogen exposed fish per exposure tank (6 x 4 x 30 = 720). Analysis could indicate whether depuration occurred after removal of chemical exposure..
Chemistry	Livers	PCBs, DDTs, PAHs, % lipids, lipid class	720	archived	6exposure groups x 4 replicate tanks x 3 composites per replicate (10 fish per composite) **after 5 weeks of exposure**
Growth	Whole bodies	Weight monitoring to adjust food quantity	200	(same number as in other tanks at start of feeding study)	Frequent measurements of fish from an additional control tank will be used to calculate growth rate and amount of food to add to each tank as fish grow. Fish do not need to be archived at the end of the test.
Growth	Otoliths	Microstructural analysis/archive	720	720	6 exposure groups x 4 replicate tanks x 30 fish per replicate (same fish used to create chemistry composites).
Growth	Whole bodies	Weight/length	1400	1400	When fish are removed after 5 weeks, all fish will be weighed and measured before analysis or archiving (7 exposure groups x 4 replicate tanks x 50 total fish per replicate). 720 of these fish will be chemically analyzed (described above).
Immunotoxicity	Pathogen Disease Challenge	Pilot study to characterize lethal concentration curve (survival)	900		6 pathogen concentrations (5 pathogen and 1 control (in triplicate tanks), 50 fish per tank. These fish will not be archived.
Immunotoxicity	Pathogen Disease Challenge	Survival, weight/length	2400	archive (720)	6 pathogen treatments, 4 replicates treated with pathogen, 4 replicates untreated, 50 fish per tank at the start of phase 2. 720 non-path fish will be archived (30 from each non pathogen tank).

Immunotoxicity	Pathogen Disease Challenge	Presumptive identification of *A. salmonicida* exposure	unknown		All mortalities will be examined for the presence of *A. salmonicida*

6 SOP for Fish Processing (measurement, dissections, archival), Tissue Sample Handling, and Record Keeping

The SOP outlined below describes the gear and procedures to be employed for measurement, processsing to transfer to NWFSC for dissection or archival. After sacrifice and collection, all fish samples should be maintained in a controlled environment when they are not being processed (locked room or cabinet).

6.1 Equipment, reagents, and supplies

- Dry ice
- Buckets, fish transfer and sacrifice
- Aeration: bubblers, airlines, airstones
- Tricaine Methanesulfonate (MS222) anaesthetic
- Shipping coolers
- PTFE (polytetrafluoroethylene) cutting boards or boards covered with clean aluminum foil
- Metric measuring board – for measuring fish
- Electronic balance accurate to 0.001 g, calibrated annually, for fish wet weight, liver weight
- Weigh boats
- Paper towels
- Kimwipes™
- Dissection kit with stainless steel scalpel, scissors, and forceps, plus additional scalpel blades (enough to change between each site) for collection of liver samples and fin clips
- Magnifying glass on stand, with light
- Tap water
- Deionized water
- Isopropyl alcohol
- Dry ice
- Aluminum foil – heavy duty
- Squeeze bottles
- Ziploc® bags
- Micro brand soap for cleaning lab surfaces and instruments
- Thin tip black Sharpies
- Lab tape, different colors
- Nitrile exam gloves – talc-free (S,M,L,XL)
- Chain of custody forms
- Chain of custody tape
- Sample Processing Form (printed on waterproof paper)
- Sample Processing Notes form (printed on waterproof paper)

- I-CHEM Certified 200-0250 series, 20 mL jars (for rinsate blanks if there is concern about cross contamination from reuse of sampling equipment)

Tissue for analytical chemistry

- Sampling jars – 20 mL jars, I-CHEM Certified 200-0250 series, Type III glass (solvent rinsed) with Teflon-lined polypropylene lids]
- Solvent rinsed aluminum foil [for whole bodies]
- Sample jar labels – cryogenic, laser ready
- Pre-label all jars

Liver samples for archival and possible analytic chemistry

- 1.5 mL polypropylene SnapTop tubes [no preservative or solvent needed]
- Sample tube labels - cryogenic, laser ready
- Pre-label all tubes
- Kimwipes™

Otolith

- 1.5 mL polypropylene SnapTop tubes [no preservative or solvent needed]
- Label placed inside tube

Disease Challenge

- Lab tape, different colors
- Nitrile exam gloves – powder-free (S,M,L)
- 100x15 mm sterile Petri plates
- Alcohol burner
- Lighter
- Ethanol
- Beakers, 250ml
- Bleach
- Spray bottles
- Electronic balance with USB/RS232 communication
- 1- µl sterile disposable inoculating loops
- Necropsy cutting boards
- Necropsy instruments
- Paper towels
- Disposable plastic bags (12”x14”)
- Bacteriological Media - Tryptic Soy Agar (TSA)
- Parafilm
- Incubator
- Autoclave
- Tricaine Methanesulfonate (MS222) anaesthetic
- Aquaculture Supplies
    6” Fishnets with PVC handle extensions
    Aquaculture disinfectant: I-O-Safe or Virkon Aquatic

Buckets, fish transfer and sacrifice
Aeration: bubblers, airlines, airstones

- Wet ice
- Autoclave bags and biohazard buckets
- Recordkeeping:
  Laptop computer with P3/P4 software and digital communication adapters
  Digitizer board and pen
  Sharpies
  Waterproof paper

6.2 Lab setup and preparation

Fish to be processed for transfer to the NWFSC or other analyses will be removed from their source tank with a small dip net. Fish will be euthanized with a lethal dose of MS222 in a small container. Fish will be measured (weight and length) and placed in individual plastic bags labeled with the sample number on the inside and outside of the bag. Fish samples will be placed in a cooler with dry ice until being transferred to a locked -80 deg C freezer for archival or later dissection.

Fish to be dissected to remove otoliths and separate tissues for chemical analysis will be placed on dry ice with a paper towel below the ice to label with the sample number. All dissections will be performed on dry ice to keep fish frozen during the dissection process Following the completion of the dissection, all fish will be placed in a cooler with dry ice until being transferred back to a locked -80 deg C freezer.

6.3 Documentation

When fish die after the beginning of the study (in either phase), a sample ID, date of death, weight, and length should be recorded on a Fish Data Form. Separate fish data forms should be completed for each phase. When fish are euthanized at the end of each phase, a fish data form will be completed where sample IDs are assigned sequentially accounting for any fish that have previously died during that phase. Fish measurements (weight and length) should be documented on fish data forms and fish IDs and death dates should also be recorded on Chain of Custody forms (unless fish are to be immediately destroyed (for example, path samples)).

When fish are dissected to remove otoliths, stomach contents, or livers, fish dissection information must be documented on a Sample Processing Form, and individual component Chain of Custody (COC) forms. Separate forms should be used for each phase.

A sample Fish Data Form, Sample Processing Form, and Chain of Custody form are provided in Appendix A.

6.3.1 Sample identification

To facilitate data interpretation, a numbering system has been developed to track the phase, treatment, replicate tank, and number for each individual fish and tissue type. To track physical samples, each sample container (bags for whole bodies and containers for livers and otoliths) will be labeled with the sample ID attached to the bag, a label placed inside the bag, and both on the lid and on the side of containers. All labels will be cryogenic, laser printer ready labels that are written using permanent marker or preprinted. Below is a description of the five concatenated components that make up the SampleID for whole fish and the individual fish parts after dissection.

- Phase – There are two phases in the lab study. They are designated as Feeding and Disease. In the SampleID, the phase is represented as a single character, either F (Feeding) or D (Disease). The Phase designation is at the start of the SampleID. This single letter will be the first component of the SampleID.
- Treatment – There are 7 different feeding treatments (described in table 3) that fish will be exposed to. They are designated via a two digit code described as SC (Solvent Control), CC (Clean Control), T1, T2, T3, T4, T5. This two digit letter and number combination will be the second component of the SampleID.
- Tank – Each treatment will be replicated 4 times. This necessitates four tanks be used for each treatment. The tanks will be designated as A, B, C, & D. This single character will be the third component of the SampleID.
- Pathogen – After the completion of the Feeding phase, a subset of surviving fish will be transferred to the disease phase of the study. Fish from each tank from the feeding phase will be split into two groups. One tank for each treatment will be exposed to pathogens (path) and one not exposed to pathogens (non-path). Sample IDs for the disease challenge phase will include a designation to indicate whether they have been exposed to pathogens or not. Fish exposed to pathogens will be designated with a P and Non-Pathogen exposed fish are designated as NP. This is single or double digit letter code is the fourth component of the SampleID.
- Number – The final component of the SampleID is a three digit number representing an individual fish. There are two potential number ranges for the fish depending on the phase when the number will be assigned. Fish euthanized or dying during the Feeding phase, could be assigned a number between 001 and 200. For fish euthanized or dying in the Disease phase, the fish number range is 001 – 075.
- The Sample ID for fish before dissection consists of Phase (one character), Treatment (2 characters), Tank (one character), Pathogen (one or two characters), & number sequence (three digits). Upon dissection, the sample number will be followed by two or three characters qualifying the type of tissue (WH: whole body minus stomach contents, otoliths, and liver; OTO: Otoliths, and LI: Liver)
- Capitalization must be maintained when recording SampleIDs on field/sample data sheets

- Example of Fish Sample IDs:
    - FCCA001 – F (Feed), CC (Clean Control), A (Tank 1), 001 (Fish number 1)
    - DT1BNP001 – D (Disease), T1 (Treatment 1), B (Tank B), NP (Non-Pathogen), 001 (Fish number 1)
    - DT2CP006OTO – D (Disease), T2 (Treatment 2), C (Tank 2), P (Pathogen), 006 (Fish number 6), OTO (Otolith)

- Whole body sample ID example: DT5CNP001WH
- Otolith sample ID example: DT5CNP001OTO
- Liver sample ID example: DT5CNP001LI

6.3.2 Fish Data and Sampling Processing Forms and Notes

Entries for Fish Data and Sample Processing Forms and Notes will be made with indelible ink. Forms and notes should be printed on waterproof paper and clearly state the date and processor name. No erasures should be made; all corrections should consist of a single line-out deletion, followed by the processor's

initials and the date. The processor should initial and date each page at the end of the day. See Appendix A for example forms.

Additional **Sample Processing Notes** can be used to record the following additional information:

- Date and time
- Sample dissection team names
- Description of activity and method
- Time of beginning and end of activity
- Sample identification numbers as on the labels for the individual fish tissue samples
- Fish physical examination comments (general comments only, specific comments will be on sample processing forms)
- Fish dissection comments (general comments only, specific comments will be on sample processing forms)
- Any deviations from the fish processing will be recorded, along with the reason for the changes
- Unusual circumstancs that may affect interpretation of results.

6.3.3 Chain of Custody Procedures

Chain of custody procedures are followed to authenticate a sample from the time it is taken until the results are introduced as evidence. For the purposes of litigation, agencies must be able to prove the legal integrity of all samples and data introduced as evidence. This means that it is necessary to have an accurate written record to track possession, handling, and location of samples and data from collection through reporting. Chain of custody facilitates this verification process. Failure to follow COC procedures in this guideline does not necessarily render data unusable; however the Assessment Manager should be notified of any deviations from the COC guidelines. Assuring that proper COC guidelines are followed is vital to assuring the integrity of the samples, and the data generated by the analysis of those samples.

A Chain of Custody (COC) Form will be initiated when fish are collected to be transferred between labs for processing or archival to track location, disposition, entity responsible for each fish, and, subsequently, individual or composite tissue containers. The COC Form will be completed in indelible ink, scanned, and a copy will accompany the shipment to the laboratory (COC Form, Appendix B). The COC Forms will be enclosed in resealable plastic bags and taped to the inside lip of coolers. The information on this Form will be used to track all samples from field collection to receipt at the analytic laboratory. Upon delivery and receipt of coolers, the COC Forms must be signed and dated by the recipient (analytical laboratory) and the individual (field NOAA staff) that relinquishes the samples. The laboratory is required to log in samples and note non-conformances. Temperature exceedances, or absence of dry ice that may indicate thawing of tissue, will be immediately reported to the Laboratory QA Office and Field Coordinator (Table 1). These samples will be processed with the other samples and flagged in the final data tables. Sample processing and analysis will not proceed until permission from the Chemistry QA Manager or Field Coordinator is given.

Samples are considered to be in custody if they are 1) in the custodian's possession or view; 2) in a secured location and in a locked compartment; or 3) in a container that is secured with an official seal(s) such that the samples cannot be reached without breaking the seal(s). The sample custodian will check that all COC Forms are filled out properly and completely, and that the samples are stored in the appropriate conditions. See Appendix A for form.

6.4 Fish Processing and Handling

The subheadings below outline the procedures and methods used to process the target juvenile Chinook salmon. Fish will be dissected at the Northwest Fisheries Science Center in Seattle after all contaminated food exposures and fish collections are complete. Staff will record the weight and length of the fish, extract the otoliths, and remove and weigh stomach contents and liver as described below. After the completion of the disease challenge phase, any mortalities that occurred during the test will be processed for presumptive identification of pathogen induced mortality. This will occur at the Newport Research Station in Newport, Oregon.

6.4.1 Fish dissection/necropsy overview

Dissection of fish will be conducted by or under the supervision of an experienced fisheries biologist. Fish will be processed on a "clean" work-surface with "clean" instruments as described in Section 6.5, Field lab equipment cleaning and decontamination procedure. Separate tools (scissors and forceps) will be designated for use on outer tissue ("outside") and use on internal tissue ("inside") in order to minimize cross-contamination.

6.4.2 Length and weight

- **Equipment/supplies**
  - Measuring board
  - Scale
- **Protocol/procedures**
  - Target fish will be weighed (to the nearest 0.001 g)
  - Total length will be measured by placing fish flat on a measuring board
    - The measurement will be from the tip of the snout to the posterior end of the longer lobe of the caudal fin (to the nearest mm)
  - Both measurements will be recorded on the Sample Processing Form
- **Decontamination protocol**
  - Between fish, rinse instruments with water
  - Between treatments, follow instrument and work area decontamination protocol below

6.4.3 Collection of otoliths

- **Equipment/supplies**
  - Scalpel
  - Fine tipped forceps
  - Magnifying glass with light
  - 1.5 mL polypropylene SnapTop tubes
- **Protocol/procedures**
  - Make a dorsal to ventral cut from top of operculum, about half way down
  - Extend head forward to expose tissue
  - Extract both of the biggest otoliths (sagittae) from each fish using forceps
  - Place both otoliths in the same tube (just 1 sagittal otolith will be used in the analysis, but in the event it is cracked the other sagittal otolith will be available)
  - Record on the Sample Processing Form
- **Decontamination protocol**

- Between fish, rinse instruments with water
- Between treatments, follow instrument and work area decontamination protocol below

- **Storage and handling of samples**
  - Store at room temperature

6.4.4 Access to internal organs

- **Equipment/Supplies**
  - "outside" scissors and "outside" forceps
  - "inside" scissors and "inside" forceps
  - Scalpels (optional)
- **Protocol/procedures**
  - Internal organs will be accessed by opening the fish with a pair of fine scissors
  - Use "outside" scissors to make incision just anterior to anus and cut straight towards gills
  - Using the "outside" scissors and "outside" forceps, cut out a "window" in the flesh by cutting an arch dorsally beginning and ending at the edges of the incision – try to keep the tissue attached for ease in transferring to the sample container for chemistry analysis
- The internal organs will be gently removed from the internal cavity onto a clean cutting board using "inside" scissors and "inside" forceps
- The liver will be isolated (section 6.4.5).
- The stomach contents will be removed and archived (section 6.4.6).

6.4.5 Collection of liver for archival and possible chemical analysis

- **Equipment/supplies**
  - "inside" scissors and "inside" forceps
  - 1.5 mL polypropylene SnapTop tubes
  - Scale
- **Protocol/procedures**
  - Isolate liver with cleaned "inside" forceps and remove from other internal organs with scissors or scalpel blade
  - If the gall bladder can be identified, do not include it with the liver sample, place it with the whole body composite
  - Tare the 1.5 mL SnapTop tube
  - Place the liver in the 1.5 mL SnapTop tube, no solvent or preservative necessary
  - Weigh the liver to the nearest 0.001 g
  - Close tube securely (audible snap)
  - Record on the Sample Processing Form
- **Decontamination protocol**
  - Between fish, wipe any tissue from tools with Kimwipes™, rinse thoroughly with ethanol, rinse thoroughly with de-ionized water, then dry with clean Kimwipes™
  - Between tanks, follow instrument and work area decontamination protocol below
- **Storage and handling of samples**
  - Samples will be placed on dry ice until being transferred to a -80 deg C freezer

6.4.6 Removing stomach and intestinal contents

- **Equipment/supplies**
  - "inside" scissors and "inside" forceps (x2)
- **Protocol/procedures**
  - The stomach and intestines will be gently separated from the other organs
  - The stomach will be lifted using designated "inside" forceps
  - Using a second "inside" forceps, the contents will be expelled and discarded
  - The stomach tissue (i.e., empty, scraped out stomach) will be rinsed with DI water to remove any traces of contaminant laden food, returned into the body cavity and included with the rest of the fish tissue sample
- **Decontamination protocol**
  - Between fish, wipe any tissue from tools with Kimwipes™, rinse thoroughly with ethanol, rinse thoroughly with de-ionized water, and dry with clean Kimwipe
  - Between tanks, follow instrument and work area decontamination protocol below
- **Storage and handling of samples**
  - Samples of fish without stomach contents will be stored on dry ice until being transferred to a -80 deg C freezer

6.4.7 Remaining whole bodies minus stomach contents, livers, and otoliths returned to labelled bag

- **Equipment/supplies**
  - "outside" scissors and "outside" forceps
  - Composite vial
- **Protocol/procedures**
  - Remaining whole body will be returned to the labelled bag, with the second label that indicates SampleID number with "WH" (e.g., 200001WH, 200002WH, etc.)
- **Decontamination protocol between fish**
  - Between fish, wipe any tissue from tools with Kimwipe™, rinse thoroughly with ethanol, rinse thoroughly with de-ionized water, and dry with clean Kimwipe™
  - Between treatments, follow instrument and work area decontamination protocol below
- **Storage and handling of samples**
  - Samples will be placed on dry ice until being transferred to a -80 deg C freezer

6.4.8 Presumptive identification of pathogen induced mortalities

- **Equipment/supplies**
- Buckets, fish transfer and sacrifice
- Tricaine Methanesulfonate (MS222) anaesthetic
- PTFE (polytetrafluoroethylene) cutting boards or boards covered with clean aluminum foil
- 95% and 75% Ethanol
- Agar plates
- Loops
- Parafilm
- Alcohol burners
- Autoclave bags
- Paper towels

- Kimwipes™
- Dissection kit with stainless steel scalpel, scissors, and forceps, plus additional scalpel blades (enough to change between each site) for collection of liver samples and fin clips
- Magnifying glass on stand, with light
- Tap water
- Deionized water
- Isopropyl alcohol
- Squeeze bottles
- Micro brand soap for cleaning lab surfaces and instruments
- Nitrile exam gloves – talc-free (S,M,L,XL)
- Sample Processing Notes form (printed on waterproof paper)

- **Protocol/procedures**
    - Remove dead fish from tank using a net specific to the tank sitting in disinfectant. Rinse off all disinfectant from net prior to dipping net into tank water.
    - Place dead fish in plastic bag and record tank number, date, and time on the bag using a Sharpie marker.
    - Place the bag in a cooler or bucket with ice.
    - Put net back in disinfectant, disinfect net handle and gloves.
    - Record count of mortalities for that day and tank on the daily mortality log sheet located within the Fish Disease Lab
    - Bring the cooler containing mortalities to Necropsy Lab (NAL 117).
    - Remove the proper number of Tryptic Soy Agar (TSA) plates from the fridge, bring to room temperature
        - Four fish per TSA plate, maximum
    - If not already labeled, label plates with mortality numbers and date – make sure plate is dry
    - Place individual fish on digitizer board and spray with 70% ethanol
    - Log fish ID, tank number, time, date, length, and weight with P3/P4 digitizing software using the specific P3/P4 formatting directions
    - Move fish to cutting area and necropsy. Spray and wipe-down digitizer with alcohol/10% bleach between tanks
        - Necropsy station has 1 beaker of ethanol plus an alcohol flame. Tools need to be dipped in alcohol and flamed before use.
        - Wipe fish from head to tail with an alcohol sprayed paper towel
        - Flame sterilize the instruments before each use and carefully necropsy the fish to expose the kidney (section 6.4.4)
        - Aseptically streak head kidney with a sterile loop on to the labeled quarter of a TSA plate

- **Decontamination Protocol between fish**
    - Dispose of pathogen treated fish, loops, gloves, paper towels, and mortality bags in an autoclave biohazard bag
    - Take all mortalities back to the Fish Disease Laboratory FDL and place in the waste freezer for autoclaving prior to disposal
- **Storage and handling of samples**

- After 24-96 hours record the presence/absence of growth and color of bacterial colonies on the TSA plates next to the mort number on the disease challenge logs and the plates autoclaved
- Autoclave and discard fish exposed to pathogens

6.5 Equipment cleaning and decontamination procedure

When processing specimens for contaminant analysis, anything (work-surfaces, instruments, etc.) that may contact those portions of a specimen that are subject to contaminant analysis must be cleaned according to the sequence below before fish from each treatment is processed.

Between treatments:

A "clean" work-surface (lab counter, cutting board, sorting tray, etc.) and "clean" instruments (stainless steel dissection tools) means they have been:

- wiped and cleared of any tissue or residue
- washed in warm soapy water (Micro brand soap)
- thoroughly rinsed three times using running tap water
- solvent rinsed using isopropyl alcohol (held in a Teflon squeeze bottle)

Field/Lab personnel must change nitrile gloves between treatments**.**

Between fish from the different tanks of the same treatment:

The work surface is wiped of any tissue or residue and rinsed with water. Tools should be wiped with a Kimwipe™ to remove any tissue. Tools used for fin clips or extraction of stomach contents and liver tissue should be rinsed thoroughly with ethanol and de-ionized water. All other tools should be rinsed thoroughly with de-ionized water, and dried with a clean Kimwipe™.

Gloves: Gloves will be worn whenever handling fish. Lab personnel must change nitrile gloves between sampling units, or more often as needed. Gloves will be talc- or dust-free nitrile.

Quality assurance/control. Rinsate blanks should be collected if there is a risk of cross contamination from reuse of sampling equipment. After cleaning the equipment in accordance with the procedures described in this method, rinse the clean equipment with solvent or cleaning solution and collect the rinsate in a sample jar (20 mL jars, I-CHEM Certified 200-250 series). Note on the field sample form when, from which site, and how rinsate blanks were collected.

6.6 Sample handling and storage procedures

- **Whole fish tissue samples for chemistry or archival.** Tissues will be kept frozen during processing, and placed back on dry ice immediately following processing. All tissues will be maintained on dry ice during this time, and placed in a locked -80 deg C freezer or on dry ice at the end of the day.
- **Otoliths**. Otoliths will be placed in dry microtubes and kept at room temperature. At the end of each sample processing day, all otoliths will be placed in a locked drawer at room temperature until processed.
- **Sample archiving.** All excess sample material remaining after laboratory analysis will be archived (with the exception of any samples exposed to pathogens, which will be destroyed due

to biohazard concerns). The laboratory will maintain COC procedures and sample integrity for the entire time the samples are in their possession. The laboratory will store the excess samples until otherwise notified by the Assessment Manager (Table 1).

6.7 Health and Safety

NWFSC (2019) describes health and safety protocols for handling chemicals under laboratory conditions.

7 Analytic methods

The methods used for contaminant analysis of tissues (whole body and liver), otolith microstructural analysis, and confirmation of *A salmonicida* are described below.

7.1 Chemical analysis, fish tissue

Whole body tissue composites (less stomach contents, otoliths, and livers) will be created from juvenile Chinook salmon. The goal is to obtain three whole body composite samples of 10 fish each for DDTs, PCBs, and PAH contaminant analysis from each dietary treatment tank.

The mass requested by the NOAA NWFSC analytic lab for DDTs, PCBs and PAH analysis is a minimum of 4 g of fish in each whole body composite. This mass is to ensure 2 g is available for extraction after potential mass loss following the necropsy and homogenization.

All measurements of DDTs, PCBs, and PAHs in fish tissue composites for this study will be conducted by NOAA NWFSC (Seattle, WA) according to Sloan et al. (Sloan et al. 2004, Sloan et al. 2014). In brief, juvenile salmon bodies with stomach contents, livers, and otoliths removed will be homogenized and extracted with dichloromethane, using an accelerated solvent extractor. The sample extracts will be precleaned on an alumina–silica column, and then further cleaned using size-exclusion liquid chromatography. The sample extracts will be analyzed by low-resolution gas chromatography/mass spectrometry. Measured concentrations in fish tissue will include 45 PCBs (PCBs 17, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 82, 87, 95, 99, 101/90, 105, 110, 118, 128, 138/163/164, 149, 151, 153/132, 156, 158, 170/190, 171, 177, 180, 183, 187, 191, 194, 195, 199, 205, 206, 208, and 209), six DDTs (o,p'-DDD; o,p'-DDE; o,p'-DDT; p,p'-DDD; p,p'-DDE; p,p'-DDT), and 24 PAHs [naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, biphenyl, 2,6-dimethylnaphthalene, acenaphthylene, 2,3,5-trimethylnaphthalene, acenaphthene, fluorene, retene, phenanthrene, 1-methylphenanthrene, anthracene, fluoranthene, pyrene, chrysene + triphenylene (coelute), benzo[*a*]pyrene, benzo[*e*]pyrene, perylene, dibenz[*a,c+a,h*]anthracene (coelute), benzo[*b*]fluoranthene, benzo[*j+k*]fluoranthene (coelute), indeno[1,2,3-*cd*]pyrene, benzo[*g,h,i*]perylene]. Percent lipids will be measured gravimetrically following extraction in dichloromethane, and lipid class determinations will be conducted using thin-layer chromatography/flame ionization detection (Iatroscan; wax ester and sterol esters, triglycerides, free fatty acids, cholesterol, phospholipids and other polar lipids).

7.2 Chemical analysis, liver tissue (optional)

Liver composites will be created using the same fish and composite assignments as used for the whole body composites. Samples will be analyzed if whole body PAH concentrations are not sufficient to judge whether laboratory fish accumulate contaminants similarly to field collected fish. The mass required for

each liver tissue composite is 50 mg. Liver tissue removed from juvenile salmon will be analyzed for hydroxylated PAH metabolites (OHPAHs) by NOAA NWFSC using a modified method described in Ylitalo at al. (Ylitalo et al. 2017). The list of the 30 individual OHPAHs are shown in Table 6. Liver samples will be mixed with water, spiked with surrogate standard, then extracted using methanol by agitation. Liver mixtures will then be centrifuged and supernatant added to a phospholipid removal cartridge, in order to remove proteins and highly polar lipids. Subsequently, a buffer solution containing β-glucuronidase and sulfatase will be added to the treated liver extract in order to cleave the phase II conjugates (glucuronide- and sulfate-conjugates) from the OHPAH metabolite, transforming them into phase I metabolites to be measured. Studies have shown that a portion of phase II PAH metabolites can be formed with glutathione (James 1986, Collier and Varanasi 1991). However, our enzymatic hydrolysis method does not include an enzyme to cleave the bond between glutathione and the PAH moiety. Thus, levels of OHPAH metabolites measured by our method only include unconjugated OHPAH forms as well as glucuronide and sulfate conjugates. The hydrolyzed liver solution will be cleaned and the targeted OHPAHs will be extracted via solid phase extraction (SPE) using methanol/diethylether as the final solvent. The SPE extract will be concentrated and an aliquot of the final methanolic extraction will be injected into the LC-MS/MS (liquid chromatography-tandem mass spectrometry). The 30 individual OHPAHs will be separated through a reverse-phase column (150 mm x 2.1 mm, 1.7 µm particle size) using water and methanol as the mobile-phase in a linear gradient. The 31 analytes will be detected using electrospray ionization in negative mode and monitored/quantified using multiple-reaction monitoring of molecular fragments in negative mode. The final LC-MS/MS analysis will be conducted using an ultra-performance liquid chromatography (UPLC) system (Waters Acquity UPLC) for the separation and a triple quadrupole mass spectrometer (AB Sciex QTRAP 5500) equipped with Turbo-V ion source for the OHPAH detection and quantitation.

Table 6. List of individual hydroxylated PAH metabolites (OHPAHs) analyzed by LC-MS/MS

Individual OHPAHs	**Abbreviation**
2-hydroxynaphthalene	2-OHNPH
1-hydroxynaphthalene	1-OHNPH
6-methyl-2-hydroxynaphthalene	6-CH3-2-OHNPH
1-methyl-2-hydroxynaphthalene *(a)*	1-CH3-2-OHNPH
2-methyl-1-hydroxynaphthalene *(a)*	2-CH3-1-OHNPH
4,4'-Dihydroxybiphenyl	4,4'-OHBPH
2-hydroxydibenzothiophene	2-OHDBT
3-hydroxyfluorene	3-OHFLU
2-hydroxyfluorene	2-OHFLU
trans-9,10-dihydroxy-9,10-dihydrophenanthrene	9,10-OH-9,10-HPHN
trans-1,2-dihydroxy-1,2-dihydrophenanthrene	1,2-OH-1,2-HPHN
3-hydroxyphenanthrene *(b)*	3-OHPHN

Individual OHPAHs	Abbreviation
2-hydroxyphenanthrene *(b)*	2-OHPHN
9-hydroxyphenanthrene	9-OHPHN
1-hydroxyphenanthrene	1-OHPHN
4-hydroxyphenanthrene	4-OHPHN
1,8-*bis*(hydroxymethyl)anthracene	1,8-OHMeANT
2-hydroxy-9,10-anthraquinone	2-OH-9,10-ATQ
1,5-dihydroxy-1,2-dihydro-9,10-anthraquinone	1,5-OH-9,10-ATQ
trans-2,3-dihydroxy-2,3-dihydrofluoranthene	2,3-OHFLA
trans-5,6-dihydroxy-5,6-dihydrochrysene	5,6-OHCHR
trans-3,4-dihydroxy-3,4-dihydrochrysene	3,4-OHCHR
trans-1,2-dihydroxy-1,2-dihydrochrysene	1,2-OHCHR
cis-5,6-dihydroxy-5,6-dihydrobenz[a]anthracene	5,6-OHBaA
trans-8,9-dihydroxy-8,9-dihydrobenz[a]anthracene (c)	8,9-OHBaA
trans-10,11-dihydroxy-10,11-dihydrobenz[a]anthracene (c)	10,11-OHBaA
cis-4,5-dihydroxy-4,5-dihydrobenzo[a]pyrene	4,5-OHBaP
cis-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene	7,8-OHBaP
r-7,*t*-8,*t*-9,*c*-10-tetrahydroxy-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrobenzo[a]pyrene (+/-)	7,8,9,10-OHBaP
trans-4,5-dihydroxy-4,5-dihydrobenzo[e]pyrene	4,5-OHBeP

Otolith microstructure will be analyzed to estimate recent somatic growth using methods described previously (Chittaro et al. 2018). Sagittal otoliths will be embedded in crystal bond and polished in a sagittal plane using slurries (Buehler©'s 600 grit silicon carbide, 5.0 alumina oxide and 1.0 micropolish) and a grinding wheel with Buehler©'s 1500 micropolishing pads. Polishing will cease when the core of the otolith is exposed and daily increments are visible under a light microscope. Otoliths will be photographed using a digital camera (Leica DFC450) mounted on a compound microscope (Zeiss©). Using Image Pro Plus© (version 7, Mediacybernetics), measurements will be taken from each otolith, including distance from otolith core to edge (i.e., otolith radius at the time of capture) and distance from otolith core to daily increments in from the otolith edge (i.e., otolith radius measured at n days before sacrifice).

7.3 Presumptive identification of A. salmonicida

The pathogen *A. salmonicida* produces a brown pigment on bacterial agar and this is considered a presumptive identification of *A. salmonicida* subspecies *salmonicida*. Dead fish will be removed throughout the observation period and weight and length collected. Using sterile technique, kidney tissue from the dead fish will be streaked on to growth agar, and allowed to incubate until colonies appear. Colonies will be checked visually for the presence of brown coloration which is presumptive identification for *A. salmonicida*.

7.4 Laboratory quality assurance

7.4.1 Chemical analysis, fish tissue analytical quality assurance criteria

Quality assurance criteria for DDTs, PCBs and PAHs analyzed in salmon samples for this study are summarized in Table 7 (taken from Sloan et al., 2019).

Table 7. Minimum analytical quality assurance criteria for DDTs, PCBs, and PAHs by gas chromatography/mass spectrometry (from Sloan et al. 2019)

Quality assurance element	Minimum frequency	Acceptance criteria
Instrument calibration	Each calibration standard is analyzed at the start of every batch of samples, or once every two batches in one continuous analytical sequence.	Analyte concentrations must be calculated using point-to-point calibration with at least five concentration levels of calibration standards. Each surrogate standard in the calibrations standards must have an RSD of its response factors (response area divided by the concentration) that is ≤15%.
Continuing calibration	One at start and end of every analytical sequence and between every 10 or fewer for field samples.	The relative standard deviation (RSD) of the analyte responses relative to the internal standard must be ≤15% for the repetitions. This criterion does not apply to Nonachlor III, PBDEs, or PCBs 11, 196, 200, 201, 202, or 207.
Reference material: National Institute of Standards and Technology (NIST) standard reference material (SRM) 1946, 1947, 1974c	One appropriate SRM with every batch of 20 or fewer for field samples.	The concentrations ≥70% of individual analytes, as well as the gravimetric percent lipid, if requested, must be within 30% of either end of the 95% confidence interval range of the certified values. These criteria do not apply to analytes with concentrations below their lower limit of quantification (LOQ) when the lower LOQ is within or greater than the 95% confidence interval, nor to those analytes known to have coeluting compounds.
Laboratory method blank	One with every batch of 20 or fewer for field samples.	No more than 10% of the analytes' concentrations can exceed 2 x lower LOQ. Samples are not corrected for analytes found in the blank.

Quality assurance element	Minimum frequency	Acceptance criteria
Laboratory sample replicates (i.e., duplicates or triplicates)	One with every 26 or fewer field samples.	The RSDs of analyte concentrations must be ≤15% for triplicates, or percent differences must be ≤30% for duplicates, for ≥90% of the analytes that have concentrations > 1 ng/g.
Surrogates (internal standards)	At least one internal standard/ surrogate is added to every sample.	The surrogate recoveries must be between 60–130%.
Interlaboratory comparison	At least one per year, if available.	In conjunction with NIST or the IAEA, accuracy-based solutions, sample extracts, and representative matrices are analyzed. Acceptance criteria are the same as those for reference material. All results are sent back to NIST or IAEA for comparison across laboratories.

Measurement quality objectives for bias associated with measurement of percent lipids are that each NIST standard reference material (SRM) result should be within its control limits (Sloan et al, 2019):

- Upper control limit = [1.3 × (certified concentration + uncertainty value for 95% confidence)]
- Lower control limit = [0.7 × (certified concentration – uncertainty value for 95% confidence)]

Precision

Precision represents the reproducibility of the individual measurements from the same sample. Precision is monitored and controlled within batches using laboratory replicates of field samples and across batches by analyzing SRM of applicable matrix i.e., tissue. For this study, a National Institute of Standards and Technology (NIST) mussel SRM 1974c will be used as the reference material for PAH analyses, and a NIST fish tissue SRM 1947 will be used for DDT and PCBs analyses [Note, SRM 1974b was previously used, but is no longer available from NIST]. Cross-batch precision is expressed as the relative standard deviation (RSD) for repeated measurements. The RSD of analyte responses relative to the internal standard must be ≤ 15% for the repetitions.

Bias (Accuracy)

Bias demonstrates the degree to which the measured value represents the true value. Bias or accuracy of samples is evaluated by comparing measured SRM values with NIST certified values. Concentrations of ≥70% of individual analytes are to be within 30% of either end of the 95% confidence interval of the reference values. Results of QA analysis will be reviewed by the Laboratory QA Officer.

Sensitivity

The limit of quantitation (LOQ) for all organic chemicals in this study is “the concentration that would be calculated if that analyte had a detector’s response area equal to its area in the lowest-level calibration standard used in the instrument calibration. When an analyte is not detected in a sample or it has a response area that is smaller than its area in the lowest-level calibration standard used, the concentration of the analyte in that sample is reported to be less than the value of its

LOQ" (Sloan et al., 2019). Typically LOQ values in 2 g fish whole-body composites range from 0.65 to 1.5 ng/g wet weight for PAHs and 0.15 to 0.50 ng/g wet weight for DDTs and PCBs.

Representativeness

Representativeness is the degree to which data represent a characteristic of an environmental condition. In the laboratory this is ensured by the proper handling and storage of samples and initiation of analysis within holding times. The procedures for this study include standardizing initial size of fish and sufficient replication to determine variability of exposure and accumulated concentrations within and between dietary treatment groups. These practices will allow practitioners to evaluate representativeness of this study to field conditions.

Comparability

Comparability is the similarity among different datasets for use in combining or comparing data. The methods used in this analysis follow similar protocols with previous studies, with comparable or lower limits of detection. One distinction in the protocol described in this study will be chemistry measures on whole bodies minus stomach contents, otoliths and livers, whereas previous studies may have retained the stomach contents, liver, and otoliths in the whole body analyses. This may bias study samples to underrepresent the contamination profiles of the fish sampled. The extent to which removing the stomach contents and livers may modify the contaminant concentration of the whole body fish composites is not known at this time.

7.4.2 Chemical analysis, liver tissue analytical quality assurance criteria

The minimum analytical quality assurance criteria for salmon liver analysis for hydroxylated polycyclic aromatic hydrocarbon metabolites (OHPAH) are summarized in Table 8.

Table 8. Minimum analytical quality assurance criteria for OHPAHs by liquid chromatography-triple-quadrupole mass spectrometry

Quality assurance element	Minimum frequency	Acceptance criteria
Instrument calibration	Each calibration standard is analyzed at the start of every batch of samples.	Analyte concentrations must be calculated using a Wagner calibration curve with at least five concentration levels of calibration standards. Concentrations of analytes in the calibration standards as measured using the calibration curve must be 70–130% of the actual concentration.
Continuing calibration verification	One at start and end of every analytical sequence and between every 15 or fewer for field samples.	The RSD of each analyte's responses relative to the internal standard must be ≤20% for the repetitions.
Reference material: NIST SRM 3672—smokers' urine	One with every batch of 20 or fewer for field samples analyzed for OHPAHs.	The concentrations ≥70% of individual OHPAHs must be within 15% of either end of the 95% confidence interval range of the reference values. These criteria do not apply to analytes with concentrations below their lower LOQ when the lower LOQ is within or greater than the 95% confidence interval.

Laboratory method blank	One with every batch of 20 or fewer for field samples.	No more than 10% of the analytes' concentrations can exceed 2 × lower LOQ in a method blank.
Laboratory sample replicates (i.e., duplicates or triplicates)	One with every 26 or fewer for field samples, as amount of sample available allows.	The RSDs of analyte concentrations must be ≤15% for triplicates, or percent differences must be ≤30% for duplicates, for ≥90% of the analytes that have concentrations >LOQ.
Surrogates (internal standards)	At least one internal standard/ surrogate is added to every sample.	The surrogate recoveries must be 60–130%.
Interlaboratory comparison	No intercomparison studies are available at present.	

Precision

Precision represents the reproducibility of the individual measurements from the same sample. Precision is monitored and controlled within batches using laboratory replicates of field samples and across batches by analyzing SRM. For this study NIST SRM 3672 (human smoker's urine) will be used as the reference material for OHPAH analyses. Cross-batch precision is expressed as the RSD for repeated measurements. The RSD of analyte responses relative to the internal standard must be ≤ 15% for the repetitions.

Bias (accuracy)

Bias demonstrates the degree to which the measured value represents the true value. Bias or accuracy of samples is evaluated by comparing measured NIST SRM 3672 values with NIST certified values. Concentrations of ≥70% of individual analytes are to be within 30% of either end of the 95% confidence interval of the reference values. Results of QA analysis will be reviewed by the Laboratory QA Officer (Table 1).

Sensitivity

The LOQ for this method is compound dependent and ranges from 0.15 – 4.5 ng/g of liver. Each LOQ was calculated based on LC-MS/MS limit of detection (LOD) for each analyte in ng/mL of methanol (lowest concentration of the calibration curve that produced a signal-to-noise ratio of approximately 3-5) and converted to concentration in liver using the regular volume of final extract and amount (wet weight) of liver extract. The conversion is performed according to the following equation: LOQ = LOD (ng/mL MeOH) x $V_{extract}$ / V_{sample}, where $V_{extract}$ is the final volume of extract in MeOH and V_{sample} is the wet weight of each liver sample extracted. This LOQ provides a representation of the lowest amount of a particular OHPAH that would be in a specific liver sample that the applied method would be able to detect in the final extract. When an analyte is not detected in a sample or it has a response area that is smaller than its area in the lowest level calibration standard used, the concentration of the analyte in that sample is reported to be less than the value of its lower LOQ.

Representativeness

Representativeness is the degree to which data represent a characteristic of an environmental condition. In the laboratory this is ensured by the proper handling and storage of samples and initiation

of analysis within holding times. The procedures for this study include standardizing initial size of fish and sufficient replication to determine variability of exposure and accumulated concentrations within and between dietary treatment groups. These practices will allow practitioners to evaluate representativeness of this study to field conditions.

Comparability

To the best of our knowledge, no previous data for OHPAHs in liver of juvenile Chinook salmon exposed to contaminants are available. However, the analytical method used in this analysis is a modification of the method reported by Ylitalo et al. to measure hydroxylated PAH metabolites in bile of visibly oiled and unoiled sea turtles collected after the Deepwater Horizon oil spill (Ylitalo et al. 2017), as well as in previous studies to determine OHPAHs in fish embryos exposed to PAHs (NWFSC unpubl. data). Similar sensitivity is expected for analysis of liver samples, however levels of chemical contaminants in liver samples are usually found to be lower than levels in bile (Varanasi et al. 1989, Krone et al. 1992).

7.4.3 Otolith analysis

Precision

Precision represents the reproducibility of the individual measurements from the same sample. Precision is monitored and controlled by having the same person read each otolith, and a minimum of 10% of the otoliths two times, with each reading occurring on a different day. If the distance from the otolith core to the edge of the otolith (i.e., otolith radius at the time of capture) and distance from otoliths core to seven daily increments in from the otolith edge (i.e., otolith radius measured at 7 days before capture) match then the associated data is assigned to that otolith. A match is considered a RSD ≤ 15% for the measurements from the same otolith. If the readings do not match then all otoliths will be read twice, and a third reading will be performed for any otolith where the repeated measurement RSD is > 15%. If none of the three readings match then the otolith will be excluded from the statistical analyses.

Bias (accuracy)

Bias demonstrates the degree to which the measured value represents the true value. Each otolith will be read without any knowledge of fish sample location. Bias of samples will be minimized through consistency in the measurement protocols, ensuring the increment being measured is in optimum focus, and ensuring the otolith is mounted so that the incremental plane is as close to horizontal as possible.

Completeness

Completeness is the ratio of usable data from the otolith analyses. It is fully expected that all otoliths will be processed and read, producing a reliable data point from each fish.

Representativeness

Representativeness is the degree to which data represent a characteristic of an environmental condition. In the laboratory this is ensured by the proper handling and storage of samples and initiation of analysis within holding times. The procedures for this study include standardizing initial size of fish and sufficient replication to determine variability of exposure and accumulated concentrations and growth measurements within and between dietary treatment groups. These practices will allow practitioners to evaluate representativeness of this study to field conditions.

Comparability

Comparability is the similarity among different datasets for use in combining or comparing data. However, the methods used in this analysis follow similar protocols with previous studies using different species.

8 Description of the interpretation techniques to be used

Phase I of the Chinook laboratory feeding study will be conducted as a one-way experimental layout with 4 replicate fish tanks per each of 7 experimental levels: control, reference and 5 feeding doses. This experimental design for Phase I essentially follows the experimental design for dose response studies described by Meador et al. (2005) and statistical analyses described therein will be applicable to some aspects of Phases I and II after preprocessing measurement endpoints so that each primary experimental unit represents one degree of freedom in statistical analyses. Phase II will be a disease challenge study where the fish from each of the reference and treatment tanks will be split and half of the fish will be exposed to the disease. Treatments will be applied at the fish-tank level, and measurement endpoints will be generated at the individual fish level as well as at an intermediate level (i.e., composites) for endpoints such as chemical analysis of body burdens. Statistical analyses will be conducted in a phased approach, starting with simple descriptive plots and summaries of measurement endpoints (i.e., survival and growth). Kaplan-Meier (Kaplan and Meier, 1958) survival estimates will be developed to represent survival rates for each treatment in Phase I and Phase II.

Because the experimental design also incorporates a nested component with repeated measurement of endpoints at the fish-tank, composite, and individual fish level, a mixed effects approach to the survival analysis is anticipated. In particular, estimating the association between contaminant exposure and chosen endpoints (i.e., survival and growth) will require incorporation of predictor variables measured at multiple levels, from the tank to the composite to the individual fish. Therefore, statistical models suitable for multiple levels of experimental units will be applied so the data can be analyzed with an underlying statistical model formulation supporting inference beyond the descriptive approaches described above. These analyses will rely on mixed effects model framework. For example, Austin (2017) provides an overview of the use of mixed effects models for survival analysis, including modifications of the Cox proportional hazards model to accommodate experimental designs with fixed and random effects. Further, Gelman and Hill (2007) and Little et al (1996) detail the application of mixed effects models to accommodate multiple levels of covariate measurements from the primary experimental unit (e.g., fish tank) to group level covariates (e.g., composites of fish) to individual subjects (e.g., fish).

It is anticipated that the effects of chemical contamination on the chosen endpoints are likely to act in concert through the complex mixture of contaminants in Portland Harbor. In such situations, it is generally untenable to isolate the independent effects of individual contaminants. Because chemicals with similar fate and transport properties tend to be correlated in sample data, we anticipate development of a principle components analysis to summarize groups of contaminants into composite variables (i.e., principle component scores; Harrell, 2001) which are by definition mutually independent and therefore appropriate for inclusion in mixed effects multiple regression models. This approach avoids arbitrary scaling and conversion to toxic equivalents and provides a means to associate measured endpoints directly with identified mixtures of co-occurring contaminant mixtures.

9 Data management

9.1 Documentation and records management

Records will be maintained documenting all activities and data related to sample collection as well as to laboratory analyses. Results of data verification and validation activities will also be documented. All of these documents and data will be stored in NOAA's DIVER (Data Integration Visualization Exploration and Reporting), a NOAA application for the integration and distribution of NRDA-related response, assessment, and restoration data. All publicly available documentation will be available through NOAA's DIVER tool (https://www.diver.orr.noaa.gov/). The public can access these data using the DIVER Explorer query tool that allows users to search, filter, and download data. A complete collection of records will be kept in the DIVER Portal, which is the log-in side of DIVER, and requires a username and password in order to access the information.

9.2 Data records available in DIVER

A key objective of DIVER is to accommodate the storing and organizing of data and information. This allows for the querying of sample data along with associated non-sample data (e.g., field measurements, continuous-read instruments, photos) to occur and helps case teams members answer a variety of case related questions. To pursue this objective, DIVER data managers identify the overlapping concepts generally implicit in each data set, defined as the core fields (listed in Appendix D, Table D1). The core field information makes the related data available for searching and download.

9.2.1 Field data and fish dissection documentation

For field sampling efforts and fish processing (dissections), all data will be stored electronically. Upon the return of the field sampling team to the field lab each day, data intake and processing will occur for the all cameras, GPS units, and field forms used during the field sampling and these will be uploaded into DIVER. Similarly, after each day of performing fish dissections, fish processing forms and related laboratory notes will be uploaded into DIVER. See Appendix D for details on data intake and processing.

Accurate transcription and review of field and fish processing information is critical for data usability. Data transcription will be reviewed by a second party on at least ten percent of forms to verify accurate transcription. Valid values ranges will be identified for key fields and values outside of those ranges will be flagged for field or processing team review. During the field sampling, any changes will be noted on the raw data sheets with a line through the original, initials of the editor, and the corrected value noted. Validation comments should be noted on the data sheet. Revised sheets will be re-scanned and added to the appropriate DIVER file collection.

Information on the field and fish processing forms will be transcribed into ORR Electronic Data Delivery (EDD) template formats. These template formats allow the data to be integrated and queried in DIVER. These templates also have functions that allow for QA/QC of the data and additional error checking. A list of the templates that could be used can be found in Table D2, Appendix D.

9.2.2 Laboratory data documentation

The data management team will assemble all of the information reported by the laboratories once the survival, physical measurements (fish weight and length), chemical, and otolith data have been appropriately validated. The laboratory data and documentation will be included in the project's file collection within DIVER for data archiving, data analyses, and use with GIS. References and/or links to the following types of data set documentation, if available, will include: all quality assurance documentation for the original data set; validation reports; laboratory analytical reports; and final project reports summarizing the data. The database structure (Table B3) and database rules and specifications (Table B4) are further described in Appendix B.

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Appendix A. Forms

Fish Data, Sample Processing and Chain of Custody forms are provided in this section.

- Print the form on water-resistant paper (if available). Make more than enough copies of the form for each day's work.
- Fill out forms with waterproof pen or permanent marker. Do not use pencil, or biro (erasable) ink.
- Fill in blanks with "N/A" if data are not applicable or not available. Avoid leaving blank values on data forms.
- Do not erase or black out erroneous entries on the forms. Errors should be corrected by crossing out the entry with a single line and signing and dating the strike-through.
- Original chain of custody forms should always stay with the samples. Make a copy of the chain of custody form before sending it with the samples.

Attached forms:

-Fish Data Form (to record sample IDs, date of death or sacrifice, fish weights, lengths, and important notes)

-Sample Processing Form (to record sample IDs for tissues, date of processing, and important notes)

-Chain of Custody Form (to inventory and transfer fish samples between locations or labs)

NRDA Chain of Custody Form

Page 1

Sampler Information		Lead Contact Information	
Contact/Phone/Email:		Contact/Phone/Email:	
Affiliation:		Affiliation:	
Incident Name:		Survey/Project Name	

Special Instructions	Turn Around Time:

Analyses requested							Lab Use Only	
A	B	C	D	E	F	# of containers	Lab Name:	
							Waybill Number:	
							Lab Report #:	
							# of Coolers:	
							Cooler Temp:	

Sample ID	Sample Date	Sample Time	Matrix	ID	Comments
	mm/dd/yyyy	(24-hr local)		#	
				1	
				2	
				3	
				4	
				5	
				6	
				7	
				8	
				9	
				10	
				11	
				12	
				13	
				14	
				15	
				16	
				17	
				18	
				19	
				20	
				21	
				22	
				23	
				24	
				25	
				26	

Enter Analyses above, with preservative specified, if needed.
Enter x's in boxes below.

Relinquished by				Received by			
Date	Time	Signature	Printed Name/Org.	Date	Time	Signature	Printed Name

Appendix B. Data management

This document contains supplemental material for the data management considerations for the lab study.

- Data intake and processing protocol outline used upon completion of
- Table B1. DIVER environmental data specifications – core fields
- Table B2. A list of the templates that may be used to transcribe the data from the Fish Data and Sample Processing Forms
- Table B3. Database table types and descriptions
- Table B4. Laboratory data and documentation: database rules and specifications

Data intake and processing

Upon the completion of the lab study, data intake and processing will occur for the all lab notebooks and forms used during the study. This involves scanning all relevant lab notebooks and uploading to DIVER File Collections. Chemical analysis and other results will also be transferred into DIVER using information found in the following sections.

Table B1. DIVER environmental data specifications – core fields

The core fields identify overlapping concepts generally implicit in each data set. The core field information makes the related data within DIVER available for searching and download. If a specific core data field is not applicable to a particular data set, it is assigned a default value (typically "Not Defined") so that comprehensive data searches return full results.

Field Name	Field Definition	Field Set Within DIVER Explorer	Field Value Source
Case/Activity	The name of the case incident or the activity used to collect data.	Case/Activity Overview	User-Generated
Collection Workplan	The workplan under which the field data were collected.	Case/Activity Overview	User-Generated
Region	Region	Case/Activity Overview	User-Generated
Workgroup	The Technical Working Group under which the field data were collected.	Case/Activity Overview	User-Generated
Workplan Topic Area	The main resources of focus of a Collection Workplan.	Case/Activity Overview	User-Generated
Workspace Name	Name of the Portal Workspace where data were entered.	Case/Activity Overview	User-Generated
Collection Form	The type of the data submission form used by the field team to submit raw field data.	Collection Summary	User-Generated
Collection Study Name	The name of the study under which the field data were collected.	Collection Summary	User-Generated
Data Category	General category of data collection (e.g., Instruments, Photographs, Samples, or Visual Observations).	Collection Summary	User-Generated
Data Classification	The purpose for which data was collected within the case incident or activity.	Collection Summary	User-Generated
Data Source	The originating owner of the dataset.	Collection Summary	User-Generated
Source Type	General owner/source of the data (e.g., NRDA, Response, Responsible Party).	Collection Summary	User-Generated
Collection Matrix	The type of sample or record collected (e.g., Sediment, Water, Photograph, Wipe).	Field Data	User-Generated

Field Name	Field Definition	Field Set Within DIVER Explorer	Field Value Source
SampleID	Unique ID assigned to each sample by the field sampler.	Field Data	User-Generated
Station/Site	Station or site identifier. This is often defined by the workplan and/or recorded by the field team, but may be standardized to database requirements.	Field Data	User-Generated
Date	Data collection date, as year, month, and day.	Location/Date/Time	User-Generated
End Latitude	End Latitude	Location/Date/Time	User-Generated
End Longitude	End Longitude	Location/Date/Time	User-Generated
Start Latitude	Start Latitude	Location/Date/Time	User-Generated
Start Longitude	Start Longitude	Location/Date/Time	User-Generated
State	The state where the field event took place.	Location/Date/Time	User-Generated
Analysis Category	General category of analysis performed (e.g., Plankton_Nekton, Visual Observation, Contaminant Chemistry). For additional detail, see Analysis Type and/or Analysis.	Results: All Data Types	User-Generated
Analysis Status	Status of samples in the analysis process as reported by laboratories or through results (e.g., Archived, Results Available, In Analysis Queue etc.).	Results: All Data Types	User-Generated
Analysis Type	Subcategory (i.e., type) of analysis performed, such as Biomass, Hematology, Genetics, etc. For additional detail, see Analysis.	Results: All Data Types	User-Generated
Review Status	Extent of data quality review performed.	Results: All Data Types	User-Generated
Sharing Status	Identifies extent of data distribution (e.g., Publicly Available).	Results: All Data Types	User-Generated
Region ID	Region ID	Case/Activity Overview	DIVER-Created
Station Group List	Predefined sets of grouped stations/locations	Case/Activity Overview	DIVER-Created
DIVER Dataset	DIVER's internal database table name	Collection Summary	DIVER-Created
File Collection ID	Record identifier for the corresponding DIVER file collection.	Collection Summary	DIVER-Created

Field Name	Field Definition	Field Set Within DIVER Explorer	Field Value Source
Record ID	Identifier for each observation data sheet entered into the DIVER database.	Collection Summary	DIVER-Created
Trip ID	Identifier for tracking field collection events and the way data files were provided to the Data Management Team (one Trip ID per file collection or zip file).	Collection Summary	DIVER-Created
Image Id	Record identifier for a particular photograph.	Results: All Data Types	DIVER-Created
Link to Related Files	Link to source files for related data	Results: All Data Types	DIVER-Created
Photo URL - Midsize	Mid-sized image	Results: Photographs	DIVER-Created
Photo URL - Original	Original image	Results: Photographs	DIVER-Created
Photo URL - Thumbnail	Thumbnail sized image	Results: Photographs	DIVER-Created
QM Site ID	Identifier for a site in the Query Manager database.	Results: Samples	DIVER-Created

Table B2. A list of the templates that may be used to enter lab data.

Information from this study will be transcribed into ORR Electronic Data Delivery templates. These templates are set up to allow the data to be arranged in a format that allows a streamlined workflow to be integrated in DIVER. These templates also have functions that allow for QA/QC of the data and additional error checking.

Descriptive name	Name for Reference	Example data	File name	Description
TEMPLATES				
Chemistry/Toxicity Results	ChemTox	Tissue chemistry	NOAA_Template_ChemTox_Excel_V3.0_20180301.xlsx	Laboratory or field results for contaminant chemistry. Toxicity data from studies conducted in a laboratory.
Biological and other non-chem laboratory analysis (sample-based)	BioLab	Fish measurements and samples	NOAA_Template_BioLab_V1.2_20180301.xlsx	Measurements related to biological activity (either individual organism or community metrics), using field-collected or lab-derived samples and measured in a laboratory.
ANCILLARY FILES				
Study Notes tool	NA	Study meta-data	NOAA_StudyNotes_V2.8_20170320.accdb	A stand-alone Study Note application has been developed to assist the Template user in developing study meta-data. This application can be opened in another instance of MS Access while working on the template population.
Template Tester	Tester	NA	NOAA_Tester_V3.0_20180301.accdb	The Template Tester is a Microsoft Access VBA (Visual Basic for Applications) application that has been designed with the objective of identifying errors and omissions in completed Template files.
Template Guidance	NA	NA	NOAA_Templates_Guidance_20180301.xlsx	Guidance and instructions on the different templates, and their interoperability.

Table B3. Database table types and descriptions (bold text indicates main data tables; other tables are supplementary tables)

The database structure will have a ten-tier hierarchy, i.e., ten major table types that are split into a relational structure. The ten types include the study table, station table, sample tables, chemistry tables, and bioassay tables. Data captured will adhere to rules and specifications listed in Table C4, "Laboratory Data and Documentation: Database Rules and Specifications."

Table Type	Description
study	The study table provides basic information regarding the study (e.g. name, contact, etc.) and identify the multiple sample collection events. Each study is assigned a unique, two-character StudyID, which is used to link to tables in the other tiers of the database hierarchy. **studynot** Contains information regarding the document(s) associated with the study and data. **studyref** Contains study-specific meta-data for specific topics.
station	The station table identifies locations for samples that were submitted for chemical and/or toxicological analyses. Each record of the table has a unique combination of SiteID + StudyID + StationID. Stations are defined for each study by a unique set of geographic coordinates reported as latitude and longitude. **stnlist** Contains a list of stations in each Station Group inlcuding historical Query Manager Watersheds **stnxtra** Contains additional attribute data for stations.
smpmaster	The sample tables provide information about the samples collected for chemical and/or toxicological analyses, including collection date, depth (if relevant for the matrix type), and sample type (e.g., field sample, field duplicate, composite sample). The master sample table stores all matrix types. Each record within the sample tables is unique based on SiteID + StudyID + StationID + SmpCode. **smpxtcoord** Contains additional coordinates associated with a sample, for example composited sub-sample locations. **smpxtra** Contains additional attribute data for samples. **tissrep** Sample information for part samples that make up composited tissue samples. **sedrep** Sample information for part samples that make up composited sediment samples.

chemmaster	The chemistry tables store the results for chemical analyses, for all matrix types. Supplementary chemistry tables store additional information related to analytical chemistry results. Each record is unique, based on SiteID + StudyID + StationID + SampleID + Labrep + Chemcode. Chemcodes are ten-character codes assigned to analytes. Using chemcodes eliminates the potential confusion associated with the multiple ways in which an analyte name might be written (e.g., dibenzo(a,h)anthracene versus dibenzo[a,h]anthracene) or with chemical synonyms used by different laboratories (e.g., 2-methylphenol versus o-cresol). Different Labrep codes are used for results where a duplicate chemical record might otherwise occur in the chemistry table. For example, if a sample was analyzed by the same analytical method and two different laboratories, the results may be distinguished by Labrep. **chemqc** Stores quality control samples, such as field blanks, that are not included in the chemmaster table. **chemns** Stores Tentatively Identified chemicals (TICS) and originally reported sums that are not included in the chemmaster table.
biosumm	Mean of sediment bioassay results, with one record per sample tested. **biorep** Contains replicate data from the sediment bioassay results.

Table B4. Laboratory data and documentation: database rules and specifications

Laboratory data will adhere to the following rules and specifications:

- For consistency and compatibility with legacy systems (based on an Xbase format), the tables are created with a structure requiring that the key fields used to link related tables have matching field sizes and the content of these fields must match between tables, in terms of upper and lower case lettering.
- If two or more organisms of the same species are collected for the same study at the same location (lat/long coordinates) and share the same matrix they are assigned different field SampleIDs. The samples will be assigned a unique composite ID number if combined as a composite. Thus, two field SampleIDs may be merged into a single unique Composite ID so that all chemical analyses are associated with a single sample record in the sample table. If not, these samples will maintain unique Sample IDs.
- As noted, a lab may split one fish into different components. In the laboratory EDD, the different components are distinguished by a suffix added to the original client sample ID. Within the NOAA Chemistry/Toxicity database, the resulting samples of different matrices will be assigned different sample IDs.

A suffix will be added to a SampleID to relate a sample that has been split into different fractions or components. The component parts are assigned the SampleID with letters qualifying the type of tissue (WH: whole body minus stomach contents, otoliths, and liver; OTO: otoliths; LI: liver) such that SampleID 200001 would be split into 200001WH (whole body minus stomach contents, otoliths, and liver), 200001LI (liver), and so on.