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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Acronyms and Abbreviations

ANOVA	Analysis of Variance
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFR	Code of Federal Regulations
CHP	Chemical Hygiene Plan
COC	Chain of Custody
CSO	Combined sewer overflow
DIVER	Data Integration Visualization Exploration and Reporting
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DMT	Data Management Team
EPA	U. S. Environmental Protection Agency
ESA	Endangered Species Act
ESU	Evolutionarily Significant Unit
GC/MS	Gas chromatography/ mass spectrometry
HASP	Health and safety plan
HSO	Health and Safety Officer
LC	Lethal Concentration
LDR	Lower Duwamish River
LOQ	Limit of Quantification
NIOSH	National Institute of Occupational Safety and Health
NIST	National Institute of Standards and Technology
NOAA	National Oceanic and Atmospheric Administration
NRDA	Natural Resource Damage Assessment
OSHA	U. S. Occupational Safety and Health Administration
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PH	Portland Harbor
PPE	Personal protective equipment
RSD	Relative Standard Deviation
QA	Quality assurance
QC	Quality control
QAPP	Quality assurance project plan
SRM	Standard Reference Material
TBT	Tributyl tin

1 Abstract

This study is intended to determine whether dietary exposure to contaminants of concern 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, the association of growth and contaminant concentration was also evaluated. 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).

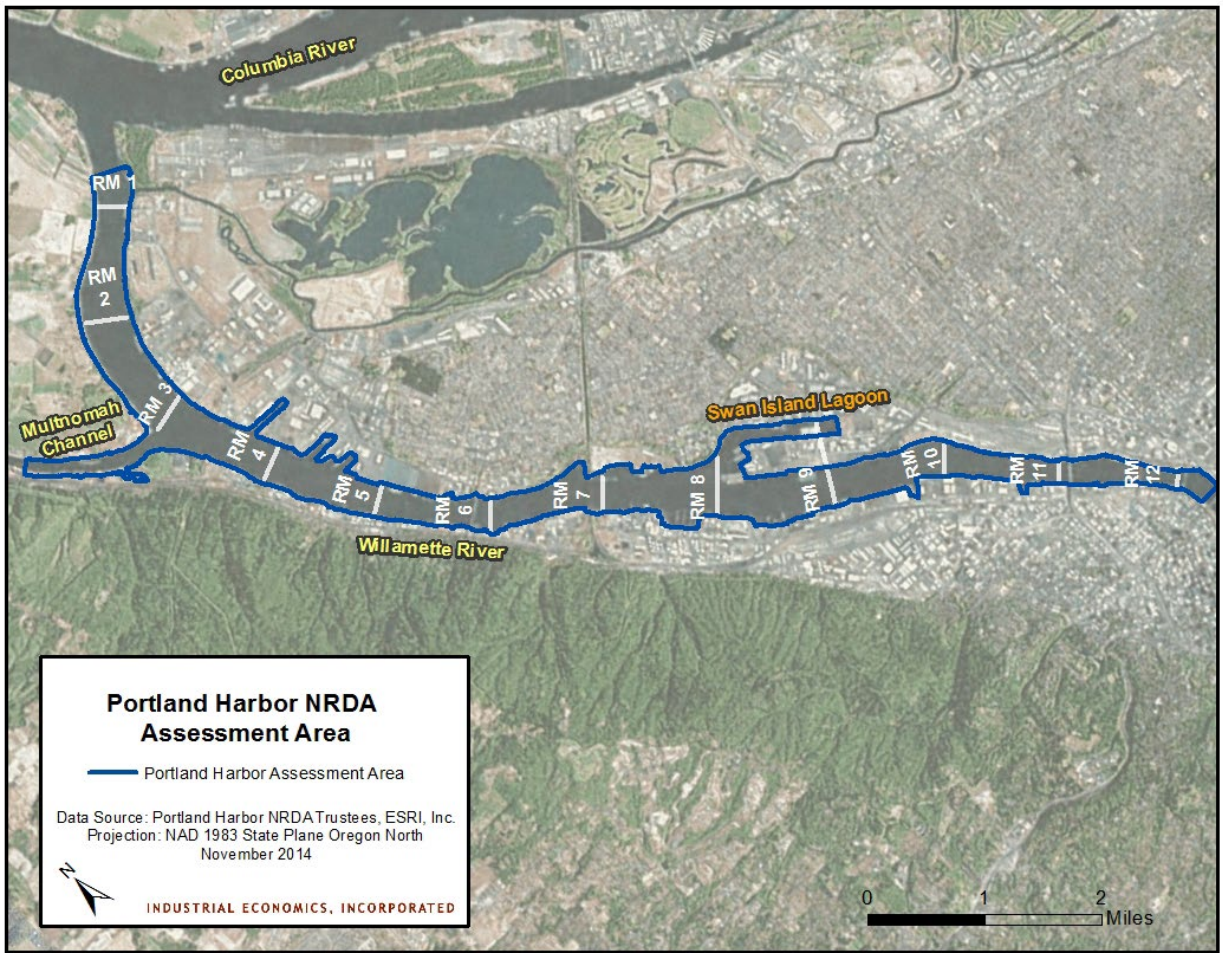
2 Background: Dietary exposure study rationale

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 that time, numerous industries have released potentially toxic chemicals into the river. Common sources of pollution have included permitted and non-permitted end-of-pipe discharges, accidental spills and releases, 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 Portland Harbor Superfund site extends from river mile 2 to 11, inclusive of upland areas (Figure 1).

Contaminant exposures to juvenile Chinook salmon in the Lower Duwamish River were also considered when designing this study because of the similar industrial histories, contaminants of concern, and indicator species.

Figure 1. Map of Portland Harbor NRDA Assessment Area



2.2 Contaminants of concern

Focal contaminants for this study include polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethanes (DDTs) and related metabolites, and polycyclic aromatic hydrocarbons (PAHs). Many other contaminants of concern, such as tributyltin (TBT), are known to be present in the Natural Resource Damage Assessment (NRDA) area (as described in IEC 2019) but are not considered in the present study design.

2.3 Results from previous studies

Previous literature has summarized the state of knowledge on the effects of PAHs, PCBs, DDTs, and other pollutants on fish with endpoints including growth, immunotoxicity, and reproduction (Johnson et al. 2014; Collier et al. 2014). Adverse health effects were observed in field assessments as well as in controlled laboratory studies after contaminants exposures ranging from 24 hours to several weeks (Collier et al. 2014).

In 2018, juvenile (sub-yearling) out-migrating Chinook salmon were collected from the lower mainstem of the Willamette River, Oregon as part of an NRDA study. Their Willamette River origin was subsequently confirmed by genetic analysis. Otoliths from these fish were extracted for determination of growth rates, and tissues were analyzed for contaminant concentrations. All collections and analyses were done as described in the QAPP (NOAA NMFS 2018). Growth and contaminant concentrations from 38 whole body tissue composite samples (less stomach contents and liver tissue), composited by site, were evaluated for an association between modified growth and tissue contaminant concentrations (DIVER 2020) as the juvenile Chinook salmon moved from upstream reference locations through downstream contaminated sites. Whole body (less livers, otoliths, fin clips, and stomach contents) contaminant concentrations were the following: total PAH concentrations ranged from 5 to 36 ppb wet weight (ww); total DDT concentrations ranged from 8 to 497 ppb ww; and total PCB concentrations ranged from 12 to 391 ppb ww. Lipids in whole bodies (less livers and stomach contents) ranged from 0.8-3.0%. Six site-based composite samples of stomach contents were also analyzed for contaminant concentrations (DIVER, 2020). Concentration of contaminants in the stomach contents were the following: total PAH concentrations ranged from 74 to 834 ppb ww; total DDT concentrations ranged from 13 to 142 ppb ww; and total PCB concentrations ranged from 26 to 58 ppb ww.

A similar field study was conducted in the lower Duwamish River, Washington, in 2018. Twelve composite samples of stomach contents of out-migrating, hatchery-origin Lower Duwamish River Chinook salmon were chemically analyzed in 2018 (Washington Department of Fish and Wildlife unpublished data). Total PAH concentrations in stomachs ranged from 43 to 8000 ppb 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 2 to 23 ppb ww. Total PCB concentrations ranged from 15 to 78 ppb ww. Lipids in whole bodies (less livers and stomach contents) ranged from 1.7-3.1%. Although stomach contents from these fish collected in 2018 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 100 and 260 ppb ww of total PCBs (Varanasi 1993; Ylitalo unpublished data, *pers comm*).

3 Project description

3.1 Purpose and overview

This Final Quality Assurance Project Plan and Workplan (QAPP-WP) supplements the earlier Interim Final QAPP-WP dated December 20, 2019. It refines study details regarding the feeding and disease challenge phases by incorporating results of additional review of exposure concentrations, selection of disease organisms, and analysis of tissues.

This study is focused on the health and survival of juvenile Chinook salmon. The goal is to determine whether dietary exposures to environmentally-relevant mixtures of PAHs, PCBs, and DDTs cause growth impairment (primary objective) or immunotoxicity resulting in increased mortality in response to a challenge with pathogenic bacteria (secondary objective). Although the experimental design for this laboratory study is premised primarily on contaminant exposure information obtained from the Willamette River field assessment in 2018, corresponding exposure data from the lower Duwamish River were also considered in selecting chemical mixture compositions and relative concentrations.

To evaluate growth, Chinook salmon will be exposed to five doses of a defined mixture of PAHs, PCBs, and DDTs. The range and relative proportions of contaminants are based on concentrations previously measured in the stomach contents of juvenile fish collected from the Portland Harbor area and the Lower Duwamish River. Two control treatments will also be tested. One "solvent-treated" control group will be fed food treated with the solvent used to dissolve chemical standards (but no other chemicals added). One untreated control group (no chemicals or solvent added to food) will also be tested. Growth metrics will include weight and length at the time of sacrifice (after five weeks of exposure to contaminated diet). In addition, otolith microstructural analysis will be used to determine average growth rates over three time periods at the end of the feeding phase (the 7-, 14-, and 21-day intervals prior to sacrifice). Whole body composite samples (less stomach contents, livers, and otoliths) will be analyzed for contaminant concentrations to relate tissue concentrations in this controlled feeding study to those previously measured from juvenile Chinook salmon captured in Portland Harbor and the lower Duwamish River.

For the bacterial disease challenge, fish surviving contaminant exposure will be exposed to either *Aeromonas salmonicida* or *Vibrio anguillarum,* using conventional methods (Arkoosh et al. 2005). Both bacteria have a large host range. *A. salmonicida* and *V. anguillarum* are etiological agents of furunculosis and Vibriosis, respectively. Both diseases are systematic diseases known to cause high mortality in salmonids (Feckaninova et al. 2017; Frans et al. 2011). The bacteria which causes mortality with the least amount of induced temperature, density, and handling stress will be used for both the generation of the lethal concentration response curve and at the end of the five-week dietary exposure phase. The determination of the appropriate bacteria and exposure time will be made through testing conducted before the disease challenge phase begins. Six exposure groups, including the five contaminant mixture doses and the solvent treated controls (but not the untreated controls), will be evaluated. The juvenile Chinook salmon will be divided into two disease treatments of either bacteria or sterile nutrient media alone. Fish will be subsequently held and monitored for daily survival (Arkoosh et al. 2005).

3.2 Focal salmonid species

This study is primarily intended to generate targeted health and survival information for the Upper Willamette River Spring Chinook salmon ESU juveniles that outmigrate through the Portland Harbor Superfund site as sub-yearlings. Given practical limitations on working directly with wild Endangered Species Act (ESA)-listed fish, a hatchery stock was chosen based on the following criteria (in order of

importance): (1) an established Chinook salmon brood stock; (2) availability of fertilized embryos by early January 2020; (3) an inland (vs. coastal) stock (4) ocean-type (immediate seaward-migrating) life history, to match the Upper Willamette River Chinook salmon Evolutionarily Significant Unit (ESU). In accordance with the above, Chinook salmon from the Little White Salmon National Fish Hatchery (operated by the US Fish and Wildlife Service will be used for this study.

3.3 Practical constraints

In addition to the aforementioned limits on working with wild, ESA-listed Chinook salmon, the primary constraints for this study were the availability of wet laboratory space and supporting facilities for holding, exposing, growing, and subsequently challenging fish with an infectious agent. Other constraints included the small sizes of the fish (field collected fish weighed, on average, less than 2 g each) and the corresponding sample mass available for whole body less stomachs, livers, and otoliths (4 g minimum for PAH, PCB, and DDT analyses) composites. Finally, budgetary limitations for chemical analyses precluded the inclusion of additional contaminant classes such as TBT in the study.

4 Project tasks and schedule

This timeline for this study will extend from approximately January, 2020 through October, 2020.

Table 1. Proposed schedule for completing laboratory work and analysis

Task	Dates	Lead staff (all NOAA)
Prepare diet formulations	February 2020	Gina Ylitalo Irvin Schutz
Acclimate fish	January 2020-February 2020	Joe Dietrich
Feeding Trial-growth	March 2020-April 2020	Jessica Lundin Joe Dietrich
Disease challenge	April 2020-May 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	Nat Scholz Jessica Lundin Mary Arkoosh Others to be determined
Report final	February 2021	Same as above

5 Overall study design

5.1 Methods

15,000 Chinook salmon will be obtained from the Little White Salmon National Fish Hatchery as button-up fry (see section 3.2 for source considerations and section 5.6 for requirements for number of fish). Fry will be transferred to the Northwest Fisheries Science Center's Fish Disease Laboratory (FDL) at the Newport Research Station in Newport, Oregon, and then grown in circular fiberglass tanks. During this

initial acclimation to hatchery conditions, fish will be fed untreated (i.e., unaltered by chemical dosing) larval feed from a commercial source (Otohime), beginning with pellet sizes B1 and B2. Fish will be held in indoor tanks individually supplied with flow-through dechlorinated municipal water. Conventional water quality parameters (e.g., pH, temperature, chlorine, dissolved oxygen) will be routinely monitored throughout the study. The building in which the wet lab is located will be secured in the evenings and on weekends, with access limited to those with keycards. During the week, the wet lab room will be unlocked and lab security will be monitored by staff involved with the study.

As described above, the first phase of the study design includes seven distinct treatment groups: an untreated control, a solvent-treated control, and a range of five distinct contaminated diets. The switch to PAH, PCB, and DDT-amended pellets will begin when fish are large enough (~ 1.5 g ww) to consume the contaminant-treated pellets (Otohime C1 size). Prior to the onset of the contaminant feeding phase of the study, the juvenile Chinook salmon will be sorted by size for consistency of length and weight across treatment groups. To determine a representative pre-exposure size range, 100 individuals will be randomly selected from the rearing tanks. Means and standard deviations for length and weight for this sample will be used to estimate the size class of fish within and across each treatment group. This group of fish will then be sacrificed. A floating fish grader will be used to restrict fish to a minimum and maximum size determined from the 100 fish sampled. All exposure groups will have the same total number of fish at the beginning of the study, with 4 replicate tanks per group and 180 fish per tank.

During the contaminant feeding phase of the study, groups of fish pre-sorted into consistent size ranges will be transferred to circular fiberglass exposure tanks (28 total; 400 L, 1.0 m in diameter). The tank assignments will be distributed randomly. In addition, a single tank of 180 unexposed fish will be maintained to monitor growth (henceforth referred to as the "growth tank"). Fish from this growth tank will be subsampled each week to determine average weights and adjust daily ration across the 28 experimental treatment tanks as needed. During the acclimation interval (i.e., the first three days after transfer, prior to delivery of contaminated feed), a second tank of fish will be maintained as a reserve source of Chinook salmon to replace any mortalities following transfer to the tanks. After three days of acclimation (unaltered diet, mortalities replaced as noted above) exposure to the seven diets will begin. Thereafter, mortalities will be removed daily but not replaced, and the ration of feed to each tank will be adjusted to maintain consistent rations per fish and reflect reductions in the total number of fish and estimated growth in surviving fish.

Juvenile Chinook salmon will be fed approximately 1.9% body weight (bw) day^{-1}, with individual body weights estimated from the average wet weight at the time of fish transfer to each of the 28 exposure tanks. Thereafter, the feeding regimen will be maintained at 1.9% bw day^{-1} by adjusting to the growth trajectory of the 50 fish weighed weekly from the parallel growth tank.

Fish will be fed at least twice daily over a minimum of six days per week throughout the exposure period. At the end of the five-week exposure interval, 50 fish per tank will be collected over the course of a single day, in a sequence determined by a random number generator. The fish will not be fed on the day of collection to allow gut clearance. Fish will be removed from their treatment tank with a small dip net and euthanized with MS222 (500 mg/L). Fish will be measured for weight to the nearest 0.01 g, fork length to the nearest mm, and then be immediately frozen on dry ice for subsequent shipping (frozen) to the NOAA Northwest Fisheries Science Center (Seattle, WA) for storage at -80 °C. Thereafter, 30 individual fish per tank will be dissected on dry ice to remove stomach contents, livers, and otoliths (see Section 6). Otoliths will be processed for microstructural analyses to determine growth rates. The stomach contents will be removed and discarded. The livers will be removed and archived for potential chemistry analyses. The remaining whole bodies (minus stomach contents, liver and otoliths) will be

combined into one 30-fish composite for chemical contaminant analyses. Of the 50 Chinook salmon sampled from each tank, the remaining 20 will be stored in a -80 °C freezer for later analysis if necessary. Fish from the solvent-treated control tank replicates will be used for statistical analyses (see Section 8); fish from the untreated control tank replicates will be archived in a -80 °C freezer. Growth, as determined from measures of length and weight, will be compared between the two control groups (solvent and untreated diets only) using an ANOVA. If length and weight are not significantly different, the solvent treated control will be used for statistical comparisons across treatments. If lengths or weights are significantly different between the two control treatments, otolith and tissue samples will be analyzed from both groups and used for statistical comparisons across treatments.

At the end of the five-week dietary exposure phase, the disease challenge (second phase) will begin. In advance, during the prior exposure interval, a pilot experiment using a subset of control fish fed unaltered diet, will be implemented to generate a lethal concentration-response curve to determine a target exposure concentration for the bacterial pathogen (see section 5.4). At the beginning of the disease challenge phase of the study, fish from each dietary exposure replicate tank will be randomly divided into either a 400 L pathogen tank or a parallel 400 L non-pathogen tank. This design will yield 48 tanks – i.e., six diet treatments times four replicates each with two disease treatments (pathogen or no-pathogen). The unaltered control feeding treatment will be discontinued due to limitations in tank space. Fifty fish will be placed in each of the disease challenge tanks unless there are insufficient surviving fish for a particular dietary contaminant treatment. Excess fish not needed to populate phase 2 tanks will be archived. All fish will be provided unaltered Otohime feed pellets (not amended with solvent or contaminants) at a rate of 1% bw day^{-1} for the duration of the disease challenge study phase.

After transfer to the 48 tanks, juvenile Chinook salmon will be temporarily removed and placed into a separate exposure vessel containing aerated fresh water and aliquots of either bacteria or sterile nutrient media will be added. Thereafter, fish will be returned to their 400 L tank, and observed daily for mortalities until three consecutive days are observed without additional die-offs. Dead fish will be removed daily throughout the observation period and weight and length will be recorded. Using sterile technique, kidney tissue from all dead fish will be streaked on to growth agar and allowed to incubate for colony bacterial growth. Colony growth will be checked for the target pathogen using a presumptive identifier (Noga 1996) or polymerase chain reaction (Arkoosh and Dietrich 2015). At the end of the observation period, all surviving fish will be measured for length and weight. A target of thirty (30) fish from each non-pathogen treatment tank, depending on survivorship, will be archived for possible later analysis. All fish exposed to pathogens will be autoclaved due to biohazard concerns.

All tissue samples will be maintained and stored accordingly to standard procedures. Chain of custody will be initiated when fish are collected and samples are prepared for transport between laboratory facilities (Section 5.7). Analytical methods and associated 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.

5.2 Tasks, analyses, and anticipated timelines

		Samples	Analysis	# Fish/ # samples for analysis	Notes	Jan	Feb	Mar	Apr	May	Jun to Aug
Fish: initial size and subsequent growth											
1	Obtain approximately 15,000 fertilized Spring Chinook salmon button-up fry from Little White Salmon National Fish Hatchery.					x					
2	Acclimate fish to laboratory conditions in tanks.					x	x				
3	Begin feeding fry with unmodified (clean) commercial diet to acclimate fish to pelletized food.					x	x				
4	Weight monitoring to adjust food quantity. Weekly measurements of fish will be used to calculate the amount of food to add to each tank as fish grow. Fish do not need to be archived at the end of this rearing period.			Min 10 per wk/ 0	Growth tank; fish not archived	x	x	x			
Contaminated diet formulation											
5	Mix target contaminants (standards) into commercial pelletized food to create 5 target doses, 1 clean control, and 1 solvent control. Confirm concentrations through chemical analysis; also analyze control feeding treatments (1 clean control, 1 solvent control). Run samples in duplicate.	Diet	PCBs, DDTs, PAHs, % lipids	diet/ 14	5 doses, 1 control, 1 solvent control (in duplicate)		x				
Phase 1, controlled dietary exposures											
6	Select fish within a target initial size range. Distribute fish randomly to 7 exposure groups (5 contaminated diet exposure doses, 1 clean control, 1 solvent control), each exposure group will have 4 replicate tanks, with 180 fish per tank. A total of 28 tanks and 5,040 fish will be used for this phase of the study.			5,040/ NA				x	x		
7	Archive un-dissected whole body samples at the NOAA NWFSC lab for possible compositing and chemical analyses prior to feeding contaminated diet.	Un-dissected whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class, otolith microstructural analysis (optional)	30/ archive				x	x		
8	Feed contaminated diet for 5 weeks. All mortalities will be removed from tanks, lengths and weights recorded.	Un-dissected whole bodies (mortalities)	Weight and length	Unknown/ NA	Mortalities			x	x		
9	Weight and length measurement on all fish saved for analysis or archive after the 5 weeks of exposure to the contaminated diet.	Un-dissected whole bodies	Weight and length	1400/ NA	7 exposure groups x 4 replicate tanks x 50 total fish per replicate				x		
10	Immediately freeze fish on dry ice for shipment to NOAA NWFSC Seattle, WA								x		

	Samples	Analysis	# Fish/ # samples for analysis	Notes	Jan	Feb	Mar	Apr	May	Jun to Aug	
11	Extract otoliths, stomach contents, livers from 30 fish from each tank (50 fish from each tank collected).								x		
12	Submit remaining whole body samples to the NOAA NWFSC lab for compositing and chemical analyses after 5 weeks of exposure to the contaminated diet.	Whole bodies (less stomach contents, livers, otoliths)	PCBs, DDTs, PAHs, % lipids, lipid class	720/ 24	6 exposure groups x 4 replicate tanks x 1 composites per replicate (30 fish per composite)				x		
13	Submit remaining liver samples to the NOAA NWFSC lab for possible compositing and chemical analyses after 5 weeks of exposure to the contaminated diet. Number of composites will be determined by total mass of liver available (target 2g/sample)	Liver tissue	PCBs, DDTs, PAHs, % lipids, lipid class (optional)	720/ 6-12	6 exposure groups x 4 replicate tanks x 1-2 composites per treatment (60-120 fish per composite)				x		
14	Submit otoliths to the NOAA NWFSC lab for microstructural analysis after 5 weeks of exposure to the contaminated diet.	Otoliths	Microstructural analysis	720/ 720	6 exposure groups x 4 replicate tanks x 30 fish per replicate (same fish used to create chemistry composites)				x		
15	Archive un-dissected whole body samples at the NOAA NWFSC lab for possible compositing and chemical analyses and otolith microstructural analysis after 5 weeks of exposure.	Un-dissected whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class, otolith microstructural analysis (optional)	480/ archive	6 exposure groups x 4 replicate tanks x 20 additional fish				x		
16	Archive un-dissected whole body samples at the NOAA NWFSC lab for possible compositing and chemical analyses and otolith microstructural analysis after 5 weeks of exposure.	Un-dissected whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class, otolith microstructural analysis (optional)	200/ archive	Clean control group x 4 replicate tanks x 50 fish per tank				x		
16B	Archive un-dissected whole body samples at the NOAA NWFSC lab after 5 weeks of exposure.	Un-dissected whole bodies	Archive	840/ archive	7 exposure groups x 4 replicate tanks x 30 fish per tank				x		

		Samples	Analysis	# Fish/ # samples for analysis	Notes	Jan	Feb	Mar	Apr	May	Jun to Aug
Phase 2, disease challenge											
17	Pilot study to calculate the lethal concentration (LC) response to the pathogen using subset of control fish.		Pilot study to characterize lethal concentration curve (survival)	900/ 0	6 pathogen concentrations (5 pathogen and 1 control (in triplicate tanks), 50 fish per tank. These fish will not be archived.		x	x			
18	Redistribute remaining fish from phase 1 to 400 L tanks for phase 2, disease challenge. This will include 6 feeding study treatment groups (clean control feeding group discontinued). Each of the 4 replicates will be divided into two new tanks, 1 treated with the target concentration of pathogen and 1 treated with the bacterial media only (non-pathogen) for a total of 48 tanks. Each new tank will start phase 2 with a target of 50 fish.								x	x	
19	Expose fish in respective tanks to desired LC of pathogen or non-pathogen.								x	x	
20	Track survival.								x	x	
21	All mortalities will be removed from tanks, lengths and weights recorded.	Un-dissected whole bodies (mortalities)	Weight and length	Unknown/ NA	Mortalities				x	x	
22	All mortalities will be removed from tanks, kidneys struck on to agar plates, plates examined for growth and pathogen identified.	Kidneys	Examine for presence of pathogen	Unknown/ NA	Mortalities				x	x	
23	End disease challenge trial, sacrifice surviving fish and record length and weight measurements. Any pathogen exposed fish will be autoclaved due to biohazard concerns after weight and length measurements are recorded.	Un-dissected whole bodies	Weight and length	Up to 2400/ NA	Any pathogen exposed fish not archived for possible dissection will be autoclaved due to biohazard concerns.				x	x	
24	Archive 30 un-dissected whole body samples per exposure group replicate tank of **non-pathogen** exposed fish surviving after the phase 2 disease challenge at the NOAA NWFSC lab for possible compositing and chemical analyses and microstructural analysis of otoliths. Analysis could indicate whether depuration occurred after removal of chemical exposure.	Un-dissected whole bodies	PCBs, DDTs, PAHs, % lipids, lipid class, otolith microstructural analysis (optional)	720/ archive	6 exposure groups x 4 **non-pathogen** replicate tanks x 30 fish per tank.					x	

		Samples	Analysis	# Fish/ # samples for analysis	Notes	Jan	Feb	Mar	Apr	May	Jun to Aug
Data, report(s), and peer-reviewed manuscript(s)											
25	Complete QA/QC review of data.								x	x	x
26	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).					x	x	x	x	x	
27	Analyze data, prepare report(s) and peer-reviewed publication(s).								x	x	x

5.3 Exposure considerations across treatment groups

5.3.1 Determination of treatment doses

DDT, PCB, and PAH stomach content data (ng/g ww) from juvenile Chinook salmon collected from Portland Harbor in 2018 (n=6), and Duwamish River in 2006 (n = 1) and 2018 (n = 10 for PAHs only) were used to select target dietary treatments intended to mimic stomach contents of field-collected fish (Table 2). Median concentrations from both sites were used to select concentrations for treatment concentration 2 (T2) (Table 2). The target total PAH concentration of treatment 5 (T5) represents the highest concentration of PAH-contaminated diet used in Meador et al. (2006). Target concentrations for other dietary treatments were established by maintaining the relationship between each compound in treatment concentration 2 (T2) and creating log-based dilution concentrations.

5.3.2 Selection of analytes

The stomach content data described above were used 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 four 2018 Portland Harbor sites (DIVER, 2020), and one 2006 Duwamish River site were used. For the PCB analyte selection, stomach content data from four 2018 Portland Harbor assessment area sites (DIVER 2020), and one 2006 Duwamish River assessment area site were used. The PAH data from four 2018 Portland Harbor assessment area sites (DIVER 2020) plus ten 2018 WDFW Duwamish River stomach content samples collected from six Duwamish River assessment area sites (WDFW unpublished data) were used for the PAH analyte selection. Data from fish collected outside of the Natural Resource Damage Assessment areas (as defined in IEC 2018 and Elliott Bay Trustees 2019) were excluded from this analysis.

For each of the six DDTs analyzed in the stomach content samples, analytes that contributed greater than or equal to 5% to the sum of (∑) DDTs (on either a mass or molar basis) were selected for inclusion in the feeding study.

For each of the 40 PCB congeners analyzed 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) analyzed 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 known toxicity (benzo[*a*]pyrene).

5.3.3 Calculation of percent contribution of individual analytes

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 3. This list of compounds and relative proportions of each represent what is to be added to the prepared diet. 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.

5.3.4 Preparation of contaminated diet

For preparation of the experimental diet, wet weight stomach content values for class of compound and individual analyte were converted to dry weight values assuming a 20% dry to wet weight ratio, consistent with previous studies (Arkoosh et al. 2010). Target concentration in the feed was also calculated based on dry weight of the feed pellets, using 7% maximum moisture content reported by Otohime for the C1 pellets.

The final total PCB, total DDT, and total PAH target concentrations in T2 feed were 167, 76, and 4550 ng/g dry weight (33, 16, and 910 ng/g ww), respectively (Table 2). Chemical diet treatments will be prepared at the NWFSC lab in Seattle, WA. Preparation of the contaminated diet will involve a dilution of two concentrated custom stock solutions prepared by Accustandard Inc. (New Haven, CT) in dichloromethane ($MeCl_2$) and applied to the feed pellets for each of the five target treatment concentrations. Additional diet will be prepared using dichloromethane only, without the addition of the chemical stock solution, as a control for solvent treatment. A second control ('clean control'), with no addition of dichloromethane or chemical stock solution, will be used in parallel with the other treatments using the same handling and storage protocol. All fish food for a given treatment will be made at one time. Otohime C1 larval feed pellets will be treated using a stock solution of a contaminant mixture in dichloromethane. Fish pellets will be placed in a stainless steel bowl, a calculated amount of stock solution will be added to 4 L of $MeCl_2$, and the entire amount will be added to the bowl covering all the pellets. The mixture will be stirred at least four times per day, with equal stirring frequency and duration for all treatments, in a fume hood with a blacked-out window sash, at room temperature until the feed visually appears dry. The feed will continue to be stirred two times per day for 3 additional days to ensure dryness. Once dried, the pellets will be placed into amber glass jars with PTFE-lined tight-fitting lids, and stored at -20 ^{o}C until needed to feed fish. Concentrations will be verified by chemical analysis of a sample of food for each treatment.

Table 2. Exposure groups - target concentrations in contaminated diet

	ng chemical class/ g dry weight		
Dose	**PCBs ng/g**	**DDTs ng/g**	**PAHs ng/g**
Clean 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	24.0	11.0	651
T2	167	76	4550
T3	1170	531	31800
T4	8170	3720	223000
T5	57100	26000	1560000

Table 3. List of compounds and relative proportions of each analyte to be added to the prepared diet

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 additional parent PAHs and alkylated PAH homologs were 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.

5.4 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 are described below:

- Prepare bacterial 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 the bacteria and carefully transfer the fish back to the flow through 400 L tank
- Collect and verify mortalities daily
- Prepare an LC-response curve of Bacteria Exposure Dilution vs. Percent Cumulative Mortality.

5.5 Parameters to be determined

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

- Lab environmental monitoring
    - Ambient temperature
    - Water temperature system wide
    - Dissolved oxygen in each tank
    - pH and chlorine system wide
- Biological metrics at the end of 5-week dietary exposures (50 individual fish per tank)
    - Fish fork length (mm)
    - Fish body mass (g)
    - Liver whole weight (mg) (hepatosomatic index)
    - Body condition (e.g., Fulton's condition factor)
- Biological metrics after disease challenge (following five-week dietary exposure)
    - Survival
    - Fish fork length (mm)
    - Fish body mass (g)
- Tissue chemistry
    - Composite samples of whole bodies (less stomach contents, otoliths, and liver tissue) tissue analyses for PCBs, DDTs, PAHs, gravimetric percent lipid determination, and lipid class (wax ester and sterol esters, triglycerides, free fatty acids, cholesterol, phospholipids and other polar lipids) –analysis after 5-week dietary exposures (1 composite analysis per feeding treatment tank)

    - (Optional) PAHs and POPs in liver tissue after dietary exposure (number of composites contingent on total mass of liver tissue available (2 g minimum needed)
- Growth
    - Otoliths (microstructure analysis) at the end of 5-week dietary exposures (30 individual fish per tank) will be used to quantify the average daily growth in most recent 7-, 14-, and 21-days
- Verification of infection
    - Identification of bacteria in kidney tissues after disease challenge (mortalities only)

5.6 Number of fish required

Based on the analyses to be performed, described in Section 5.2, 6,150 fish of similar size are needed to complete the study. For each of the 28 tanks for phase 1 (feeding contaminated diet), 50 fish will be removed after five weeks of feeding for analysis. One hundred surviving fish are needed for phase 2 (disease challenge). Each tank will be populated with 180 fish at the beginning of phase 1, allowing for 30 extra fish in each tank. The target is to start each tank with the same number of fish of similar size. Additional fish will be needed to determine the target lethal concentration for the disease challenge (n=900), verify tissue concentrations before the test begins (30), and to monitor growth rates (180) bringing the total to 6,150 fish of similar size. A total of 15,000 button-up fry were requested from the Little White Salmon National fish hatchery to ensure adequate numbers of fish of similar size will be available at the beginning of the study.

5.7 Chain of custody

Chain of custody (COC) 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. COC facilitates this verification process. Failure to follow COC procedures in this guideline does not necessarily render data unusable; however, NRDA case managers should be notified of any deviations from the COC guidelines. Assuring that proper COC guidelines are followed is important to assuring the integrity of the samples and the data generated by the analysis of those samples.

A 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 permanent ink, scanned, and a copy will accompany the shipment to the laboratory (COC Form, Appendix A). A scanned copy of the COC form will be loaded into DIVER for archival and tracking. The COC Forms will be enclosed in resealable plastic bags and taped to the inside lip of coolers. COC forms should be printed on waterproof paper when possible to ensure the information is not lost due to water or condensation. The information on this Form will be used to track all samples from collection to receipt at the analytical laboratories. Upon delivery and receipt of coolers, the COC Forms must be signed and dated by the recipient (analytical laboratory) and the individual (NOAA staff) who relinquishes the samples. This signed COC should be scanned, and uploaded into DIVER for archival and tracking. The laboratory is required to log in samples

and note non-conformances with any shipping conditions. Temperature exceedances, or absence of dry ice that may indicate thawing of tissue will be immediately reported to the NRDA case managers.

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 archived under the appropriate conditions. See Appendix A for form.

6 SOP for fish processing (measurements, dissections, and archival), tissue sample handling, and record keeping

The SOP outlined below describes the gear and procedures to be employed for measurements, dissections, and archival procedures for collected fish.

6.1 Documentation

Separate fish data forms should be completed for each phase of the study. When fish die after the beginning of the study (in either phase 1 or phase 2), a sample ID, date of death, weight, and length will be recorded on a Fish Data Form. 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 IDs, fish measurements (weight and length), and dates of death will be documented on fish data forms; fish IDs will be recorded on Chain of Custody (COC) forms.

When fish are dissected to remove otoliths, stomach contents, and livers, fish dissection information must be documented on a Sample Processing Form, and individual component COC forms. Separate forms should be used for each phase (phase 1 and phase 2 of the study).

A sample COC form is provided in Appendix A.

6.1.1 Sample identification labels

To facilitate data interpretation, a numbering system has been developed to track the 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 a sample identification number. All labels will be printed on waterproof paper able to withstand freezing and written using permanent marker or preprinted. Below is a description of the four concatenated components that make up the full SampleID for whole fish and the individual fish parts after dissection.

- Treatment – There are 7 different feeding treatments (described in table 2) that fish will be exposed to. They are designated via a two digit code described as CC (Clean Control), SC (Solvent Control), T1, T2, T3, T4, T5. This two digit letter and number combination Treatment designation is at the start 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 second component of the SampleID.
- Phase/Disease challenge – Fish removed from the experiment at the end of the feeding phase (due to mortality or sampling) will be assigned an "F" character as the third component of the

sample ID. 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 (pathogen) and one not exposed to pathogens (non-pathogen). 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; non-pathogen exposed fish are designated as N. This single character letter code is the third component of the SampleID for fish in the disease challenge phase.

- Number – The final component of the SampleID is a three digit number representing an individual fish. A number between 1 and 80 will be sequentially assigned as fish are removed from each tank in the feeding phase, and between 1 and 50 in the disease challenge phase.
- The full SampleID for fish before dissection for fish collected during the disease challenge phase consists of Treatment (2 characters), Tank (one character), Pathogen (one character), & number sequence (three digits). Upon dissection, the sample number will be followed by two characters qualifying the type of tissue (WH: whole body minus stomach contents, otoliths, and liver; OT: Otoliths, and LI: Liver). Fish IDs for fish collected during the feeding challenge phase will have four components (treatment, tank, the character "F" indicating the phase, and number).
- Capitalization must be maintained when recording SampleIDs on sample data sheets.

- Example of full SampleIDs include:
    - Example 1: CC (Clean Control), A (Tank 1), F (feeding phase), 001 (Fish number 1)
        - Full SampleID: CCAF001
    - Example 2: T1 (Treatment 1), B (Tank B), P (Pathogen), 001 (Fish number 1)
        - Full SampleID: T1BP001
    - Example 3: T2 (Treatment 2), C (Tank C), N (Non-Pathogen), 006 (Fish number 6), OT (Otolith)
        - Full SampleID: T2CN006OT

- Whole body SampleID example: T5CF001WH
- Otolith SampleID example: T5CF001OT
- Liver SampleID example: T5CF001LI

6.1.2 Fish data form, sample processing form, and sampling processing notes

Entries for Sample Processing Forms and Sample Processing Notes will be made with permanent 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.

Two forms will be used for recording information regarding the fish and subsequent samples.

Fish Data Form – This form is filled out upon the death or euthanization of a fish. At this point the fish is given a Sample ID to identify the fish throughout the rest of the dissection process. The date the fish died or was euthanized is recorded. The length (mm) and weight (g) of the fish is recorded. Additionally,

the Treatment, Tank, and Pathogen status of each fish is also recorded on this form. The Tissue Type and Species is included as well. Finally, any Sample Notes or Sample Photos taken at the time of the death are recorded to document any unusual features and/or occurrences that happened to the fish.

Sample Processing Form – This form is filled out during the dissection of individual fish. Information recorded on this form include Sample ID, Start time of processing, and Otolith number. Additionally information recorded include Liver Weight, Stomach Contents Weight, Stomach Swabs number, Sample Notes, and Sample Photo numbers.

Forms are provided in Appendix A.

6.2 Fish processing and handling

The subheadings below outline the procedures and methods to be used to process the juvenile Chinook salmon.

6.2.1 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
- Electronic balance accurate to 0.001 g, for liver wet weight
- Weigh boats
- Paper towels
- Kimwipes™
- Dissection kit with stainless steel scalpel, scissors, and forceps, plus additional scalpel blades
- Magnifying glass on stand, with light
- Tap water
- Deionized water
- Isopropyl alcohol
- 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 (XS,S,M,L,XL)
- 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 labels – cryogenic, laser ready
- 1.5 mL polypropylene SnapTop tubes [no preservative or solvent needed]

- Chain of custody forms
- Chain of custody tape
- Sample Processing Form (printed on waterproof paper)
- Sample Processing Notes form (printed on waterproof paper)

6.2.2 Length and weight

Staff will record the weight and length of all collected fish during the feeding and disease challenge phases of the study at the NWFSC's Newport Research Station in Newport, Oregon.

- **Equipment/supplies**
  - Measuring board
  - Scale accurate to 0.01g
- **Protocol/procedures**
  - Target fish will be weighed (to the nearest 0.01 g)
  - Fork 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 middle caudal ray (to the nearest mm)
  - Both measurements will be recorded on the Sample Processing Form
- **Decontamination protocol**
  - Between tanks, follow instrument and work area decontamination protocol below

6.2.3 Fish dissection/necropsy overview

Fish collected during phase 1 or phase 2 for dissection will be processed at the NWFSC in Seattle, Washington after the completion of the exposure phases. Dissection of fish will be conducted by or under the supervision of experienced NWFSC personnel. Fish will be processed on a "clean" work-surface with "clean" instruments as described in Section 6.5, 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.

All dissections will be performed on dry ice to keep fish and removed tissue frozen during the dissection process. The otoliths will be extracted, the liver will be removed, weighed, and placed in liquid nitrogen. Following the completion of the dissection, all fish whole bodies (less liver, stomach contents, and otoliths) will be placed in a cooler with dry ice until being transferred back to a locked -80 °C freezer.

Collection of otoliths

- **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 1.5 mL polypropylene SnapTop tubes (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

Access to internal organs

- **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 discarded.

Collection of liver for archival and possible chemical analysis

- **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 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 in a tared SnapTop tube
    - Close tube securely (audible snap)
    - Place tube containing liver in liquid nitrogen
    - 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 in liquid nitrogen until being transferred to a -80 °C freezer

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

- **Protocol/procedures**
    - Remaining whole body will be returned to the labelled bag, with the second label that indicates SampleID number with "WH"
- **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 °C freezer

6.2.4 Identification of pathogen induced mortalities

After the completion of phase 2 (disease challenge), any mortalities that occurred during the test will be processed for presumptive identification of pathogen induced mortality. This will occur at the NWFSC's Newport Research Station in Newport, Oregon.

- **Equipment/supplies**
  - Lab tape, different colors
  - Nitrile exam gloves – powder-free
  - Kimwipes™
  - Tap water
  - Deionized water
  - Micro brand soap for cleaning lab surfaces and instruments
  - 100x15 mm sterile Petri plates
  - Agar plates
  - Alcohol burner
  - Lighter
  - 95% and 75% Ethanol
  - Isopropyl alcohol
  - Beakers, 250ml
  - Bleach
  - Spray bottles
  - Electronic balance with USB/RS232 communication
  - 1- µl sterile disposable inoculating loops
  - Cutting boards
  - Necropsy instruments
  - Paper towels
  - Disposable plastic bags
  - Bacteriological Media - Tryptic Soy Agar (TSA)
  - Parafilm
  - Incubator
  - Autoclave
  - Aquaculture Supplies
    - 6" Fishnets with PVC handle extensions
    - Aquaculture disinfectant: I-O-Safe or Virkon Aquatic
  - Wet ice
  - Autoclave bags and biohazard buckets
  - Recordkeeping:
    - Laptop computer with P3/P4 software and digital communication adapters
    - Digitizer board and pen
    - Permanent markers
    - 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 permanent 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 (similar to 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 pathogen challenge logs and the plates autoclaved
        - For *V. anguillarum* exposures, follow protocols described in Arkoosh and Dietrich (2015) for pathogen confirmation by polymerase chain reaction.
    - Autoclave and discard fish exposed to pathogens

6.3 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. Processing of fish will proceed beginning with control fish and ending with most highly contaminated treatments.

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)

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 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 sample form when and how rinsate blanks were collected. Rinsate samples, if collected, will be archived for potential chemical analysis.

6.4 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 °C freezer or on dry ice at the end of the day.
- **Otoliths**. Otoliths will be placed in dry SnapTop tubes 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 archival.** 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 staff will maintain COC procedures and sample integrity for the entire time the samples are in their possession. The laboratory staff will store the excess samples until otherwise notified.

6.5 Health and safety

The NWFSC Fisheries Science Center (NWFSC) established a Chemical Hygiene Plan (CHP) as required by the Occupational Safety and Health Administration (OSHA) standard titled "Occupational Exposure to Hazardous Chemicals in Laboratories" (29 CFR Part 1910.1450). The CHP (NWFSC 2019) is intended to protect staff from potential health hazards associated with the handling, use, and storage of hazardous chemicals at facility laboratories. Personnel exposure to hazardous chemicals in laboratory activities will be maintained at the lowest practical levels, using administrative and/or engineering controls, and at no times will the Permissible Exposure Limits established by OSHA (29 CFR 1910.1000 Subpart Z) be exceeded.

The safe storage, use and disposal of chemicals in the laboratory requires policies and procedures for the protection of staff and the environment. The purpose of the CHP is to provide the chemical user with basic safety information regarding the use of chemicals. This CHP forms the foundation for the safe use of chemicals in the laboratory and is an adjunct to the NWFSC Hazard Communication Program. The NWFSC Safety & Environmental Compliance Officer (ali.bahrami-bayeh@noaa.gov) is responsible for recommending the minimum requirements of the CHP that laboratories must follow and for providing project safety related guidance and oversight.

7 Analytical methods

The methods used for contaminant analysis of tissues (whole body and liver), otolith microstructural analysis, and identification of bacteria 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 one whole body composite samples of 30 fish for DDTs, PCBs, and PAH contaminant analysis from each feeding study treatment tank.

The mass requested by the NWFSC analytic lab for DDTs, PCBs and PAH analysis is a minimum of 4 g of fish in each whole body composite (less stomachs, otoliths, and livers). 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 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 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].

All above analytes will be measured; only the analytes that are added to the contaminated diet will be reported unless any of the measured analytes that are not added to the diet are detected in the analyzed samples. 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 tissue will be removed, handled, and preserved according to Section 6 and may be analyzed at a later date. If analysis is performed, method details and quality assurance criteria will be reported with findings. Liver analysis may be performed if whole body tissue results (less stomachs, livers, and otoliths) are not sufficient to judge whether laboratory fish accumulate contaminants similarly to field collected fish.

7.3 *Otolith analysis*

Otolith microstructure will be analyzed to estimate recent somatic growth using methods described previously (Chittaro et al. 2018; 2020). 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).

7.4 Identification of bacteria

Dead fish will be removed throughout the observation period and weight and length recorded. 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* (Noga 1996), or polymerase chain reactions will be used to confirm *V. anguillarum* presence (Arkoosh and Dietrich 2015).

7.5 Laboratory quality assurance

7.5.1 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 4 (taken from Sloan et al. 2019).

Table 4. 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 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 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 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.
Laboratory sample replicates (i.e., duplicates or triplicates)	One with every 26 or fewer 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 NWFSC QA Officer (Jennie.bolton@noaa.gov).

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 ww for PAHs and 0.15 to 0.50 ng/g ww 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 feeding study 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. Removing liver tissue may 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.

7.5.2 Chemical analysis, liver tissue analytical quality assurance criteria

Liver tissue will be removed, handled, and preserved according to Section 6 and may be analyzed at a later date. If analysis is performed, method details and quality assurance criteria will be reported with findings.

7.5.3 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. Once the subset of otoliths has been measured twice, the average increment width across the last seven increments for both measurements of every otolith will be determined. The averages between replicate measurements will be compared using Student's t-test. The Student's t-test allows the independent readings of the same otoliths to be compared to confirm whether both provide similar results. If no significant difference is observed between replicate measurements, then the otolith measurements have a high repeatability and are thus of good quality. If significant differences are detected between replicate measurements, then a three-step process will be followed to improve otolith measurement quality (as outlined in Chittaro et al. 2020). First, the otolith(s) in the subset that show the greatest replicate measurement variability will be identified by calculating differences between replicate measurements. Second, for the purpose of identifying where the deviations in increment marks between replicate measurements arose and how to revise the otolith measurement(s), the otolith increment widths and increment markings on the otolith digital images will be marked for those otoliths that have the greatest differences between replicate measurements. Third, the Student's t-test will be repeated on the revised measurements from the subset of otoliths. If this test fails, then Steps 1–3 will be repeated on the same subset of otoliths again. If the test passes, then the above test will be repeated on a new subset of otoliths.

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 feeding study 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 as used for the 2018 field collection of juvenile (sub-yearling) out-migrating Chinook salmon from the Willamette River (discussed in Section 2.3) (NOAA NMFS 2018).

8 Description of the interpretation techniques to be used

Phase 1 of the Chinook salmon laboratory feeding study will be conducted as a one-way experimental layout with 4 replicate fish tanks per each of 7 experimental levels: untreated control, solvent treated control, and 5 feeding doses. This experimental design for phase 1 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 1 and 2 after preprocessing measurement endpoints so that each primary experimental unit represents one degree of freedom in statistical analyses. Phase 2 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 pathogen. 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 1 and phase 2.

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 Littell 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 or the Lower Duwamish River. 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 principal components analysis to summarize groups of contaminants into composite variables (i.e., principal 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 and analysis. Results of data verification and validation activities will also be documented. Copies of all of these records 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., lab measurements, continuous-read instruments, photos) to occur and helps case team 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 B, Table B1). The core field information makes the related data available for searching and download.

9.2.1 Lab data and fish dissection documentation

For lab sampling efforts and fish processing (dissections), all data will be stored electronically. After the completion of collection of samples at the end of phase 1 and phase 2, data intake and processing will occur for all forms used during the sampling and these will be uploaded into DIVER. Similarly, after each day of performing fish dissections, copies of fish processing forms and related laboratory notes will be uploaded into DIVER. Any photos taken during the study will also be uploaded to DIVER. See Appendix B for details on data intake and processing.

Accurate transcription and review of lab 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 sampling or processing team review. During the lab 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 lab and fish processing forms will be transcribed into ORR Electronic Data Delivery 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 B2, Appendix B.

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 archival, data analyses, and use with geographic information systems. 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

Chain of Custody and sample 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:

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

-Sample Processing Form (to record biological samples collected from dissected fish)

-Fish Data Form (to assign and record Sample IDs for a fish as well as document the length and weight of the fish)

-Photologger form and chain of custody

NRDA Chain of Custody Form

Page____of ____

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

Special Instructions

Please send a scan of the signed COC to Data Manager: (Enter Data Manager Email) and Lead: (Lead Contact Information Email) and keep the original form with the samples at all times | Labs - Please send results to EXA Contact: (Enter EXA Contact Email) and copy Data Manager and Lead.

Turn Around Time:

Analyses requested

A	B	C	D	E	F	# of containers

Lab Use Only	
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	
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				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/Org.

NRDA Sample Collection Form – FISH DATA

Lead Sampler Name/Phone:	Study Name: Portland Harbor Injury Assessment Juvenile Chinook Salmon Controlled Dietary Exposure and Endpoint Analysis
Lead Sampler Affiliation:	**Sample date (mm/dd/yyyy)____XX___/___XX___/2020**
NRDA Contact/Phone:	

Sample ID	**Date**	**Total Length (mm)**	**Fish Weight (g to 0.01)**	**Treatment**	**Tank**	**Pathogen**	**Tissue Type**	**Species**	**Sample Notes**	**Sample Photos**
Sample ID	*Date fish died or was euthanized*	*Total Length of Sample Fish*	*Total Weight of Sample Fish*	*SC - Solvent Control CC - Clean Control Treatment - T1, T2, T3, T4, T5*	*Tank fish located: Tank A Tank B Tank C Tank D*	N - Non Pathogen - Not dosed with a pathogen P - Pathogen - Dosed with a pathogen F - Feeding Phase Fish	*FI = Fish*	*Species common name*	*Any documentation regarding a specific fish sample.*	*Photo # for any photo taken of individual fish*
							FI	Chinook		
							FI	Chinook		
							FI	Chinook		
							FI	Chinook		
							FI	Chinook		
							FI	Chinook		
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							FI	Chinook		
							FI	Chinook		

Form filled out by:______________________________Field lab team Initials:_________ Page:__________of ____________

Sign Off:

Federal Representative/Affiliation:____________________________Date:_______________ Time (24 hr): __________

NRDA Sample Processing Form – FISH DATA

Lead Sampler Name/Phone:		Study Name:	Portland Harbor Injury Assessment Juvenile Chinook Salmon Controlled Dietary Exposure and Endpoint Analysis
Lead Sampler Affiliation:		**Processing date (mm/dd/yyyy) XX_/_XX_/2020**	
NRDA Contact/Phone:			

Sample ID	Time Begin Processing	Otoliths (#)	Liver Weight (g to 0.001)	Stomach Contents Weight (g to 0.001)	Stomach Swabs (#)	Sample Notes	Sample Photos
Sample ID	*(24-hour clock, local time)*	*Number of Otoliths Collected*	*Weight of the Liver Collected*	*Weight of the Stomach Contents*	*Number of Stomach Swabs Collected*	*Any documentation regarding a specific fish sample.*	*Photo # for any photo taken of individual fish*

Form filled out by:_____ Field lab team Initials:_____ Page:_____ of _____

Sign Off:

Federal Representative/Affiliation:_____ Date:_____ Time (24 hr): _____

NOAA OR&R PhotoLogger Form & Chain of Custody

This form must be filled out to accompany photos taken in the field, either filled out in the field or upon return to Data Intake.

Photographer Name: _____

Workgroup: _____ **Work Plan:** _____

Date of Photos (MM/DD/YYYY): _____ **Photo Range:** _____

Camera Time Zone: **AST/ADT** **PST/PDT** **MST/MDT** **CST/CDT** **EST/EDT** **Other Time Zone:** _____

Camera Date (MM/DD/YYYY): _____ **Camera Time (HH:MM:SS):** _____ **Camera Model:** _____

GPS Time Zone: **AST/ADT** **PST/PDT** **MST/MDT** **CST/CDT** **EST/EDT** **Other Time Zone:** _____

GPS Date (MM/DD/YYYY): _____ **GPS Time (HH:MM:SS):** _____ **GPS Model:** _____

Location and State where photos were taken - *Geographic area where the field work was completed (ex. Neah Bay, WA)*

General description of all photos - *If you have photos from significantly different sites / missions in the same group of photos being submitted,* please fill out this form separately for each

Keywords that describe ALL photos being submitted - *Specific keywords that describe ALL the photos this form addresses. If you choose to fill out the next section or review your photos in the PhotoLogger database you can add keywords for unique photos.*

Enter photo-specific comments here – *Provide more details to key photos of high value in the Comment section. You may also use this section if you* need to identify specific photos of sample locations or photos that are data themselves (e.g. photo plots).

Photo Number	Comment *(ex. Sample ID, significance)* and Photo-Specific Keywords

NOAA OR&R PhotoLogger Form & Chain of Custody

Suggested Keywords – *These are suggested keywords to describe your key photos. You can add others to the side. Keywords are used when importing the photos to PhotoLogger, where they will be queried by field staff, management, or outreach staff in the days and years to come. Please select keywords that are general enough to represent the photos in future queries (ex. Put species in the Comment field). More specific details can be entered into the above Comment section or later in PhotoLogger.*

Barge	Fish Kill	Oil-Sheen/Rainbow	Sediment Core
Barrel	GPS Unit	Oil-Dark	Shellfish
Barrier Island	Gravel Beach	Oil-Emulsified	Shoreline
Beach	Grounding	Oil-Tarball	Small Boat
Birds	Ice	Oil-Tarmat/Tarpatty	Snow
Boat	In-Situ Burn	Oil-Surface Residue	Source Oil
Boom	Intertidal	Oil-Stain/Coat	SAV
Container	Jar	Outreach	Subtidal
Chemical	Kelp Bed	Overflight	Sunken Vessel
Cleanup Operations	Lagoon	Pipeline	Tank
Coral	Managed Area	Pits and Trenches	Tanker/Ship
Crab	Mangrove	Quadrat	Terrestrial Turtle

Required Chain of Custody Filled Out Upon Data Intake	
Photos & GPS Data Relinquished By	**Photos & GPS Data Received By**
Name Signature:	Name Signature:
Name Printed:	Name Printed:
Agency Name Printed:	Agency Name Printed:
Date/Time:	Date/Time:

I,_____*[**Data Intake Manager print name**]*, without modification, downloaded the photographs referenced on this form in accordance with the NOAA OR&R Data Intake Protocols and uploaded without modification to DIVER in the File Collection ID number_____with the following Photo Zip file named _____ and GPS Zip file named_____.

_____ _____

Signature *Date/Time*

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 the study
- 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

During the course 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, lab forms, and chain of custody forms and uploading to lab study DIVER File Collections. Photos, chemical analyses, and other results will also be transferred into DIVER using the DIVER integration process that is described 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 from the same tank and share the same matrix they are assigned different SampleIDs. The samples will be assigned a unique composite ID number if combined as a composite. Thus, two 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 Electronic Data Deliverable, 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.