SAMPLING AND ANALYSIS PLAN FOR THE FINAL PHASE OF THE HYLEBOS WATERWAY ROUND III FISH INJURY PILOT STUDY

SALMON LABORATORY STUDIES

prepared by

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PROJECT BACKGROUND AND OBJECTIVES

Background

Juvenile chinook salmon accumulate polycyclic aromatic hydrocarbons (PAHs), and chlorinated hydrocarbons (CHs), in tissues, fluids and stomach contents while residing in the contaminated estuaries of Puget Sound, WA (e.g. Duwamish Waterway and Commencement Bay) during their out migration from freshwater to open oceans. This increased exposure in juvenile salmon is associated with increased induction of hepatic cytochrome CYP1A, higher levels of DNA damage, impaired immunocompetence, increased disease susceptibility, and growth inhibition, when compared to juvenile salmon from nonurban estuaries (McCain et al. 1990, Arkoosh et al. 1991, Stein et al. 1995, Varanasi et al. 1993).

During Round I of the Hylebos Waterway Fish Injury Assessment, it was shown that juvenile chum and chinook salmon in Hylebos Waterway had elevated concentrations of PAH metabolites in bile and CHs in liver (Collier et al., 1998). These concentrations were comparable to those associated with results from a previous study of juvenile salmon from the Duwamish Waterway and Commencement Bay (Varanasi et al. 1993) where effects had been demonstrated. It was also shown in Round I that English sole residing in the Hylebos Waterway exhibited elevated concentrations of PAH metabolites in bile and CHs in liver, elevated DNA adducts, CYP1A activity, and toxicopathic lesions in liver tissue. It was also determined that 40 - 50% of the juvenile sole displayed precocious sexual maturation. These findings demonstrated that fish from the Hylebos Waterway accumulated contaminants to levels that induce biological effects.

In Round II, three classes of compounds (PCBs, PAHs, and HCBD (hexachlorobutadiene)) and three sediment extracts [Hylebos Waterway sediment extracts (CHWSE and HWSE), and a reference sediment extract from the Nisqually River estuary (NQSE)] were tested to assess the relative toxicity of each chemical group. The results of Round II showed that chlorinated compounds and the Hylebos extracts had a negative effect on the growth of juvenile salmon over a 60-day period. These substances, along with PAHs, also increased disease susceptibility in juvenile chinook salmon. The next logical step is to conduct a detailed study of those compounds (primarily the chlorinated compounds) producing negative effects. This will be accomplished in Round III by conducting dose-response studies that will provide a statistical model for relating impaired growth, disease resistance, and pathological abnormalities to the degree of exposure to toxicants as determined by concentrations of these chemicals in the diet and tissues.

Before proceeding to the comprehensive Round III investigation, a pilot study was proposed. This pilot study is being conducted in two phases. The initial phase was accomplished in 1998, while the final phase , the subject of this SAP, was planned for 1999, but will be will be conducted in 2000.

The goals of the initial phase of the Round III Pilot Study were to document the palatability of a low-fat food pellet that was used for delivering contaminants to juvenile fish, and to ascertain that those contaminants are bioavailable when administered in this fashion. These are essential information for planning and executing the Round III comprehensive study. Juvenile chinook were fed pellets that were contaminated with known amounts of a PCB mixture (Aroclor 1254), PAHs, and CBDs. The results (see March 26, 1999 memo—Appendix A) show that the low fat pellets were palatable and are considered a successful vehicle for delivering graded doses of contaminants to juvenile salmon over a period of several weeks.

Biological Endpoints of the Final Phase of the Round III Pilot Study

Growth

Somatic tissue growth is a highly regulated process integrating the functions of numerous physiological systems. Impaired growth has been shown to be a sensitive, sublethal measure of chemical contaminant exposure, particularly in rapidly developing larvae and juvenile organisms. In 1993, we monitored growth in fish sampled from urban (Duwamish, Puyallup, Snohomish Rivers) and non-urban (Nisqually River) estuaries and their respective hatcheries (Varanasi et al. 1993). In that study, growth of juvenile chinook salmon from the non-urban estuary was comparable to fish from the respective releasing hatchery, whereas growth of juvenile chinook salmon from the urban estuary was significantly depressed relative to the growth of juveniles from its respective releasing hatchery.

Disease Susceptibility

A recent field investigation demonstrated that juvenile chinook salmon from the Duwamish Waterway (Arkoosh et al. 1991) exhibited an impaired immune system. The immunological studies examined the ability of leukocytes in juvenile salmon from the urban Duwamish Waterway, the nonurban Nisqually River estuary, and their respective hatcheries to produce a primary and secondary (memory) *in vitro* plaque forming cell (PFC) or B-cell response. Suppression of the memory (secondary) response occurred in juvenile chinook salmon from the urban estuary but not in fish from the control sites (Arkoosh et al., 1991). Additional laboratory studies demonstrated that juvenile chinook salmon exposed to either a PCB mixture (Aroclor 1254) or a PAH (dimethylbenz[a]anthracene)

exhibited a suppressed primary and secondary PFC response (Arkoosh et al., 1994). Recent studies have also shown that juvenile chinook salmon from the urban Duwamish Waterway estuary were more susceptible to the marine pathogen *Vibrio anguillarium* than were salmon from reference estuaries or hatcheries (Arkoosh et al., 1998). Therefore, chinook salmon from an urban estuary appear to be more susceptible to an infectious agent than salmon from a minimally contaminated non-urban estuary.

Increased susceptibility to an infectious agent is associated with immune dysfunction and exposure to chemical contaminants present in the Hylebos Waterway. In Round II of our fish injury studies, we showed that PCBs, HCBD, PAHs, and Hylebos sediment extracts increased the susceptibility of juvenile chinook salmon to a pathogenic bacterium, *Vibrio anguillarium*. Aspects of the final phase of the Round III Pilot Study will investigate what ranges of PCB concentrations, in association with PAHs and CBDs, affect disease susceptibility.

Overall Goals of the final phase of the Round III Pilot Study

We have established that juvenile salmon migrating through the Hylebos Waterway are being exposed to chemicals present in the Waterway, and that these compounds exert significant biological effects. This final phase of the Round III Pilot study ~~2~~ will function as a range-finding exercise to estimate the dose-response relationship between tissue concentrations and biological effects (growth and disease susceptibility). -We will conduct a dose-response study that will examine the effects of polychlorinated biphenyls (PCBs; Aroclor 1254) when given concurrently with an environmentally realistic high dose of polycyclic aromatic hydrocarbons (PAHs) and chlorobutadienes (CBDs) to juvenile chinook salmon. The PAHs and CBDs will be held constant and the PCBs will be varied to assess their effects on the ability of juvenile chinook salmon to grow normally and resist a disease challenge. This range-finding dose-response exercise (or study) will examine the worst case exposure for PAHs and CBDs within a limited range ~~for~~ of PCB concentrations. An objective of this study is to find a general response level which will allow refinement for dosing in future studies. This refinement will help ensure that the doses used in the comprehensive study will be chosen to maximize our ability to accurately define the shape of the dose-response curve.

The focus of this range-finding response study is to determine concentrations of PCBs that cause adverse effects when present in tissue with other common environmental contaminants. This is a reasonable approach for assessing the potential of one class of compounds (PCBs) to produce biological effects when present in a mixture of contaminants. Because juvenile salmon are exposed to a mixture of contaminants in the Hylebos Waterway and each contaminant may contribute to the overall toxic effect, a mixture of these contaminants must be considered when comparisons between lab and field studies are performed. Future experiments (e.g., a Round III

comprehensive study) will be needed to determine more accurately the PCB concentrations associated with biological effects and the contribution of other contaminants in the mixture.

Assessment of sublethal impacts on juvenile fish from anthropogenic contaminants is justified in determining the potential impacts to the optimal health of a population. Reductions in growth can have severe impacts on population parameters such as fecundity, the intrinsic rate of population increase, and the death rate, which have major implications on population viability (Sibly 1996). Moreover, an increase in susceptibility to infectious agents or pathogens has the potential to also regulate salmonid population abundance by altering host reproduction and survival (Gulland, 1995).

This range-finding study is one step in identifying the tissue concentrations at which contaminants in the Hylebos Waterway may cause impairments to juvenile salmon migrating through the Waterway. It will lead to a more comprehensive study that will help establish a "no observed effect tissue residue" (NOER) and provide data to determine the dose-response relationship between contaminant residues in tissue, exposure concentrations, and biological effects. Once generated, the NOER can be related to those concentrations observed in wild fish and tissue concentrations predicted with Equilibrium Partitioning models (EqP). Equilibrium Partitioning models are based on the physicochemical properties (e.g., fugacity) of neutral hydrophobic organic compounds and allow prediction of sediment concentrations when tissue concentrations are known (Mackay and Paterson 1981, Di Toro et al. 1991). With currently accepted models of sediment-to-tissue partitioning, our experimentally determined tissue-concentration based biological response can be related to contaminant concentrations that occur in the sediments of the Hylebos Waterway.

Objective 1 – Conduct a range-finding experiment to estimate the dose-response relationship for PCBs on the growth of juvenile chinook salmon.

Study Design - Part I

An objective of this range-finding pilot study is to relate the tissue concentrations of PCBs, in the presence of PAHs and CBDs, to impaired growth in juvenile chinook salmon. Fish will be fed the mixture of contaminated pellets for 45 days and clean pellets for an additional 46 days. This feeding regime is intended to mimic food consumption during the observed pattern of juvenile passage through an urban estuary and during the subsequent transition to coastal areas that are less contaminated. Growth will be measured at day 91. The experimental design and doses are shown in Table 1.

Whole-body lipid levels will be determined. These are needed for interpreting the tissue residues of organic contaminants and for predictive modeling of sediment-to-tissue concentrations.

Objective 2 – Conduct a range-finding experiment to estimate the dose-response relationship for PCBs on the disease susceptibility in juvenile chinook salmon.

Study design Part 2

An additional objective of this range-finding pilot study is to compare the tissue concentrations of PCBs, in the presence of PAHs and CBDs, to increases in disease susceptibility in juvenile chinook salmon. Juvenile chinook salmon from four of the treatment doses will be exposed to a pathogenic marine bacterium, *Vibrio anguillarum*, and tested for disease susceptibility by measuring mortality. These fish will come from two replicate tanks dedicated to this objective. Because of limited space or the disease challenge study, we will test only one of the controls (solvent) and 4 four of the treatments.

DESCRIPTIONS OF TASKS

Preparation of Fish Pellets

Most commercial fish feeds cause juvenile salmon to have unnaturally high fat content. This is a result of the high fat levels found in the (food) pellets. Consequently, juvenile salmonids raised on this diet generally contain a lipid content that is several times higher than that found in wild fish. We have prepared a low-fat fish pellet that will presumably produce a much leaner fish, similar to those found in the field. This pellet was successfully used in the initial Pilot study (Appendix A). Because the amount of chlorinated hydrocarbons, that can reach the organs and cause a deleterious effect is directly controlled by the amount of lipid in a fish, we will strive to maintain a lipid level that more accurately reflects conditions found in wild fish.

Fish pellets will be dosed following procedures successfully used during the initial Pilot work. They will be soaked in a methylene chloride solution with the desired concentrations of contaminants. Shallow trays will be used to facilitate evaporation of the methylene chloride. Pellets for each experimental treatment will be made in one batch and stored at -20° C in polyethylene containers. There will be two control treatments of food without contaminants: pellets treated with methylene chloride and pellets not treated with methylene chloride.

Determination of Dosages

We will examine 5 contaminant doses, which will represent of the range of concentrations planned for the comprehensive study. These 5 contaminant doses and two controls will constitute the Pilot study's 7 treatments; we will employ five replicate tanks per treatment (Table 1). Variable PCB concentrations will be tested in

association with constant concentrations of PAHs and CBDs. Since this is a range finding exercise,only-three of the 5 replicates will be used to study growth. The other two replicates will be used for the disease challenge work.

A mixture of wet weight and dry weight concentrations are shown in this SAP because both formats were used in previous reporting. Dry weights will be reported during the Round III Pilot Study, along with the dry/wet weight ratio for interconversion. Dry weight measurements are preferred because they reduce contaminant concentration variability generally caused by differences in moisture content between biological samples.

Fish pellets will be dosed with PCB (Aroclor 1254) at five different levels to achieve the desired tissue concentrations. During the initial phase of the Round III Pilot, we assumed a 50% absorption rate and calculated the pellet concentrations necessary to achieve the desired tissue concentrations. Many studies (Gobas et al. 1993) indicate that the absorption efficiency by fish for such hydrophobic compounds is in the range of 35 - 75%, when based on wet weight. Results from the initial phase of the Round III Pilot (Appendix A) show that the ratio between food and whole-body concentrations was approximately 1:1 for PCBs and 10:1 for CBDs, based on dry weight. We will use these ratios to achieve the desired tissue concentrations during this phase of the Round III Pilot.

A geometric factor of about three (3.4) was chosen to provide the following target whole-body PCB tissue concentrations: 0, 0.25, 0.85, 2.9, 9.8, 33.4 μg/g dry weight (0, 0.05, 0.17, 0.58, 2.0, 6.7 μg/g wet weight) (Table 1). For the growth portion of the study, there will be 7 treatments (including two control treatments) with three replicates for a total of 21 tanks. For the disease study, there will be 5 treatments (including one control) with two replicates for a total of 10 tanks. A grand total of 31 tanks will be used. Each tank will contain 100 fish. Included in the 7 treatments will be two control treatments: fish fed uncontaminated pellets and fish fed pellets treated with the solvent (methylene chloride) used to coat the pellets with contaminants.

The pellets will have a constant concentration of PAHs and CBDs over all test treatments: 25 ppm for PAHs and 0.5 ppm for CBDs. The following is a justification for using those concentrations.

- ***PAHs***—Total PAHs in the stomach contents of chinook salmon from the Hylebos Waterway have been reported to exceed 20 ppm dry weight (Collier et al. 1998). Another study found total PAH concentrations up to 500 ppm dry weight in stomach contents of chinook sampled in the Duwamish River (Varanasi et al. 1993) (assuming a dry to wet weight ratio of 0.2).
- ***CBDs***—A previous study found total CBD concentrations between 1 and 10 ppm dry weight in English sole liver captured in the Hylebos Waterway (Malins et al. 1982). More recently, Collier et al. (1998) reported that English sole liver contained up to 50 ppb wet weight of hexachlorobutadiene (HCBD) ≈ 250 ppb dry weight). In the Malins et al. (1982) study, it was reported that HCBD accounted for about 10% of the total CBDs in sediment from the 11th Street Bridge area of Hylebos Waterway. Given the

average HCBD concentration in the liver of chinook salmon collected from the Hylebos was 2.2 ppb wet weight (10 ppb liver dry weight) and our measured value of 25 ppb from a nominal value in fish pellets of 1000 ng/g, we selected a nominal fish pellet concentration for total CBDs to be 500 ng/g.

Salmonid Culture

Colleagues (Drs. Penny Swanson, Walton Dickoff, and Karl Shearer) at the Northwest Fisheries Science Center (REUT Division) have many years of experience studying salmon husbandry and have assisted us in designing this experiment to have optimal conditions for salmonid growth. High variability in growth rate and tissue concentrations of the target toxicant can occur in this type of study; therefore, measures to assure uniformity in fish husbandry are warranted.

Fish density in the experimental tanks and the feeding schedule have been selected to promote minimal variation in growth between sampling units (replicate tanks) within a treatment. Reducing replicate variability will increase the ability to detect growth rate differences among treatments. There will be 100 fish per tank (4-ft diameter, 1,500 liter tanks, filled to about 900 L). This density will reduce the tendency for dominant fish to attack others and allow a more equitable distribution of food. Also, fish will be fed once per day (at approximately two percent body weight per day) to provide a more even distribution for food and contaminant uptake and to mitigate excessive feeding by dominant fish.

Dose-Response Growth Studies

The growth of juvenile chinook salmon exposed in the laboratory to PCBs, PAHs, and CBDs will be examined at the end of the 91-day period. Juvenile salmon of approximately the same size (median ± 0.5 cm, approximately) will be fed amended fish pellets for the first 45 days (see Table 1 for doses; Figure 1 provides a schematic diagram of the study design). After this period, the fish will be fed untreated pellets. There will be two control treatments, one will include fish fed uncontaminated pellets from the same lot and the second will consist of pellets treated with the same solvent (methylene chloride) used to coat the pellets with contaminants. Each treatment will have three replicate tanks dedicated to the growth challenge. On day 0, 15 fish will be selected at random from the larger pool of fish and combined into 3 composites of 5 fish each for chemical analyses. At the end of the experiment (day 91) all fish will be weighed and measured. For the day 45 and 91 sampling, fish will be allowed to purge their stomach contents for 24 hours before sampling to avoid including contaminated food in the tissue residue determination. Five fish from selected tanks will be composited into 1 sample and analyzed for whole-body concentrations of test contaminants (see Table 1). Separate samples will also be taken for bile and

liver (Table 1). Liver samples will be composites of 5 fish and bile sample composites will contain 10 fish each. The liver samples will be taken randomly from 5 of the fish used for bile samples. Also on day 91, a composite of one fish per tank will be sampled for whole-body lipid content. Mortalities will be monitored daily (Monday – Friday) and once on the weekend during this study.

Disease Resistance Studies

The disease susceptibility of juvenile chinook salmon will be investigated for four treatments plus the solvent control. (Note: Laboratory space limitations necessitate this restriction of treatments; consequently, the two replicate tanks for Treatment 1—control and Treatment 7—highest PCB dose will not be used. See Table 1). At the end of the exposure period, (day 45) 60 fish from each of the two replicate tanks (separate from the three growth challenge tanks) will be taken for the disease-challenge study. Treatments 2, 3, 4, 5, and 6 will be tested (Table 1). The fish will be exposed to the marine pathogen, *Vibrio anguillarum*, using three concentrations (LC 0, 96 hr LC30 and 96 hr LC50), as established from an LC response study as described in Arkoosh et al. (1998). Mortality will be monitored on a daily basis for up to 14 days, and the cumulative mortality curves for each treatment group will be used to determine disease susceptibility.

Chemical Analyses

Whole-body tissue residues will be determined for contaminants according to Table 1. All fish samples will be analyzed for total PCBs and CBDs. Selected samples will be analyzed for PCB congeners. From our previous work in NOAA's Status and Trends Program, 17 PCB congeners were selected, which are representative of the congeners of interest. Concentrations of PAHs will be determined in the fish pellets and a select number of fish samples (Table 1). Bile metabolites will be determined in selected treatments from the study to determine exposure to PAHs

Before the experiment starts, 15 fish from the common pool of fish will be composited into 3 replicates for determination of the initial whole-body residues for all analytes (PCBs, PAHs, and CBDs). On days 45 and 91, five fish will be taken from selected tank replicates to form a composite sample for chemical analysis (Table 1). A total of 35 whole-body composite samples will be taken. Of these, there will be 35 total PCB, CBD, and PAH determinations, and 15 analyses for PCB congeners. A total of 14 chemical analyses will be performed on the food. Fish pellets for all treatments will be analyzed at the beginning of the exposure period (one treatment in triplicate), and the five dosed treatments again after the exposure period (day 45).

A subset of fish will be analyzed to determine liver concentrations. The purpose of this is to determine the ratio between liver and whole-body concentrations. To accomplish this analysis, 6 additional composites (two replicates from three treatments) on days 45 and 91 will be analyzed for a total of 12 samples (Table 1). Total PCBs, PAHs, and CBDs will be determined in these composites. On day 91, concentrations of individual congeners also will be determined. Because of previous work that determined concentrations of these analytes in liver tissue only, a correlation between concentrations in liver and whole-body will be needed for interstudy comparisons. Whole-body concentrations are preferred for this study because of past dose-response studies, EqP modeling, and because toxicants, such as the CBDs, may accumulate to high concentrations in organs other than the liver.

There will be a total of 35 whole-body, 14 fish pellet, 12 liver, and 12 bile composite samples. There will be a total of 64 determinations each for total PCBs, CBDs, and PAHs, and 34 analyses for PCB congeners (not including the laboratory QA replicates).

Bile Samples

Bile from 5 individual juvenile salmon per composite will be collected into a single amber vial with a glass vial insert. The bile is collected as follows: after excising the abdominal mass and separating any mesenteric attachments connecting the gall bladder to liver and upper intestine, clasp the bile duct with forceps and cut bile duct with scissors, taking care to avoid spillage of bile from bladder onto the liver. The bile can be subsequently collected by perforating the bladder with a #11 scalpel blade mounted on a scalpel handle, while suspended over the mouth of the amber glass vial. There will be 3 composite samples for bile on day 0 from the common pool of fish, 6 samples on day 45, and 6 samples on day 91.

Lipids

Lipids will be determined with a modified Bligh and Dyer technique (Herbes and Allen 1983), which uses a chloroform-methanol solvent extract system. The SOP for lipid analysis is in Appendix C of the Quality Assurance Plan (QAP). At the beginning of the experiment (day 0) three composites of three fish each from the common pool of fish will be collected for lipid determination. These results will be used to normalize tissue concentrations for reducing variability and for modeling. At the end of the contaminant exposure period (day 45), a 3-fish composite from one tank in each disease challenge treatment will be analyzed (5 composites, see Table 1). At the end of the experiment (day 91), one fish from each growth study replicate of each treatment will be composited and sampled for whole-body lipid content (7 composites). Fish pellets will also be analyzed for lipid content for relating lipid

normalized diet concentrations to field values. One sample from each treatment will be analyzed at the beginning of the test and at day 45. There will be 29 lipid determinations (not including QA replicates).

Data Analysis and Products

Statistical tests will be performed to evaluate relationships between laboratory exposure to toxicants and indicators of fish injury (e.g., impaired growth and increased susceptibility to disease). The results will be analyzed using Analysis of Variance with a multiple comparison test to determine which treatments are significantly different from the control. The criterion for significant differences between treatments will be set at $\alpha = 0.05$. The data will also be amenable to dose-response analysis with a Generalized Linear Model technique (Kerr and Meador 1996), which will provide a mathematical relationship between dose and response. This technique will also generate a 95% confidence interval for both the dose and the proportion responding. The biological relevance of any observed reduction in growth or disease resistance will be determined from other published studies and modeling exercises. To the extent possible, the results for the both tissue residues and dietary concentrations from the lab study will be compared to values observed in field collected animals. With these range-finding results, we will estimate a "no observable effect tissue residue" (NOER) for PCBs in relation to the high concentration of PAHs and CBDs. This NOER will be compared to concentrations measured in wild fish from the Hylebos Waterway to gauge the degree of impact and will be a useful value for refining the dosing scheme for the comprehensive Round 3 study. Equilibrium Partitioning (EqP) models can be used to predict tissue concentrations when sediment concentrations, total organic carbon content in sediment, and tissue lipid levels are known. The equations can be rearranged to predict a sediment concentration for a given tissue concentration.

Data acquired and analyzed as described in this SAP will be used to address the following questions:

1. Are elevated tissue concentrations of PCBs, in combination with environmentally realistic concentrations of PAHs and CBDs, related to growth impairment or increased susceptibility to pathogenic bacteria? If so, do these effects occur in a dose-response relationship?

2. At what concentrations do PCBs in the diet cause effects that are significantly different from the control?

3. After dosing the fish, will the contaminants persist in tissue for at least 45 days when fish are fed clean food?

4. After the lowest observed effect (LOER) and no observed effect tissue residue (NOER) are estimated in this range-finding test, how should the comprehensive study be designed?

5. What is the relationship between liver and whole-body concentrations of these contaminants?

6. What is the relationship between exposure concentrations, tissue residues, and bile FACs for PAHs?

SCHEDULING OF TASKS

Dose-Response Studies

Date	Task
Jan – May 2000	Revise Sample and Analysis Plan Develop a suitable fish pellet Complete construction of new area for experiment Plumb in new tanks and new chiller
End of May	Acquire fish from Soos Creek Hatchery
End of June	Complete revisions to the Pilot Study SAP
July 10, 2000	Start of weighing, measuring, and tank assignment
July 28, 2000	Start of experiment: feeding dosed fish pellets
September 11, 2000	End of 45-day dosing phase, commence 45-day grow out period with untreated fish pellets, and commence desease challenge portion of study
September 22, 2000	End disease challenge
October 26, 2000	End of grow out period and live tank portion of Pilot Study; begin weighing and measuring of fish.
November 4, 2000	Complete weighing and measuring of fish
December 21, 2000	Submit preliminary results based on statistical analysis of fish size for each treatment
January 31, 2001	Delivery of report describing results of the Pilot Study with chemistry data for pellets and Day 45 fish samples
March 1, 2001	Final chemistry data report.