

**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 HCB (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 anguillarum* 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, HCB, PAHs, and Hylebos sediment extracts increased the susceptibility of juvenile chinook salmon to a pathogenic bacterium, *Vibrio anguillarum*. 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 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 $\mu\text{g/g}$ dry weight (0, 0.05, 0.17, 0.58, 2.0, 6.7 $\mu\text{g/g}$ 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) \approx 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 \pm 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 disease 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.

STANDARD OPERATING PROCEDURES

Methods for Preparation of Test Solutions and Mixtures

Preparation of the test solutions

Analytical grade compounds (Aroclor 1254 and solutions of PAHs and CBDs) will be mixed together in a solution of methylene chloride and added to fish pellets in the proportions described above (in the section Determining Dosages) to generate the dosed food. The pellets will be mixed thoroughly and the methylene chloride will be allowed to dry completely. Individual compounds for these classes are listed in Table 2. Results of the initial Pilot study determined that this method for dosing fish pellets was successful.

Sample Analyses

Analysis of organic chemical concentrations in tissue

Fish liver or whole bodies will be analyzed for chlorinated compounds using the methods described by Krahn et al. (1988) and Sloan et al. (1993). Analytes measured are listed in Table 2. Liver or whole bodies will be extracted by grinding tissue, sodium sulfate, dichloromethane, and surrogate standards with a Tekmar Tissumizer. Tissue extracts are filtered through silica-alumina and concentrated to 1 mL for further cleanup using size exclusion chromatography. The extract is concentrated and exchanged into hexane for analysis using GC/MS for aromatic hydrocarbons and GC with electron capture detection for chlorinated pesticides and hydrocarbons.

Measurements of FACs in bile

FACs including benzo[a]pyrene (BaP), phenanthrene (PHN) and naphthalene (NPH) equivalents in bile will be analyzed by HPLC based on the methods described by Krahn et al. (1986). For each sample, 3-5 μ L of thawed, untreated bile is injected onto the analytical column and eluted with an HPLC linear gradient (flow rate of 0.7 mL/min) beginning with 100% solvent A (water containing 5 ppm acetic acid) to a final composition of 100% solvent B (methanol) during a period of 15 min. After holding the mobile phase at 100% solvent B for 10 min, solvent conditions are returned to 100% solvent A during a period of 3 min. The system is then allowed to re-equilibrate for 10 min at 100% solvent A before the next sample is injected. The total area for all peaks in the region of the chromatogram where FACs are known to elute (> 9 min) are integrated. Quantification of analytes is performed according to Krahn et al. (1986). If the fluorescence response in a sample is sufficiently high that a detector response reaches its maximum (saturated), the sample is re-analyzed using a smaller injection volume.

Lipids

Lipids will be determined with a modified Bligh and Dyer technique (Herbes and Allen 1983) and will be analyzed by Columbia Analytical Services in Kelso, Washington. The SOP for lipid analysis is in Appendix C of the QAP.

Growth Studies for Juvenile Chinook Salmon

Fish collection and maintenance

Fish will be obtained from the Soos Creek Hatchery, which is operated by the Washington Department of Fish and Wildlife. Approximately 6,000 fish will be netted from holding ponds. The fish will be transferred, about 1,000 fish at a time, in a 3000 L tank designed for transporting large numbers of juvenile salmon. The water will be constantly aerated during the one-hour transport to the Mukilteo Field Facility where they will be placed in three 6-foot diameter tanks filled with fresh water. After acclimitizing the transferred fish to the holding tanks at the field facility, the fish will be gradually acclimated to saltwater by constant addition of running seawater to the tanks reaching ambient salinity (28 - 30 ppt) in approximately 48 hours. Thereafter, the fish will be held in running seawater at ambient conditions. Fish will be acclimated for another two to four weeks before the exposure begins.

Distribution and Exposure of fish

Fish and treatments will be randomized within the prescribed area to avoid potentially confounding factors such as light and temperature gradients, differential feeding, and disturbance. Details of this are provided in the SOP on randomization of fish and treatments. The procedure for adding fish to the individual 1,500 L tanks for the pilot study is shown in Figure 2 and described as follows. Individual fish will be weighed, measured, and if within prescribed size and weight parameters (initially determined by measuring a randomly selected 100 individuals), added to 20 L buckets until 100 have accumulated. The size and weight of each fish added to the 20L buckets will be recorded onto a personal computer in the lab via a LabVIEW™ software program (Appendix B). This group of 100 fish will then be added to a tank. The order of adding the fish to the tanks will be done with a random number generator. The purpose of this is to completely randomize the fish in the experimental tanks as they are selected from a common pool of fish. After all of the fish have been dispersed to the 1,500 L tanks, the assignment of treatments and replicates will be accomplished with a random number generator.

Fish will be held for up to 91 days in ambient flowing seawater and fed once daily a ration that is approximately two percent of the estimated biomass of the tank. Biomass will be estimated based on data from previous studies and estimated from the growth rate expected in this work. The tank dimensions will be identical

for each test: 4 ft diameter circular, with a tapered bottom, and containing 1,500 L of seawater. Note: each tank has a capacity of 2,000 L; however, they will only be filled to 2/3 capacity to retain any fish that attempt to jump out of the tanks. Water temperature will be maintained between approximately 10° and 12° C. In-line chillers will be used to ensure the water temperatures do not exceed 12° C. Because water will flow through each tank at 8 liters per minute, no depletion in oxygen is expected. Water will be passed through an ultraviolet sterilizer designed to remove pathogens that may affect the fish. Natural light will determine the light/dark period. Human interaction will be kept at a minimum to reduce stress in the fish. Mortalities will be recorded daily (Monday - Friday) and once during the weekend.

Sample collection

At the end of the test (day 91), all remaining fish from each tank will be weighed and measured for fork length. Some of these fish will be composited into one sample for chemical determination. Chemical sampling will occur at time zero (start of the experiment), at day 45 (the end of the contaminated pellet feeding phase), and on day 91. For tissue residue determination, 5 fish from a selected tank will be composited to produce one composite sample. On day zero, three composites of 5 fish each will be taken from the general pool of fish for tissue residue analysis. Each tank will be sampled on day 45, producing three replicates per treatment. See Table 1 for a list of tanks that will be sampled on Days 45 and 91. Samples will be placed in methylene-chloride rinsed jars and kept frozen at -20° C in a locked freezer for storage until analysis.

Disease Resistance Studies in Juvenile Chinook Salmon

Determination of lethal concentrations (LC) of *Vibrio anguillarum*.

Lethal concentrations (LC) of *V. anguillarum* for juvenile chinook salmon will be determined as described in a previous study at the Northwest Fisheries Science Center (Arkoosh et al. 1998). This information was used to determine the appropriate *V. anguillarum* concentrations for use in the disease resistance portion of Round II studies.

Growth curve determination of *V. anguillarum*.

A growth curve for *V. anguillarum* strain 1575 at 20°C will be determined to ensure that at the time of challenging the salmon, the bacteria are approaching the peak of their exponential growth phase. In brief, 2 ml of the stock culture will be placed into 500 ml TSB supplemented with 0.5% NaCl and placed on a shaker at 20°C. After the initial 9 hours of culturing, 2.0 ml aliquots will be removed every hour from the 500 ml of bacterial suspension and the turbidity of the culture determined with a UV-VIS recording spectrophotometer (Shimadzu Scientific Instrument, Columbia, MD) at a wavelength of 525 nm until just after the beginning of the stationary phase.

Infection of salmon with *V. anguillarum*

Selected Juvenile chinook salmon will be exposed to two concentrations of *V. anguillarum* (LC30 and LC50) at the end of the 45-day contaminant dosing. The disease challenge studies will be performed in tandem with fish exposed to the treatment and control diets. A non-feeding period not to exceed 24 hours will be imposed just prior to the disease challenge. Bacteria will be grown to an optimal optical density prior to use, and duplicate tanks of 20 fish per tank will be exposed to the bacteria. Duplicate control tanks with fish that were not exposed to bacteria will also be established. Salmon will be placed in 2-gallon buckets with 4 L of seawater containing the bacteria. The fish will be exposed to the bacteria for 1 hour with constant aeration. After the 1 hour exposure, the salmon will be immediately placed back into their respective tanks. Mortalities will be collected daily for the duration of the experiment, which is up to 14 days.

Necropsy

Necropsies will be performed on one out of three mortalities to ensure that the dead fish had been infected with *V. anguillarum*. The dead fish will be sprayed with 75% ethanol. A small incision will be made ventrally with a sterile scalpel blade taking care not to damage any of the internal organs. A sterile loop will be inserted into the kidney and then aseptically struck onto a TSA plate supplemented with 3.0% NaCl. Bacterial colonies from the will then be examined for sensitivities to the vibriocidal agents novobiacin™ (Bio-Whittaker, Walkersville, MD) and O129™ (Sigma Chemical Co. St. Louis, MO). Salmon will be considered to have died from exposure to *V. anguillarum* if both novobiacin and O129 inhibit the bacterial growth.

QUALITY ASSURANCE PROCEDURES

Quality assurance procedures will be followed to monitor (1) the performance of the measurement systems in order to maintain statistical control and provide rapid feedback so that corrective measures can be taken before data quality is compromised and (2) verify that reported data are sufficiently complete, comparable, representative, unbiased, and precise so as to be suitable for their intended use.

Analysis of Dosed Fish Pellets

The Aroclor 1254, PAHs, and CBD amended fish pellets will be analyzed to determine the type and concentration of chlorinated hydrocarbon compounds present. One sample of each dose will be analyzed at the beginning of the dose-response range-finding experiments. Three of the treatments will be analyzed in duplicate. Pellets will be kept at -20° C to avoid degradation of the contaminants.

Growth Studies

Balances will be calibrated at the beginning of each measuring session and all weight and length measurements, feeding, and routine husbandry will be done by the same group of persons. Water quality will be monitored routinely at the laboratory facility. Temperature will be determined continuously. Salinity, pH, and oxygen content of the seawater will be measured at least three times per week. Tanks will be cleaned every other day to remove uneaten pellets and feces.

Disease Resistance Studies

Two control treatments will be conducted to determine background levels of mortality due to exposure to *V. anguillarum* (Table 3). One control treatment will include contaminant-fed fish not exposed to the bacteria, the second control treatment will consist of fish fed with the solvent treated pellets. The three *V. anguillarum* treatments to be tested for each PCB or CBD dose will consist of 20 fish/tank with two tanks/treatment. Every third mortality will be examined biochemically and microscopically to ensure that the deaths are from exposure to the pathogen *V. anguillarum*. Water quality will be monitored to assure that the water-flow and UV sterilization systems are working properly. The same individual will supervise the all phases of the disease challenge study.

Chemical and Biochemical Analyses

Chemical analyses of whole-body and liver

The quality assurance criteria are listed in detail in the Quality Assurance Plan (QAP). The QAP contains sections that address the assessment of data quality, the quality control procedures, data reduction methods, and the procedures for corrective action, if needed. A detailed description of the GC/MS method for analysis of organic analytes is presented in Appendix A of the QAP.

Analysis of Bile FACs

The quality assurance criteria for bile are listed in Table 2 of the QAP. Quality control procedures include analyses of High Pressure Liquid Chromatography (HPLC) calibration standards (CS), method blanks, replicates, and the bile reference materials (BRM). If the RSD for any analyte in the initial calibration is > 15% or in the continuing calibration is > 25% (during the period of analyzing a sample set), corrective maintenance will be performed and the bile samples re-analyzed. One method blank is analyzed at the beginning of each set of samples. A bile reference material (BRM) is also analyzed in duplicate with each set of samples. The concentrations of FACs from each analysis of the BRM are then compared to the results from an interlaboratory quality assurance exercise which included two laboratories from the Environmental Conservation Division and one laboratory from Texas A & M University (Krahn et al. 1991). If the BRM concentrations vary by more than ± 2 standard deviations from the historical mean of previously analyzed BRMs, corrective action is taken, including instrument maintenance or repair and re-analysis of samples. One replicate bile sample is analyzed for each group of 20 samples.

Lipid Analysis

Standard materials of known lipid content will be analyzed concurrently to assure accurate determinations. The instruments used to make lipid determinations will be calibrated before each use. See the QAP for details on the method of analysis for lipids and the quality control procedures and criteria.

Chain of Custody Procedures

Chain of custody procedures will be used for all samples and for all data and data documentation, whether in hard copy or electronic format. For samples collected as part of the dose-response study, each container is considered to be an individual sample and is assigned a unique ID number. A sample is considered in "custody" if: a) it is in the custodian's actual possession or view, b) it is retained in a secured place (under lock) with restricted access, or c) it is placed in a container and secured with an official seal(s) such that the sample cannot be reached without breaking the seal(s).

Samples are kept in the custody of designated sampling personnel until transfer to the laboratory. The original signed and dated chain of custody record accompanies the sample(s). The laboratory sample custodian or designee maintains a laboratory sample-tracking record, similar to the chain of custody record, that will follow each sample through all stages of laboratory processing.

All unanalyzed samples and unutilized sample aliquots or extracts are held by the laboratory in a manner to preserve sample integrity at a secure location with chain of custody procedures for one (1) year after the QA Contractor has validated the data package for that particular set of samples.

All data and data documentation, whether in hard copy or electronic format, is the responsibility of the QA Coordinator acting on behalf of Counsel to the Case Management Team. These materials will all be clearly marked with "Attorney Work Product."

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Table 2. List of Organic Analytes

Detailed Organic Analyses (Food, liver tissue, and whole bodies of chinook juvenile salmon will be analyzed)

PAHs

2-methylnaphthalene, acenaphthene, fluorene, anthracene, phenanthrene, pyrene, fluoranthene, chrysene, benz[a]anthracene, benzo[a]pyrene,

PCBs

PCB congeners (Nos. 18, 28, 44, 52, 66, 101, 105, 118, 128, 138, 153, 170, 180, 187, 195)

Chlorobutadienes (pentachloro-, and hexachlorobutadiene).

Figure 1.—Schematic Chart of Round III Pilot Study Design

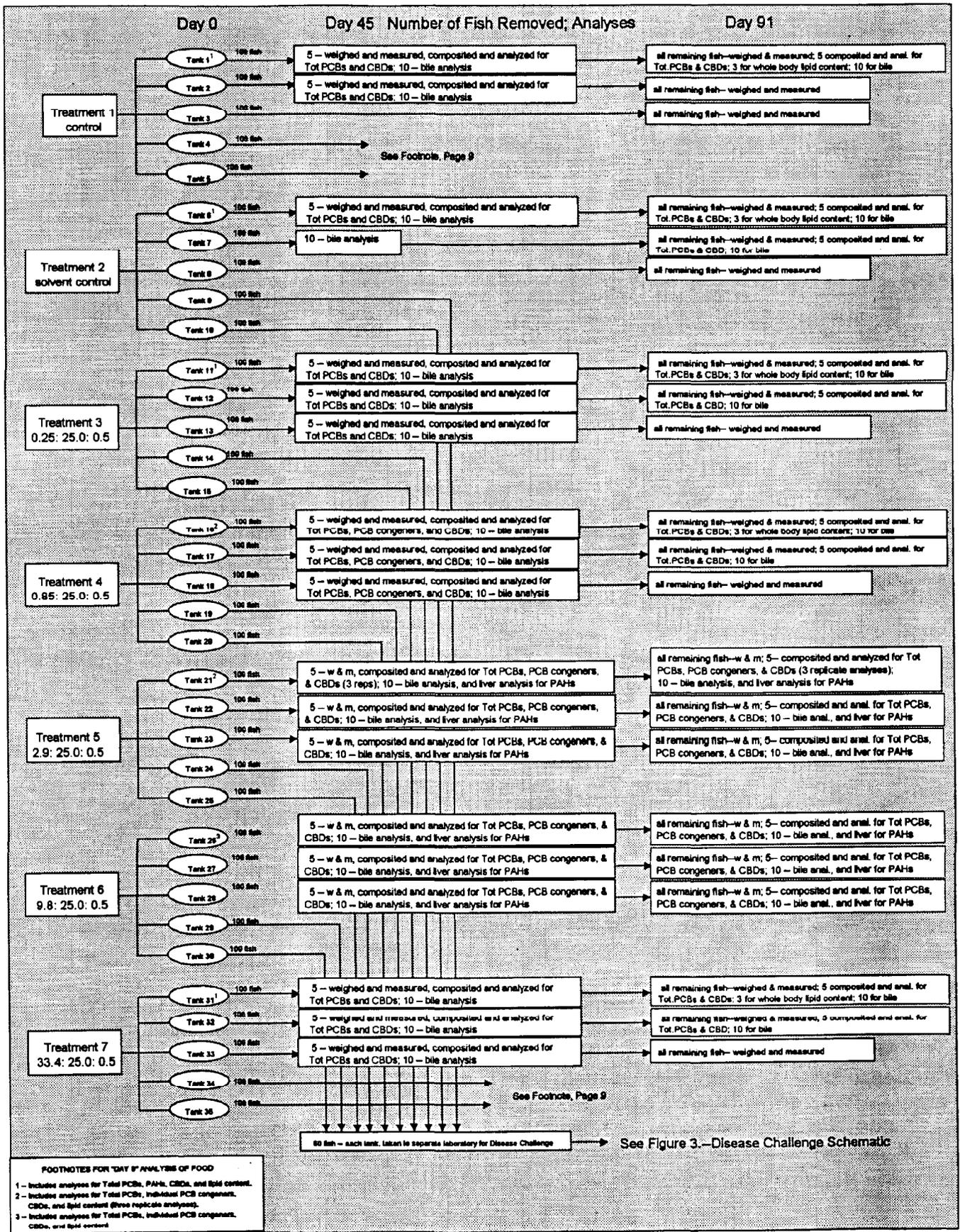


Figure 2. -Schematic chart describing how test tank populations are obtained for the Round III Pilot Study.

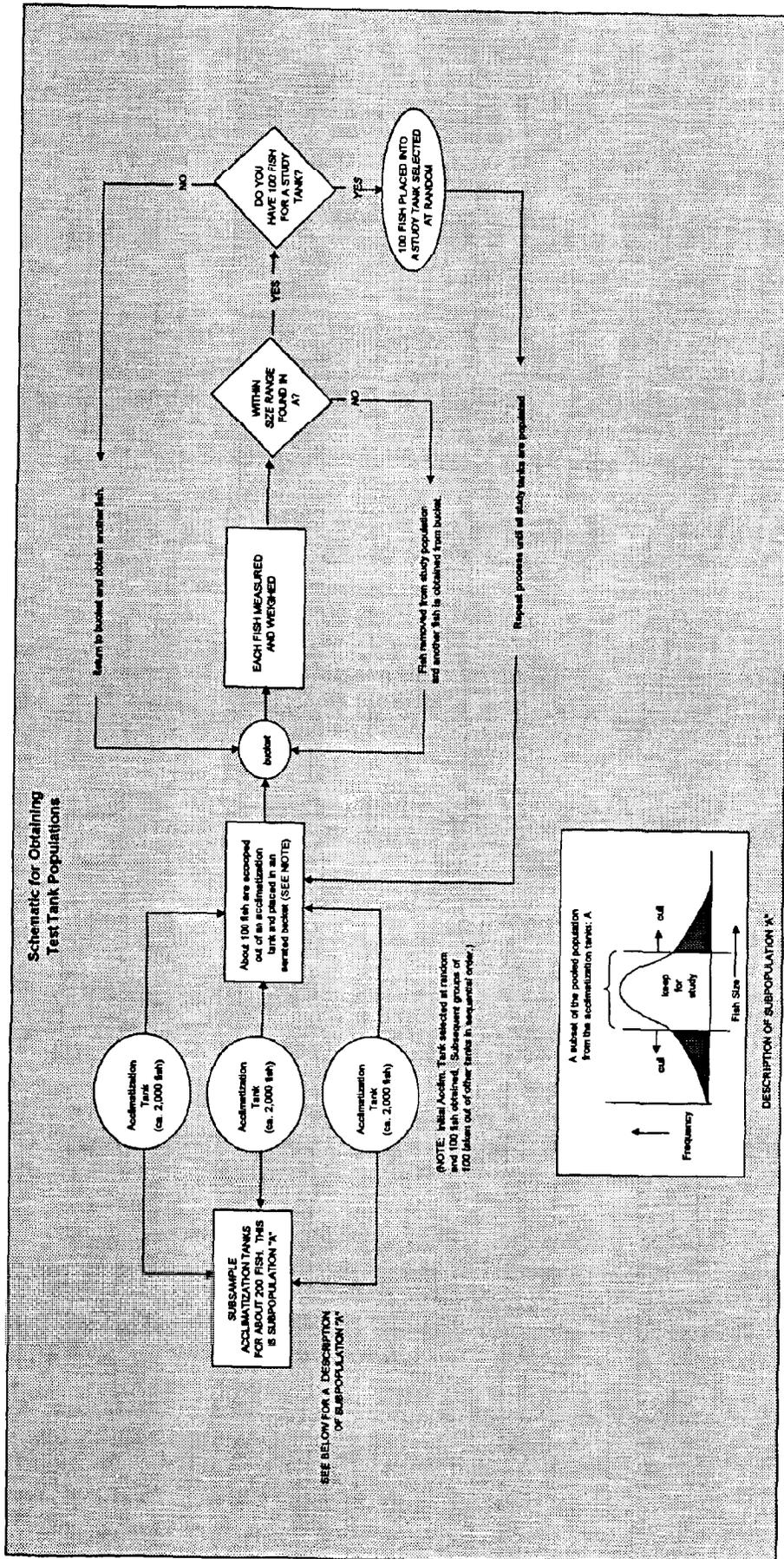
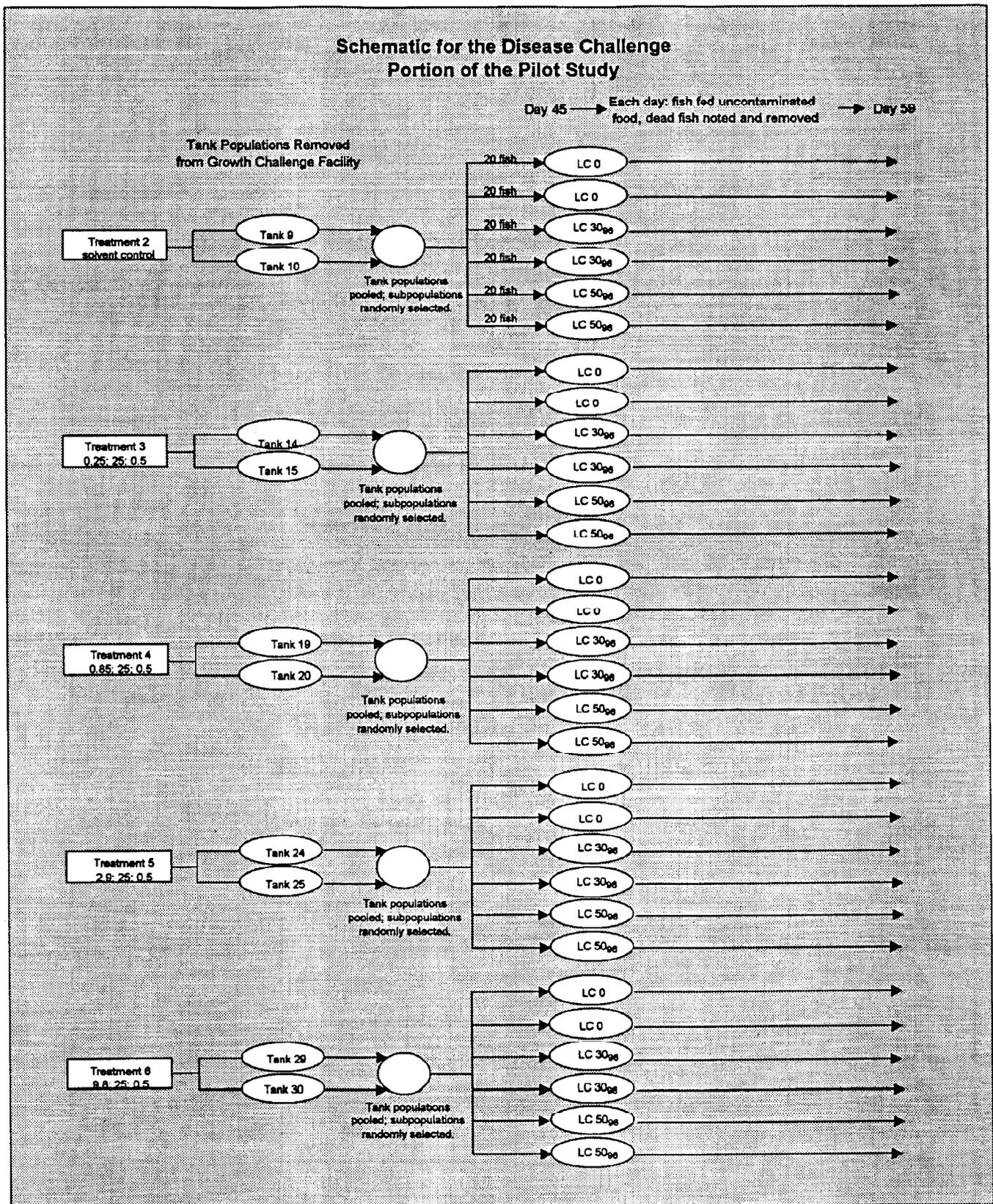


Figure 3.—Schematic diagram of the disease challenge portion of the Round III Pilot Study





UNITED STATES DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
NATIONAL MARINE FISHERIES SERVICE

Northwest Fisheries Science Center
Environmental Conservation Division
2725 Montlake Boulevard East
Seattle, WA 98112

March 26, 1999

MEMORANDUM FOR: NORCAx11 - Rob Wolotira
FROM: F/NWC5 - John E. Stein *John E. Stein*
SUBJECT: Hylebos Waterway Fish Injury Assessment - Salmon
Laboratory Studies Round III Pilot Study

This is the data report for the Hylebos Waterway Fish Injury Assessment - Salmon Laboratory Studies Round III Pilot Study as described in the Sampling and Analysis Plan (SAP) of October 1998 and recommendations for conducting pilot study 2. The goals of the Hylebos Waterway Fish Injury Pilot Study were to (1) document the palatability of the low-fat pellet that we had made for delivering the contaminants to juvenile fish and (2) to ascertain that those contaminants were bioavailable when administered in this fashion. These essential pieces of information will be used for planning and executing both the second pilot study and the proposed comprehensive study.

Experimental methods

Fish were fed contaminated fish pellets for up to 28 days; a period representative of the residence time for outmigrant juvenile salmon in river systems/estuaries such as the Hylebos Waterway. The time period for exposure was variable because of a protozoan parasite (*Cryptobia*) that caused premature death in the fish.

Following contaminant exposure, the fish were sacrificed for chemical analysis. Once the analyses were completed, bioaccumulation of the contaminants was assessed in whole fish and compared to the known amount fed to fish. Details of the chemical analyses are provided in the Quality Assurance Plan (QAP), which was submitted previously (Jan 1999).

The polychlorinated biphenyls (PCBs) and chlorobutadienes (CBDs) were measured in the pellets and fish tissue, and the data presented here are evidence of the palatability of the pellets and the degree to which the contaminants were bioaccumulated. PAHs are rapidly metabolized in fish; hence measurement in tissue represents only a small fraction of that taken up. The PAHs were measured



in a select group of fish for comparison to control values to determine if bioaccumulation occurred from the pellets. The PAH parent compounds were measured in the fish pellets, allowing for a comparison to PAHs in tissue. Whole-body PCB determinations were conducted for all samples and a select number of samples (both replicates for two treatments) were analyzed in detail for 17 individual PCB congeners. Fish tissue was analyzed for CBDs from those treatments that had CBDs in the food.

In this study, we examined 9 doses and two controls (Table 1), which are representative of the range of doses planned for the comprehensive study. The contaminant dosages (PCBs, PAHs, and CBDs) were assessed in this pilot study in the same proportions as those proposed for the comprehensive study. At the end of the exposure period, five fish were taken from each tank replicate to form 1 composite sample per tank for chemical analysis. Therefore, each contaminant dose has two chemical determinations for the sampling period.

The food and fish samples were analyzed as described in the QAP of January 1999. Briefly, aliquots of the food samples and the fish composites were solvent extracted, and the analytes were isolated by fractionating the extract using size-exclusion high-performance liquid chromatography. The analytes were quantified using gas chromatography/mass spectrometry with sequenced selected ion monitoring. All samples were analyzed for Aroclor 1254; selected samples were also analyzed for selected aromatic hydrocarbons, chlorinated butadienes, and/or selected individual polychlorinated biphenyl congeners. Quality control data for each batch of food and fish samples were acquired and evaluated according to the QAP, and the results are discussed in the enclosed Case Narrative. In addition, the SRM 1974a and the trout control material were used to validate the analytical method (see case narrative).

Results and Discussion

The quality of the chemical analyses was established by an initial demonstration of proficiency (see appendix) and by each sample batch's quality control data, all of which met the minimum quality control criteria in the QAP, as described in the case narrative (attached). The measured concentrations of the PCBs and PAHs applied to the pellets were generally very close to the nominal concentration (Table 2). The measured concentrations for the chlorobutadienes (CBDs) were consistently low compared to the nominal concentrations. Measured CBD concentrations were 37 – 38% of nominal, and if corrected for recovery, which averaged 67% for these compounds, the measured to nominal ratio would be 55 to 57%. The CBDs are likely more volatile than the PCBs, hence some of the applied material was probably lost when the solvent that was used to apply the compounds was evaporated. Such a loss does not affect our ability to conduct an experiment with CBDs added to feed at the desired dose.

The fish acquired very high concentrations of PCBs and much less of the PAHs and CBDs (Table 3). We know that the PAHs are extensively metabolized,

and the results in Table 3 are consistent with that finding. Very little is known about the disposition of CBDs in fish tissue. The PCBs were accumulated in a highly linear fashion from the fish pellets (Figure 1), producing a slope of 0.86. The CBDs were also accumulated in a dose-dependent fashion (Figure 2); however, the slope was 0.10. A comparison of this lower slope to the one for PCBs indicates either lower assimilation of CBDs from the pellets, higher metabolism, or a combination of both factors. Even though the exposure time was slightly variable between tanks, there appears to be no effect on the linear regression line for bioaccumulation.

The reason for the elevated PCBs in the solvent-control food is unknown. The PAH and CBD concentrations in food do not show the same large differences between the non-solvent and solvent controls. Moreover, because the PCB concentrations measured in fish tissue for the non-solvent and solvent controls were the same, this indicates that the solvent control food was not contaminated when it was being fed to fish. The elevated concentration in the solvent control food may have come during the sampling or analysis phases of this study. Future studies will have sample replicates that will allow detection of this type of anomalous result.

Data acquired and analyzed as described in the SAP is used here to address the following questions:

1. **Was the low-fat pellet palatable to juvenile salmon and does it allow them to thrive?** The experimental fish lived on the low fat pellets for three months in the laboratory. One month of this period was during the exposure phase of the experiment. No significant rejection of the pellets was observed.
2. **Are the contaminants (PCBs, PAHs, and CBDs) that were applied to the fish pellets and fed to fish accumulated in the tissues?** Figures 1 and 2 show a very high correlation between the dose of PCB or CBD in the food and the concentrations measured in whole fish. The PAHs show very little bioaccumulation and the results were somewhat variable over treatments. A few samples contained PAHs in the 1 $\mu\text{g/g}$ range, which may have been due to small amounts of food remaining in the stomachs of the fish sampled.

Based on the hydrophobicity of the PAHs, the uptake efficiency of the parent PAHs was probably similar to that observed for the PCBs, but extensive metabolism converted most of the parent compounds in the tissues to metabolites that are excreted. We have proposed to analyze bile for PAH metabolites to assess uptake; however, because of limited numbers of fish available in this study, we did not analyze bile.

3. **Are the tissue concentrations found in juvenile salmon fed contaminated pellets as predicted based on how much was fed to the fish?** Based on the mean fish weight of 20 grams and the amount of food received per day (3% body weight), the amount accumulated over the 28 day period was as predicted. For example, a 20 gram fish receiving 0.6 grams of fish pellets per day (3% body wt.) that contained 10.8 $\mu\text{g/g}$ PCBs, would have received 6.5 μg of PCBs per day. Over 30 days, the total amount would be 194.4 μg . This concentration of PCBs in a 20 gram fish (= 4 grams dry wt.) would equal almost 48.8 $\mu\text{g/g}$ dry weight. Reported assimilation efficiencies for PCBs in these types of studies range from 25% to 75%. If we assume an uptake efficiency of 25%, then the fish eating pellets with 10 $\mu\text{g/g}$ PCBs would be expected to have a whole body dry weight concentration of approximately 12 $\mu\text{g/g}$, which is very close to the proportion measured. We noticed during this study that not all the food given to the fish at this ration was consumed. Considering that the fish may not have eaten all of their allotment of food and the uptake efficiency is not known, the tissue concentrations can not be predicted precisely, but generally fall within the range of expected concentrations.

Conclusions

This study demonstrates that we are able to dose a low-fat pellet at desired concentrations and that the fish will consume these pellets and accumulate contaminants in their tissues in a dose-dependent fashion. The data from this pilot study on bioaccumulation provide the following recommendations for the proposed pilot study 2 and the comprehensive study that will assess the dose-response relationship between tissue residues and biological effects for these contaminants of interest.

Recommendations:

1. Maintain the dosing scheme for PCBs and PAHs.
2. Measure bile for metabolites of PAHs.
3. Adjust dosage of CBDs in the fish pellets to produce the desired tissue residue.
4. Assure that no food is present in the stomachs by dissecting the stomachs at the time of sampling.

cc: F/NWC5 – J. Meador
F/NWC5 - T. Collier
F/NWC5 - P. Krahn

Table 2. Summary results of concentrations in fish pellets

Treatment	Nominal food conc (ng/g dry wt.)			Measured food conc (ng/g dry wt.)				
	Tot PCBs	Tot PAHs	Tot CBDs	1254	Tot PAHs	HCBD	PCBD	Tot CBDs
1	control			24	33			
1	control			24	33			
2	solvent			420	70			
2	solvent			420	70			
3	1200			1500	70			
3	1200			1500	70			
4	10800			12000	42			
4	10800			12000	42			
5	1200	25000		1200	24150			
5	1200	25000		1200	24150			
6	3600	25000		4100	24850			
6	3600	25000		4100	24850			
7	10800	25000		13667	24680			
7	10800	25000		13667	24680			
8	50000	25000		64000	25320			
8	50000	25000		64000	25320			
9	1200	25000	1000	1200	23060	140	230	370
9	1200	25000	1000	1200	23060	140	230	370
10	1200	25000	4000	1333	24850	580	923	1503
10	1200	25000	4000	1333	24850	580	923	1503
11	1200	25000	16000	1100	21290	2300	3800	6100
11	1200	25000	16000	1100	21290	2300	3800	6100

Table 3. Summary results of concentrations in whole fish

Treatment	Nominal food conc (ng/g dry)			Measured fish conc (ng/g dry wt.)				
	Tot PCBs	Tot PAHs	Tot CBDs	1254	Tot PAHs	HCBD	PCBD	Tot CBDs
1	control			140	0			
1	control			150	19			
2	solvent			170	0			
2	solvent			140	16			
3	1200			1400	0			
3	1200			1500	0			
4	10800			9000	0			
4	10800			13000	0			
5	1200	25000		1200	0			
5	1200	25000		1300	193			
6	3600	25000		3300	478			
6	3600	25000		3200	80			
7	10800	25000		10000	26			
7	10800	25000		7600	1218			
8	50000	25000		55000	214			
8	50000	25000		55000	55			
9	1200	25000	1000	1500	1041	42	24	66
9	1200	25000	1000	720	159	25	10	35
10	1200	25000	4000	1500	494	120	70	190
10	1200	25000	4000	1400	976	130	84	214
11	1200	25000	16000	1100	568	350	193	543
11	1200	25000	16000	850	737	480	280	760

Figure 1. PCBs in food and whole fish

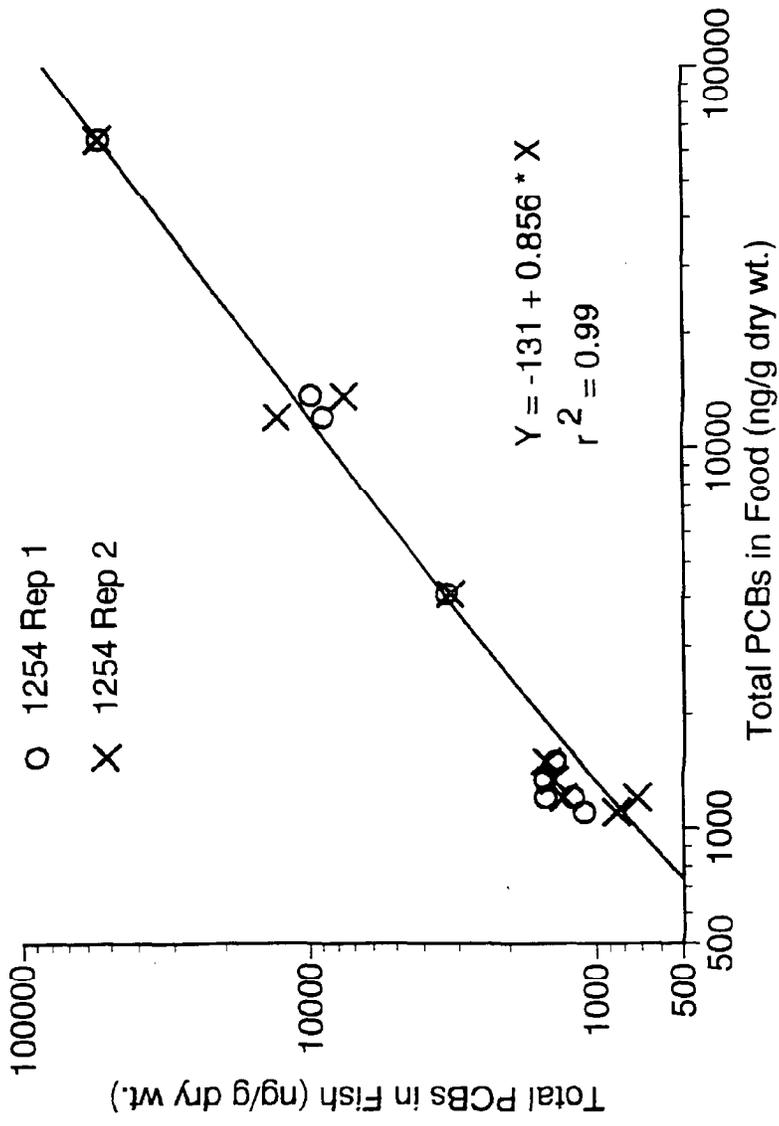


Figure 2. CBDs in food and whole fish

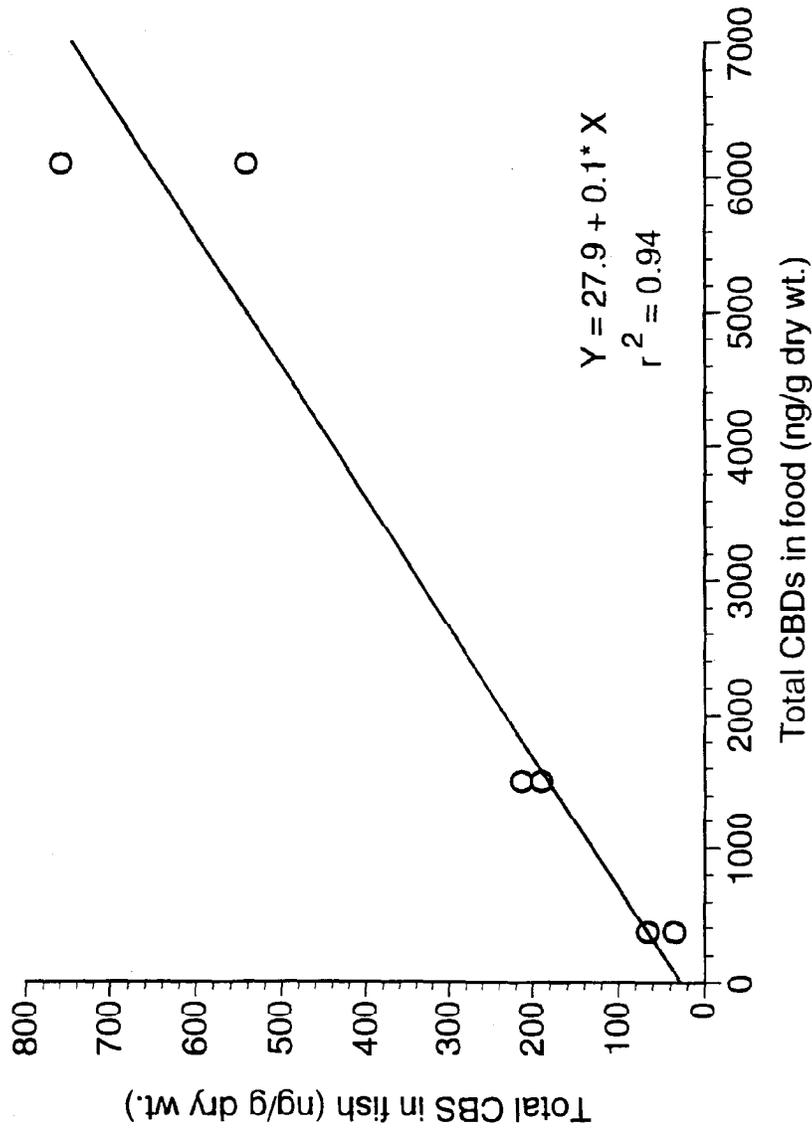
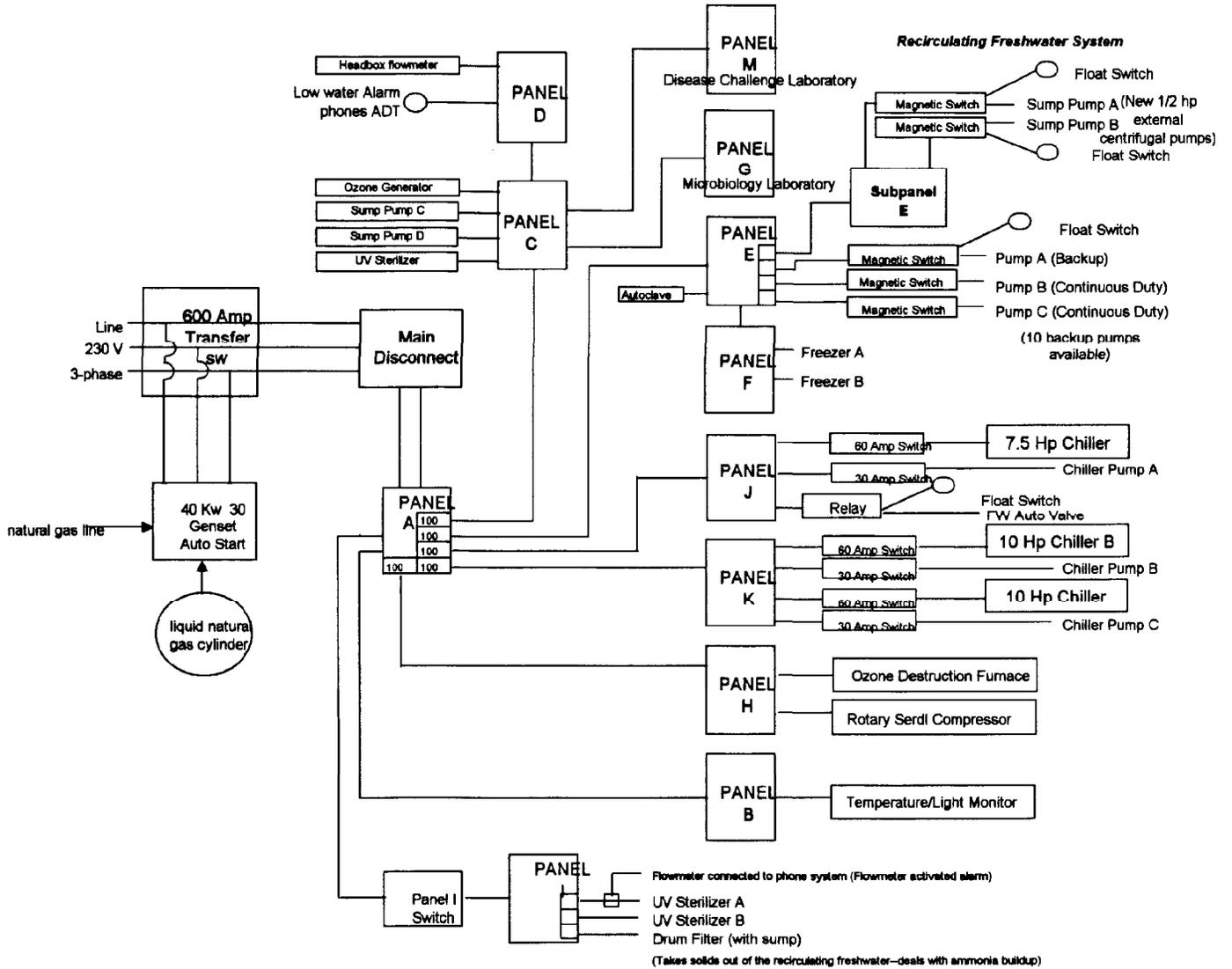
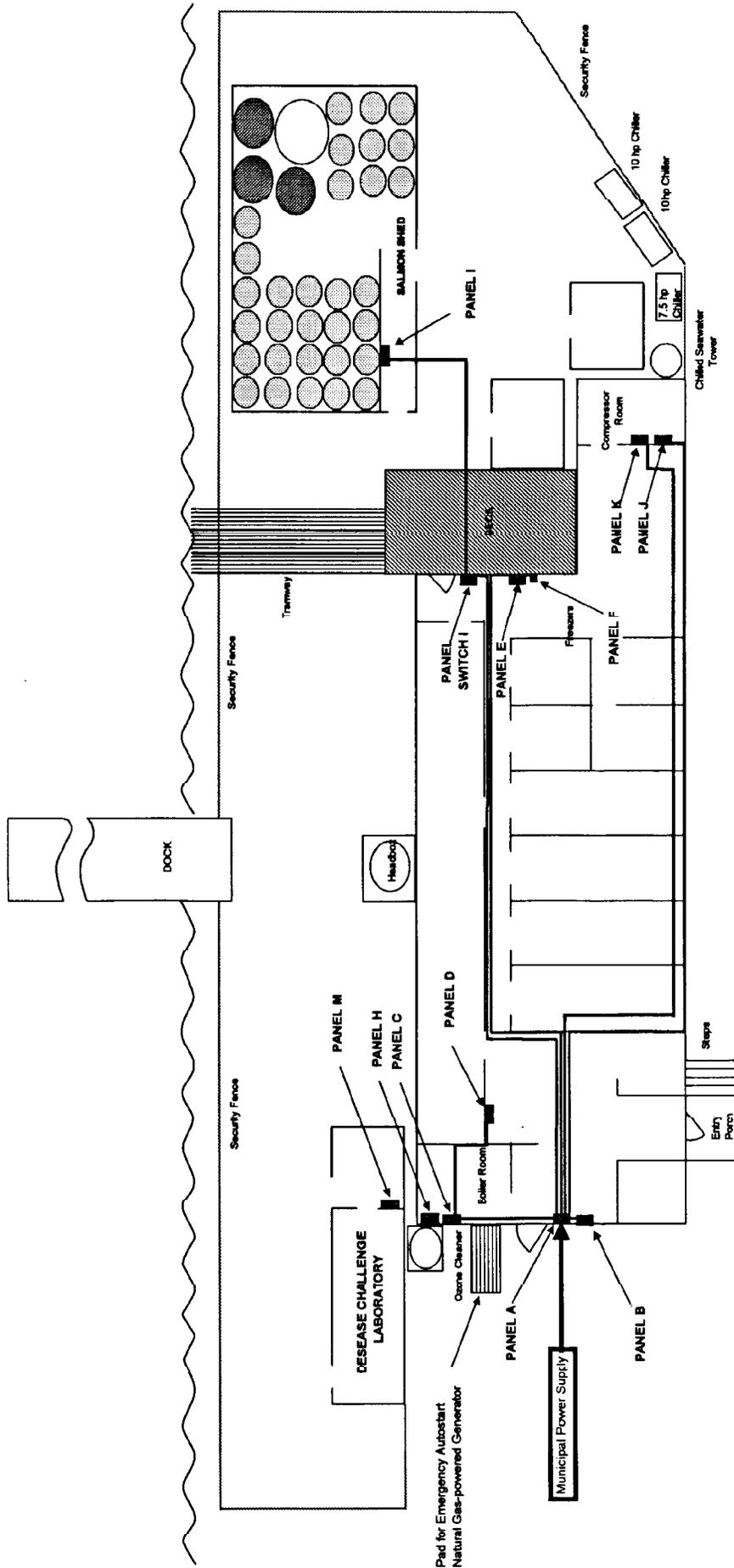


Diagram of the Mukilteo Facility Electrical System Associated with the Round III Pilot Study



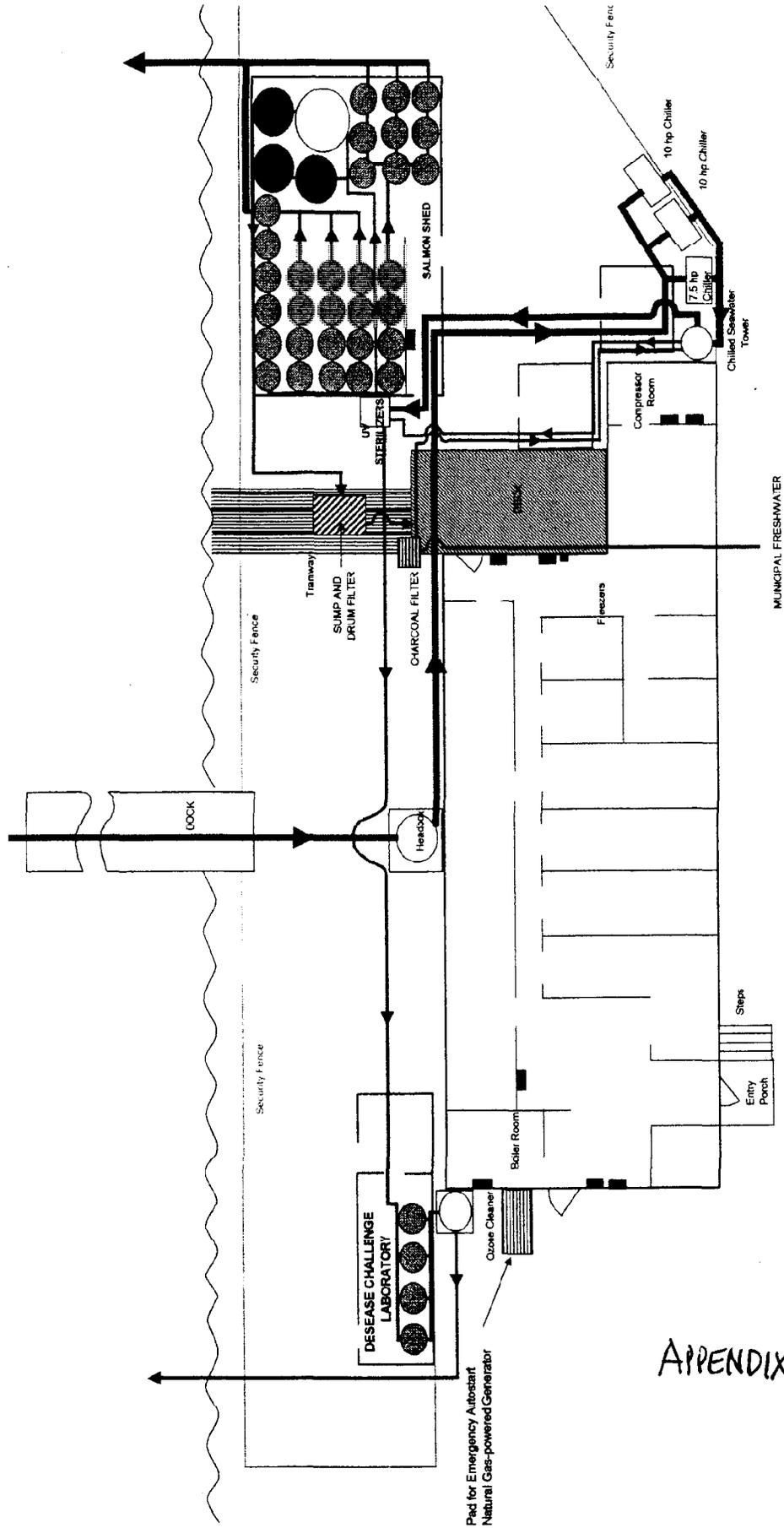
APPENDIX B

Schematic Diagram of the Electrical System Associated with the Round III Pilot Study

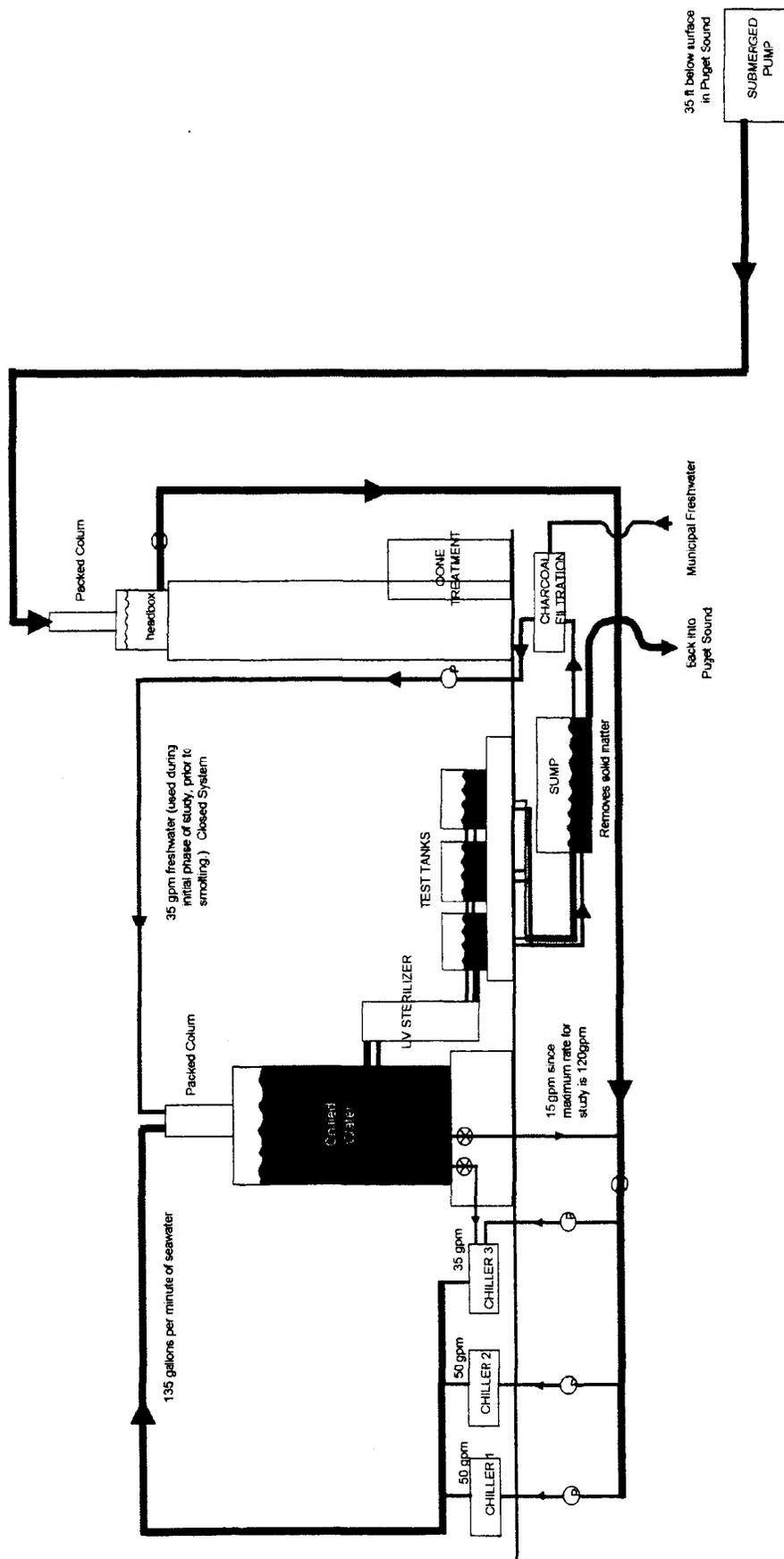


APPENDIX C

Schematic Diagram of the Circulating Water Systems Associated with Round III Pilot Study



APPENDIX D



Waterflow Schematic Diagram for the Round III Pilot Study at the NMFS NWFSC Mukilteo Facility