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 µL 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 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 I hour with constant aeration. After the I 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 1. Summary of doses and chemical analyses associated with the Hylebos Waterway Round III Pilot Study.

				Nominal in food (µg/g)			Food Day 0				Fish Day 45				Fish Day 91			
							PCBs				PCBs				PCBs			
Treat	Type	Tank	Contaminant	PCBs	PAHs	CBDs	Tot	cong	PAHs	CBDs	Tot	cong	PAHs	CBDs	Tot	cong	PAHs	CBDs
1	gr	13	control				X		X	X	X		X	X	X		X	X
1	gr	21	control								X		X	X	X		X	X
1	gr	30	control								X		X	X				
2	gr	25	solvent control				X		X	X	X		X	X	X		X	X
2	gr	19	solvent control								X		X	X	X		X	X
2	gr	14	solvent control								X		X	X				
2	dc	3**	solvent control															
2	dc	9	solvent control															
3	gr	22	PCBs, PAHs, & CBDs	0.25	25	0.5	X		X	X	X		X	X	X		X	X
3	gr	29	PCBs, PAHs, & CBDs	0.25	25	0.5					X		X	X	X		X	X
3	gr	16	PCBs, PAHs, & CBDs	0.25	25	0.5					X		X	X				
3	dc	1**	PCBs, PAHs, & CBDs	0.25	25	0.5												
3	dc	8	PCBs, PAHs, & CBDs	0.25	25	0.5												
4	gr	11	PCBs, PAHs, & CBDs	0.85	25	0.5	X*	X*	X*	X*	X*	X*	X*	X*	X	X	X	X
4	gr	23	PCBs, PAHs, & CBDs	0.85	25	0.5					X	X	X	X	X	X	X	X
4	gr	26	PCBs, PAHs, & CBDs	0.85	25	0.5					X	X	X	X				
4	dc	2	PCBs, PAHs, & CBDs	0.85	25	0.5					ψ		ψ/Φ	ψ				
4	dc	7**	PCBs, PAHs, & CBDs	0.85	25	0.5					ψ		ψ/Φ	ψ				
5	gr	15	PCBs, PAHs, & CBDs	2.9	25	0.5	X	X	X	X	X	X	X	X	X*/ψ	X*	X*/ψ*/Φ*	X*/ψ
5	gr	31	PCBs, PAHs, & CBDs	2.9	25	0.5					X	X	X	X	X/ψ	X	X/ψ/Φ	X/ψ
5	gr	24	PCBs, PAHs, & CBDs	2.9	25	0.5					X	X	X	X				
5	dc	6**	PCBs, PAHs, & CBDs	2.9	25	0.5					ψ		ψ/Φ	ψ				
5	dc	4	PCBs, PAHs, & CBDs	2.9	25	0.5					ψ		ψ/Φ	ψ				
6	gr	18	PCBs, PAHs, & CBDs	9.8	25	0.5	X	X	X	X	X	X	X	X	X/ψ	X/ψ	X/ψ/Φ	X/ψ
6	gr	17	PCBs, PAHs, & CBDs	9.8	25	0.5					X	X	X	X	X/ψ	X/ψ	X/ψ/Φ	X/ψ
6	gr	28	PCBs, PAHs, & CBDs	9.8	25	0.5					X	X	X	X				
6	dc	5	PCBs, PAHs, & CBDs	9.8	25	0.5					ψ		ψ/Φ	ψ				
6	dc	10**	PCBs, PAHs, & CBDs	9.8	25	0.5					ψ		ψ/Φ	ψ				
7	gr	20	PCBs, PAHs, & CBDs	33.4	25	0.5	X		X	X	X		X	X	X/ψ	X/ψ	X/ψ/Φ	X/ψ
7	gr	12	PCBs, PAHs, & CBDs	33.4	25	0.5					X		X	X	X/ψ	X/ψ	X/ψ/Φ	X/ψ
7	gr	27	PCBs, PAHs, & CBDs	33.4	25	0.5					X		X	X				
			Dataset Totals (liver and whole body)				9	5	9	9	29	11	29	29	22	14	24	22

* Three replicate analyses of one composite.

** Tanks from which three fish are taken and composited for whole-body lipid content on Day 45.

OTHER TABLE NOTES

--Type: gr = growth study, dc = disease challenge.

--Three of the five replicates in each treatment will be for the growth study. Two of the five replicates for Treatements 2, 3, 4, 5, and 6 will be used for the disease challenge study.

--X= whole fish for tissue chemistry, Φ = bile samples, and ψ are liver samples.

--All chemical analyses are performed on samples derived from composites of 5 fish.

--Day 0 samples for tissue chemistry are 3 composites from common pool of fish, and analyzed for whole-body lipid content, total PCBs, individual congeners (cong), PAHs and CBDs in tissue.

--Bile samples: the three composites on Day 0 are from the common pool of fish, the 6 on Day 45 are each from the two disease challenge tanks of three treatments (4-6), and the 6 composites on Day 91 are each from two growth study tanks of three treatments (5-7).

--Food pellets from all treatments will be chemically analyzed on Day 0 and Day 45.

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

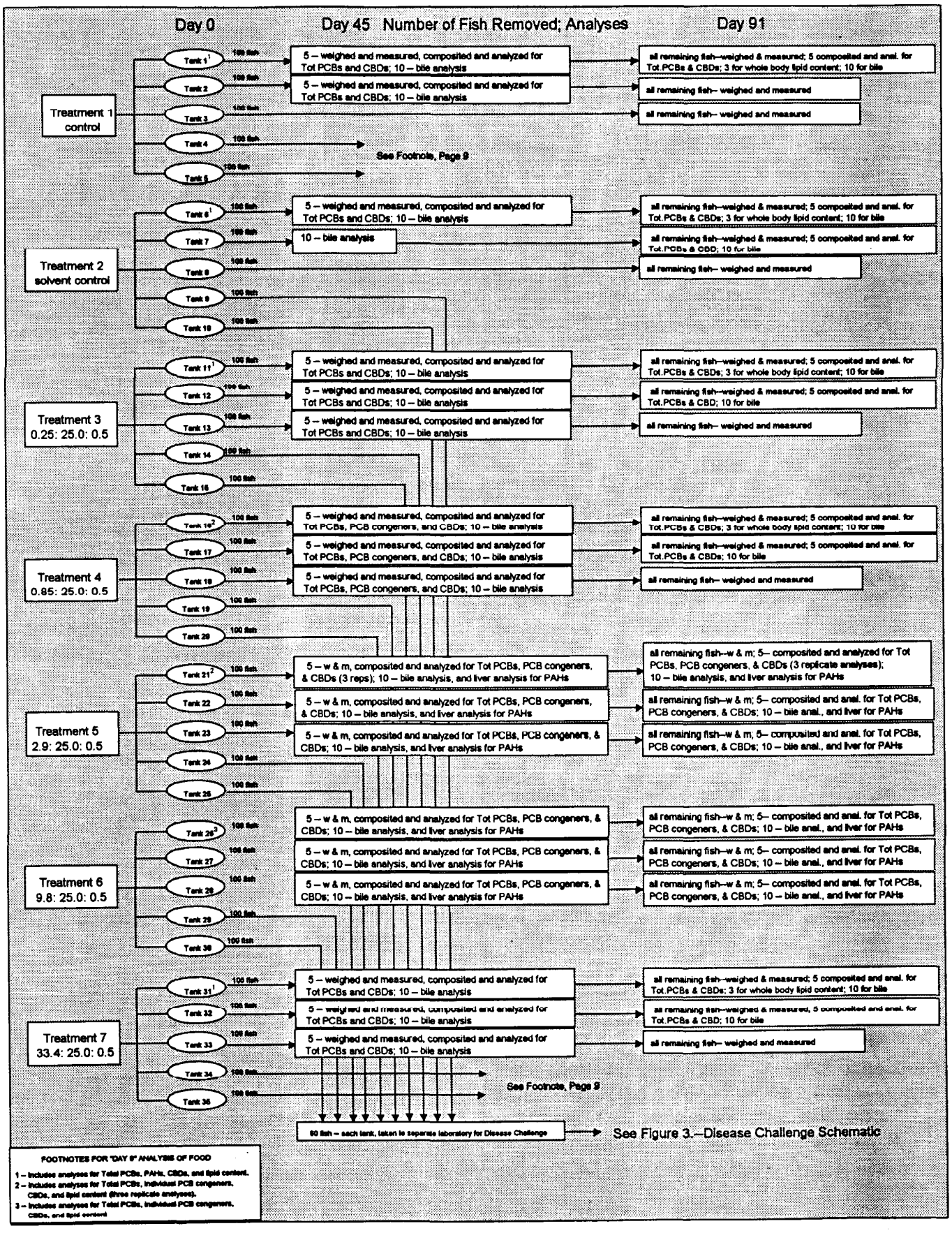

Figure 2.–Schematic chart describing how test tank populations are obtained for the Round III Pilot Study.

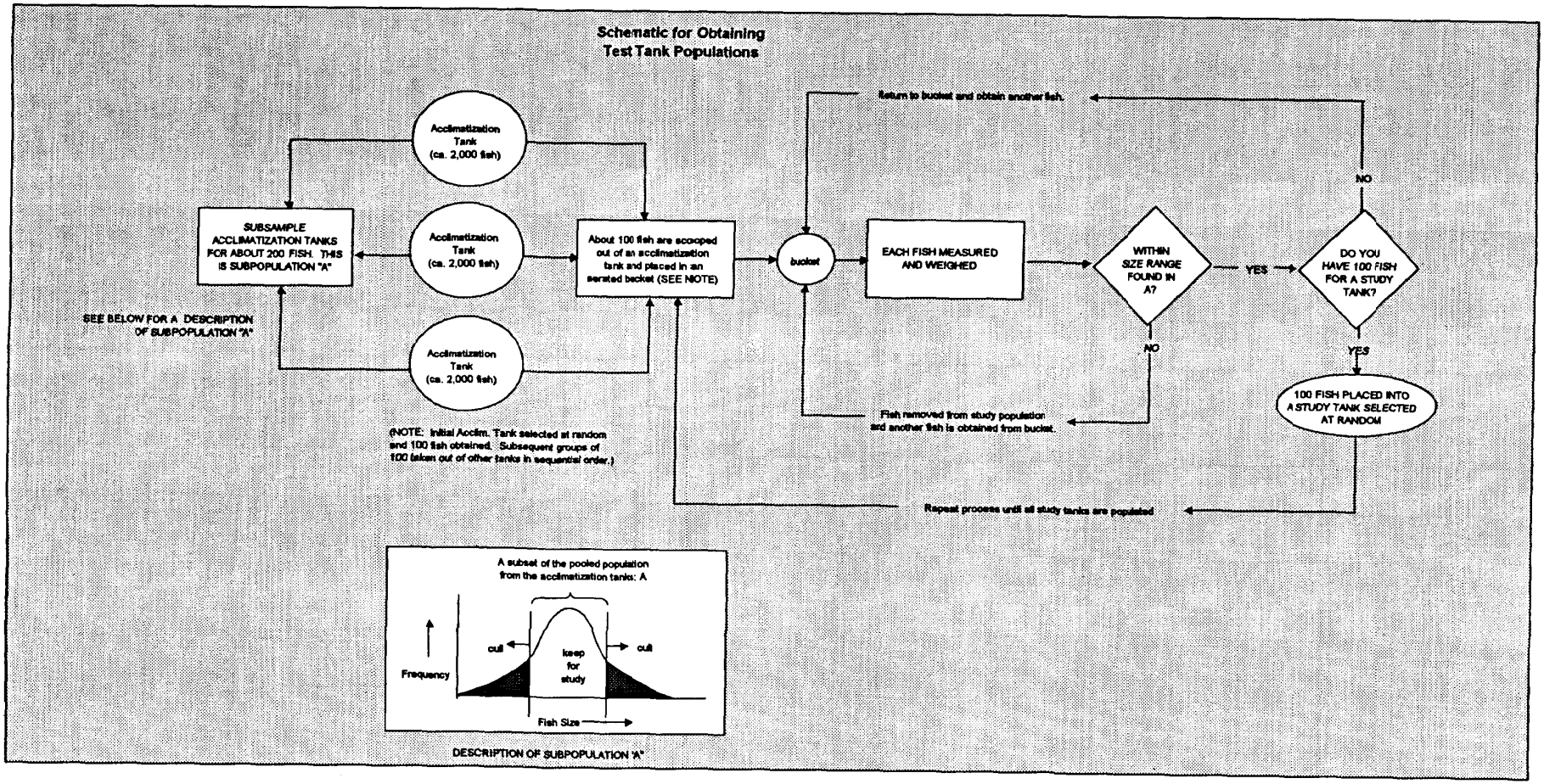

Figure 3.--Schematic diagram of the disease challenge portion of the Round III Pilot Study