APPENDIX A
SAMPLING AND ANALYSIS PLAN
FOR
HYLEBOS WATERWAY FISH INJURY STUDIES

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Project Background and Objectives

Previous investigations in the Hylebos Waterway and other waterways in Puget Sound with similar suites and levels of contaminants have identified a variety of contaminant-related injuries in indigenous fish species. In the early 1980s, elevated levels of a variety of toxic chemicals were found in tissues of English sole and rock sole from the Hylebos Waterway. These fish species also had fin erosion and a variety of liver lesions, including liver tumors (Malins et al. 1982, 1984). In studies conducted in 1989 and 1990, juvenile chinook salmon from other waterways in Commencement Bay were found to have concentrations of polycyclic aromatic hydrocarbons (PAHs), chlorinated hydrocarbons (CHs) or their derivatives in tissues, fluids or stomach contents that were similar to those found in juvenile chinook salmon from the highly contaminated Duwamish Waterway in Seattle (Varanasi et al. 1993). Juveniles from this latter urban waterway have been found to have significant concentrations of PAHs and CHs, and exhibited impaired immunocompetence and growth inhibition (McCain et al. 1990, Arkoosh et al. 1991, Varanasi et al. 1993) Another type of injury found in flatfish living in contaminated waterways in Puget Sound, such as the Duwamish Waterway, is reduced reproductive success (Johnson et al. 1988; Casillas et al. 1991). Among the reproductive effects observed are inhibition of oocyte development, inhibition of spawning, depressed plasma estradiol levels, reduced egg weight, increased larval abnormalities, and reduced viability of offspring.

More recently a number of biochemical alterations have been found in the liver of a variety of fish species from several areas in Puget Sound with elevated concentrations of contaminants in sediment, including Commencement Bay (Stein et al. 1992). Measurement of these biological markers have been used to improve the assessment of contaminant exposure and early responses in indigenous fish, and thus strengthen the link between exposure to toxic chemicals and the injuries described above. One type of alteration, known as DNA adducts, is a biomarker of exposure to potentially genotoxic compounds and is positively correlated with indicators of exposure, such as tissue levels of PCBs, and prevalences of certain liver diseases (Varanasi et al. 1989, Stein et al. 1992, 1994, Myers et al. 1995). Another alteration involves elevated levels of hepatic cytochrome P4501A (CYP1A) or associated monooxygenase activities, such as aryl hydrocarbon hydroxylase (AHH). Many studies have confirmed the induction of hepatic CYP1A activity both as an indicator of contaminant exposure and as one of the earliest physiological changes detected after exposure of fish to contaminants (Collier et al. 1991,1995; Payne et al., 1987). A number of studies have shown that measurement of these biochemical alterations, hereafter referred to as biomarkers, combined with measurement of the above-mentioned injuries, give a more consistent assessment of contaminant exposure in indigenous fish than do single bioeffects measurement.

The overall objective of the Hylebos Waterway fish injury assessment investigation is to document the current status of contaminant-associated injuries in fish indigenous to the Hylebos Waterway, to quantify the extent of these injuries, and evaluate links between these injuries and chemical contaminants in this waterway. Evidence for evaluating links will include data on concentrations of selected chemical contaminants in sediments from where fish are captured and in tissues and fluids of fish. The three primary objectives, together with more detailed background for each objective, are given below:

Objective 1—Assess Chemical Contamination and Associated Injuries in Juvenile Salmonids

In studies conducted in 1989 and 1990, juvenile chinook salmon from other waterways adjoining Commencement Bay were found to have significantly elevated concentrations of polycyclic aromatic hydrocarbons (PAHs), chlorinated hydrocarbons (CHs) or their derivatives in tissues, fluids or stomach contents that were similar to those found in juvenile chinook salmon from the highly contaminated Duwamish Waterway (McCain et al. 1990; Varanasi et al. 1993; Stein et al. 1995). Juvenile salmon from the Duwamish Waterway have also been found to have increased induction of hepatic cytochrome P450 1A and higher levels of DNA damage, and exhibited impaired immunocompetence and growth inhibition, compared to juveniles from nonurban estuaries (Arkoosh et al. 1991; Varanasi et al. 1993; Stein et al. 1995). A recent laboratory investigation demonstrated that immunocompetence of juvenile chinook salmon could be impaired by exposure to an extract of Duwamish Waterway sediment (Arkoosh et al. 1994). Because the assessment of immunocompetence and growth is very expensive, we are proposing to initially examine exposure and early biomarkers (e.g. DNA damage and enzyme induction) in the first phase (year one) of studies on juvenile salmon from the Hylebos Waterway. If significant exposure is found, then investigations to assess more serious biological effects will be recommended for the second year. Data linking specific classes of contaminants to impaired immunocompetence in salmonids is comparatively scarce, but our recent results suggest that both PAHs and chlorinated hydrocarbons may be risk factors.

Objective 2—Determine Toxicopathic Conditions in Flatfish

In the early 1980s, elevated levels of a variety of toxic chemicals were found in tissues of English sole and rock sole from the Hylebos Waterway (Malins et al. 1982, 1984). Many of these fish species also had a variety of liver lesions, including liver tumors, which are associated with exposure to carcinogenic chemicals, especially high molecular weight polynuclear aromatic hydrocarbons,
or PAHs (Myers et al. 1987, 1994a, Myers et al. 1991). These findings contributed to the eventual designation of Commencement Bay as a Superfund Site. ECD scientists also

conducted a long-term laboratory study in which a variety of unique degenerative and preneoplastic lesions were induced in English sole exposed to benzo[a]pyrene and a PAH-rich fraction of contaminants extracted from a contaminated sediment (Schiewe et al. 1991). More recently a suite of biochemical alterations have been demonstrated in a variety of fish species from several areas in Puget Sound with elevated concentrations of contaminants in sediment, including Commencement Bay (Stein et al. 1992). Measurements of these biological markers have been used to improve the assessment of contaminant exposure and early responses in indigenous fish (Krahn et al. 1986; Stein et al. 1992; Collier et al. 1993a,b; Collier et al. 1995), and thus strengthen the link between exposure to toxic chemicals and the injuries described above. For example, an investigation was conducted in the Hylebos Waterway, as well as eight other sites in Puget Sound, in 1987 and 1988 to evaluate relationships among liver lesions and these biomarkers in subadult English sole and rock sole (Myers et al. 1994b). Substantive correlations were found between the prevalences of a number of liver lesions and the levels of most of these biomarkers. We have also conducted several laboratory studies to demonstrate the time- and dose-responsiveness of a number of these biomarkers (Collier and Varanasi, 1991; Stein et al. 1993).

Objective 3—Determine Contaminant-Induced Reproductive Toxicology in Flatfish

In an effort to assess other biological impacts of the above-mentioned chemical contaminants on flatfish, the ECD developed a research program to evaluate the effects of these contaminants on the reproductive status of gonadally maturing adult female fish. Field studies showed that a significant proportion of female flatfish living in contaminated waterways of Puget Sound, such as the Duwamish Waterway in Seattle, were found to exhibit reduced reproductive success (Johnson et al. 1988; Casillas et al. 1991) compared to females from minimally contaminated sites. Another study suggested that female English sole from contaminated areas may not be making their spawning migrations at all (Collier et al. 1992). Among the reproductive effects observed were inhibition of oocyte development, inhibition of spawning, depressed plasma estradiol levels, reduced egg weight, increased larval abnormalities, and reduced viability of offspring. These field data have been incorporated into population projection models, and the results suggest that the decreased reproductive potential associated with exposure of certain flatfish species (e.g., English sole) to contaminants may be sufficient to significantly reduce the intrinsic rate of growth in populations from contaminated areas such as the Duwamish Waterway and Eagle Harbor (Landahl and Johnson, 1993). Again, similar to results for fish pathology, our data suggest that exposure to PAHs is a substantial risk factor in determining reduced reproductive success. Laboratory investigations have shown effects on circulating levels of sex hormones due to contaminant exposure, consistent with field results (Stein et al. 1991; Johnson et al. 1994).

Descriptions and scheduling of tasks

General

Juvenile Salmonids. Juveniles of two species of salmonids, chinook and chum salmon, were collected for injury assessment studies in the spring of 1994. These two species have the longest residency times in estuaries among Pacific salmon, but they have different migratory routes into the Hylebos Waterway; a large number of juvenile chum salmon are released by the Puyallup Tribe into Hylebos Creek at the head of the waterway, whereas chinook salmon are released from a state hatchery into the Puyallup River and move to the Hylebos Waterway via Commencement Bay. Fish were collected using a seine deployed from beaches or docks located around the 11th Street Bridge. Tissues collected for these studies included liver, stomach contents and bile for measurements of organic contaminants (CHs, PAHs, and their derivatives); and liver for measurements of biomarkers, e.g. DNA adducts and CYP1A. Juvenile salmon were sampled from the Hylebos Waterway approximately every two weeks from May 11 to June 29. A total of 1372 chum and 241 chinook were collected during this time. Reference fish were also sampled from hatcheries and estuaries considered to be relatively unimpacted. Reference sites for chum include the Puyallup Tribal Hatchery (n=600) and Skokomish Estuary (n=300). Reference sites for chinook include the Puyallup State Hatchery (n=200), Kalama Creek Hatchery (n=150), and Nisqually Estuary (n=170). Location of all sites are shown in Figs. 1 and 2.

Toxicopathic Conditions in Flatfish. English sole and rock sole were collected for assessment of histopathological conditions of the liver, kidney, and gonad, and measurements of organic contaminants in liver, bile and stomach contents, and of DNA adducts and CYP1A in liver. Otoliths were also collected for age determination. Flatfish were collected in the Hylebos Waterway in July and August of 1994 by otter trawl at three sites — one site near the Upper Turning Basin, one near the Lower Turning Basin, and one near the 11th Street Bridge (Fig 1). English sole were collected at all three Hylebos sites, whereas rock sole were only captured at the 11th Street Bridge site. We attempted to collect reference fish in Case Inlet in August, but were unable to locate fish of a comparable size. Therefore an alternate possible reference site at Colvos Passage (see Fig. 2 for location) was identified in October, and both English sole and rock sole were collected for reference samples. Sixty fish of each species were collected at each site.

Reproductive Toxicology in Flatfish. Adult female English sole were collected by otter trawl from the Hylebos Waterway and a reference site in Colvos Passage. Sampling was conducted approximately every 4-6 weeks from October 1994 through April 1995, during the season when vitellogenesis normally occurs in this species. Approximately 30 fish were collected at each site for every sampling period. Otoliths were collected for age determination. Blood samples were collected for estradiol analyses. Liver, kidney and ovary were collected for histopathological examination and staging of ovarian development. In January and February when yolk becomes visible in developing eggs, samples of ovary

were collected for fecundity measurements. Liver, bile and stomach contents were collected for measurement of contaminant exposure, and liver was also collected for measurement of DNA adducts and CYP1A.

Data Analysis, Products and Injury Qualification

The data from these injury assessment investigations will be placed in an integrated data management system. Statistical tests will be performed 1) to evaluate relationships between contaminant exposure and bioeffects, 2) to compare values from fish captured in the Hylebos Waterway with values for fish captured in reference areas, and 3) to compare concentrations of contaminants in liver tissue, prevalences of liver lesions and biomarker responses with similar parameters measured in previous studies.

Data acquired and analyzed through this SAP will be used to address the following questions for each species:

1. Are fish exposed to contaminants in Hylebos Waterway?
2. Are the exposures sufficient to cause injury?
3. What proportion of the population is estimated to be injured by these contaminants?
4. What might be the overall effect of this injury on the population of each species?

Table 1.

Scheduling of specific tasks

	Salmon investigations (contaminant exposure)	
Duration	**Activity**	**Numbers of fish and/or analyses**
May/June, 1994	Seining for juvenile salmon (approximately 20 days)	Approximately 1600 fish
May/June, 1994	Salmon necropsy and sample collection	Approximately 1600 fish
January-October, 1995	Chemical analyses of composites of livers, stomach contents and bile	35 comps/liver, 8 comps/stomach, 37 comps/bile
January-October, 1995	Analyses of composites of liver for biomarkers of contaminant exposure	37 comps/CYP1A 37 comps/DNA adducts
February 1996	Data reporting	N/A
June 1996	Data interpretation	N/A

	Flatfish investigations (Contaminant exposure and toxicopathic conditions)	
Duration	**Activity**	**Numbers of fish and/or analyses**
July-August, 1994	Flatfish collection aboard the RV Streeter (approximately 10 days)	Approximately 360 fish
July-August, 1994	Flatfish necropsy and sample collection	Approximately 360 fish
April-July, 1995	Sectioning and staining of fixed tissues	Approximately 360
July-November, 1995	Histological examination of tissues	Approximately 360 sections
March-October, 1995	Chemical analyses of composites of livers, stomach contents and bile	36 comps/livers 12 comps/stomach cont. 36 comps/bile
March-December, 1995	Analyses of composites of liver for biomarkers of contaminant exposure	36 comps/CYP1A 36 comps/ DNA adducts
February 1996	Data reporting	N/A
June 1996	Data interpretation	N/A

	Flatfish investigations (reproductive toxicology)	
Duration	**Activity**	**Numbers of fish and/or analyses**
October, 1994-March, 1995	Flatfish collection aboard the RV Streeter (approximately 20 days)	Approximately 360 adult female fish
October, 1994-March, 1995	Flatfish necropsy and sample collection	Approximately 360 adult female fish
May-August, 1995	Sectioning and staining of fixed tissues	Approximately 360 samples
August-November, 1995	Histological examination of tissues	Approximately 360 sections
May-December,1995	Chemical analyses of livers, stomach contents and bile.	120 indiv/liver 12 comps/stomach cont. 120 indiv/bile
May 1995 to January 1996	Analyses of liver for biomarkers of contaminant exposure	120 indiv/DNA adducts
January-August, 1995	Analyses of blood plasma for estradiol	Approximately 360 samples
January-August, 1995	Determination of somatic indices	Approximately 360 samples
February 1996	Data reporting	N/A
June 1996	Data interpretation	N/A

Standard Operating Procedures

Sample Collection and Analyses for Hylebos Waterway Fish Injury Studies 1994: Survey of Contaminant Exposure in Juvenile Salmon, Contaminant Exposure and Toxicopathic Conditions in Flatfish, and Reproductive Toxicology of Flatfish

Field collections

Juvenile Salmon

Salmon were collected with beach seines generally following the procedures as described in the Puget Sound Protocols (PTI, 1990), and by Varanasi et al. (1993). Since fish were often observed to be located near the surface of deeper water, seines were deployed from docks as well as beaches. Fish captured from the Hylebos Waterway and reference estuaries were held alive in aerated seawater, and fish from hatcheries were maintained in aerated freshwater until necropsies could be conducted. Fish processing for Hylebos collections was done either on the NOAA Ship McArthur or the RV Harold W. Streeter. Fish processing for samples from the hatcheries and reference estuaries were done at the NWFSC. Juvenile salmon were collected from the Hylebos Waterway primarily within sight of the 11th Street Bridge. Latitudes and longitudes were determined with a hand-held GPS and are listed below:

Latitudes and Longitudes of juvenile salmon collection areas in the Hylebos

47º 16.749 N 122º 23.791 W	47º 16.726 N 122º 23.661 W
47º 16.709 N 122º 23.798 W	47º 16.717 N 122º 23.662 W
47º 16.785 N 122º 23.896 W	47º 16.782 N 122º 23.719 W
47º 16.757 N 122º 23.888 W	47º 16.745 N 122º 23.799 W
47º 16.743 N 122º 23.790 W	47º 16.680 N 122º 23.961 W
47º 16.753 N 122º 23.891 W	47º 16.801 N 122º 23.836 W
47º 16.716 N 122º 23.794 W	47º 16.656 N 122º 23.651 W
47º 16.731 N 122º 23.774 W	47º 16.713 N 122º 23.808 W
47º 16.708 N 122º 23.716 W	47º 16.363 N 122º 24.005 W
47º 16.691 N 122º 23.626 W	

Flatfish for Toxicopathic Conditions and Reproductive Toxicology Studies

Flatfish were collected by 25 foot SCCWRP otter trawl similar to that described in Puget Sound Protocols (PTI, 1990) with the following differences. The net is constructed of 1.5 inch stretch mesh in the body, and 1.25 inch mesh in the cod end. Five-foot side panels have been added to increase the net opening. A cable foot rope has been added, and aluminum doors are now used instead of wood doors. The net was deployed from the 45

foot NWFSC research vessel Harold W. Streeter and towed for a duration of 5-15 minutes at a speed of 1.5-2.5 knots.

Fish were identified by trained fisheries biologists, and target species were placed in holding tanks on board the research vessel. Fish were maintained alive for no more than a few hours in tanks with flowing seawater until necropsies could be performed in the ship-board laboratory. By-catch was released.

Toxicopathic Conditions Study: Flatfish were collected from three sites within the Hylebos Waterway including locations near the Upper Turning Basin, the Lower Turning Basin and the 11th Street Bridge. Fish were also collected from the reference site at Colvos Passage. Target species were English sole and rock sole. English sole were captured at all three Hylebos sites and the reference site, while rock sole were available only at the 11th Street Bridge site in the Hylebos, and the Colvos Passage reference site.

Reproductive Toxicology Study: Beginning in October 1994, female English sole were collected near the 11th Street Bridge in the Hylebos Waterway and the reference site at Colvos Passage approximately once every six weeks until April 1995 to monitor reproductive maturation processes over time.

Necropsy and sample collections

Juvenile Salmon

At each site, or for every collection period, approximately 100-150 juvenile chum and 30-60 chinook were sampled for each composite. The number of fish needed for a composite was dependent on the size of the fish. We attempted to collect 4 composites/ species at each site for every sampling period, however the number of composites collected was dependent on the number of fish available. Thirty-seven composites of bile and liver were collected during the course of the study. This included four composites of chum from the Puyallup Tribal Hatchery, two composites of chum from the reference site at the Skokomish Estuary, twelve composites of chum collected over four sampling periods from the Hylebos, five composites of chinook from the Puyallup State Hatchery, three composites of chinook from the Kalama Creek Hatchery, three composites of chinook from Nisqually Estuary and seven composites of chinook from the Hylebos collected over four sampling periods. Fish were weighed and measured for fork length. Bile, liver and stomach contents were collected as described in Varanasi et al. (1993), except that whole livers were collected and later subdivided for each type of analysis. Liver and stomach contents were composited into glass 20 ml vials previously rinsed with methylene chloride. Bile was composited into 4 ml vials containing glass limited-volume inserts. If necropsies were conducted on the McArthur or at NWFSC, liver, bile and stomach content composites were immediately transferred to freezers. For necropsies conducted on the RV Streeter, samples were maintained on dry ice until they could be transferred to freezers for storage. Liver samples were stored at -80^{o} C, and bile and stomach contents were stored at -20° C until analyses were conducted.

Flatfish

Fish were weighed (to the nearest gram) and measured (fork length, to the nearest 1 mm), and otoliths were collected for age determination. Prior to necropsy, each fish was assigned a unique specimen number, and all sample containers were labeled with this number for sample tracking. Details of necropsy, histology sample collection and fixation, and chemistry sample collection and preservation are described in Stehr et al. (1993). Samples of liver, bile and stomach contents collected for organic chemical analysis were maintained on ice, then transferred to locked freezers for temporary storage at -80° C at the end of each day. Samples of liver for CYP1A and DNA adducts were immediately placed in liquid nitrogen and transferred to a locked -80° C freezer at the end of each day. They were temporarily stored in this freezer until they were distributed to the appropriate laboratories. Once distributed, samples for organic chemical analyses were stored at -20° C, and samples for CYP1A and DNA adduct analyses were stored at -80° C until analyses were completed. Individual samples were collected in the field, however liver, stomach contents and bile were composited in the laboratory for chemical analyses including measurements of organics, CYP1A and DNA adducts, and biliary fluorescent aromatic compounds (FACs).

Toxicopathic Conditions Study: Sixty English sole were collected at each of the three Hylebos sites (Fig. 2) as well as the reference site in Colvos Passage. Sixty rock sole were also collected at the 11th St. Bridge site in the Hylebos, and at the reference site. To determine the applicability of sample size, power analyses were conducted using lesion data from a previous study of the Duwamish Waterway (Myers et al. 1993), which is a highly industrialized waterway located about 30 miles north in Elliott Bay. The lesion type used in the analysis was foci of cellular alteration (FCA) which is considered to be a precursor to neoplastic conditions. This lesion type is less prevalent than most other contaminant-related lesion types, therefore it serves as a conservative index of power for analyses of sample size. For a sample size of 60 fish, the statistical power to detect differences in prevalence of FCA between the Duwamish and the reference site at Nisqually fell between 70% and 80% at a=0.20. For a=0.10, the power to detect fell between 55% and 60%.

Fish were necropsied and tissues preserved according to standard procedures as described in Stehr et al. (1993). Samples collected included otoliths for age determination, liver, kidney and gonad for histology; liver for organic contaminant analyses, CYP1A and DNA adducts; stomach contents for organic contaminant analyses and bile for FACs.

Reproductive Toxicology Study: Approximately thirty female fish were collected at each site during each sampling period. The test site was located near the 11th St. Bridge in the Hylebos and the reference site in Colvos passage. Samples collected included otoliths for age determination; liver, kidney and gonad for histology and to determine ovarian matu-

ration; liver for organic contaminant analyses; DNA adducts; stomach contents for organic contaminant analyses and bile for FACs. Blood was also collected for plasma 17-b estradiol concentrations, and weights of ovaries, liver, and gutted bodies were collected to determine gonadosomatic, hepatosomatic and condition indices. In January and February when yolk production becomes visible in the oocytes, one ovary was collected for fecundity determination, and portions of the remaining ovary were collected for chlorinated hydrocarbon analyses. Sample collection procedures are described in Johnson et al. (1988, 1994) and Stehr et al. (1993). Samples collected for fecundity determination are being archived, and analyses of these samples are not being performed under this SAP.

Sample analyses

Juvenile Salmon Injury (Table 2A)

Organic chemical analyses. Fish liver is analyzed for chlorinated hydrocarbons (CHs) and pesticides using the methods described by Sloan et al. (1993). Stomach contents are analyzed for aromatic hydrocarbons (AHs) as well as CHs and pesticides, also using the methods of Sloan et al. (1993). Analytes measured are listed in Table 3. Liver or stomach contents are 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.

Biliary FACs. FACs including benzo[a]pyrene (BaP), phenanthrene (PHN) and naphthalene (NPH) equivalents in bile are analyzed by HPLC according to the methods described by Krahn et al. (1986) with the following modifications.

FACs in bile are analyzed on a Waters (Milford, MA) HPLC equipped with a Waters WISP™ model 715 automatic injector, and three Perkin Elmer (Norwalk, CT) model 40 fluorescence detectors connected in series and interfaced to a Waters Millenium™ data acquisition workstation. A 0.20- x 2-cm guard column containing Perisorb™ 30- to 44-µm reverse-phase C_{18} packing (Upchurch Scientific, Oak Harbor, WA) is used in series with a Perkin Elmer HC-ODS/PAH 10-µm (0.26- x 25-cm) reverse-phase analytical column (Figure 3).

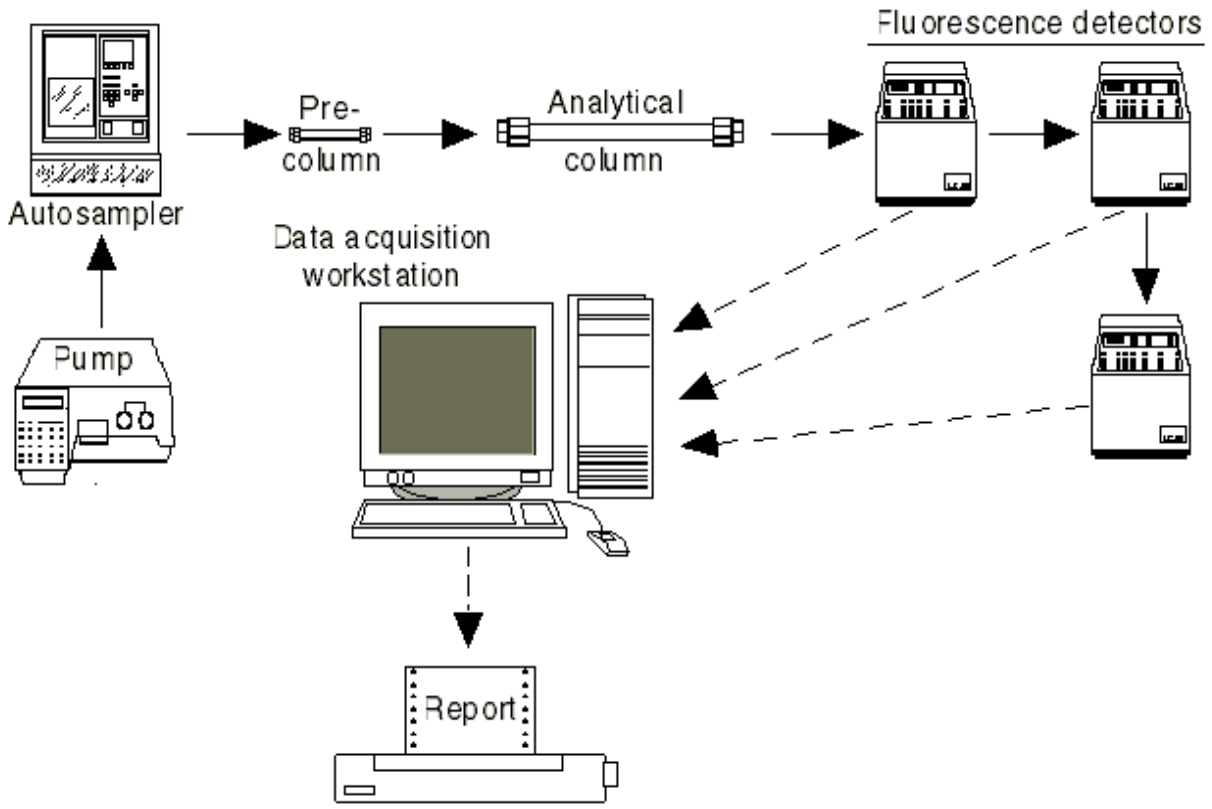

Figure 3. High performance liquid chromatography system for analyses of bile for fluorescent aromatic contaminants (solid lines indicate flow of mobile phase; dashed lines indicate electronic connections for data flow)

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 run time, including running of the linear gradient and re-equilibration of the system, is 38 min. All solvents are degassed with helium and the column temperature is held at 50°C.

Bile sample identification numbers, HPLC calibration standards (HPLC CS) and data from the bile reference pools for each analysis set are entered into a database in the Waters Millenium™2010 Chromatograpy Manager software (see below). For each set of samples, the HPLC CS, containing known concentrations of NPH, PHN and BaP, are

analyzed at the beginning of the set and after every 6 or 7 samples. A quality assurance bile reference pool is analyzed near the beginning and end of each set analyzed by HPLC. Biliary FACs are monitored by fluorescence at excitation/emission (ex/em) wavelength pairs for NPH (ex/em 290/335 nm), PHN (ex/em 260/380 nm) and BaP (ex/em 380/430 nm). The total area for all peaks in the region of the chromatogram where FACs are known to elute (> 9 min) are integrated for each wavelength pair. Quantification of analytes is performed according to Krahn et al. (1986). The concentrations (ng PAH equivalents per ml of bile or per mg biliary protein, wet weight) of FACs in the samples are calculated according to the response of each PAH in the HPLC CS. Biliary protein is measured according to Lowry et al. (1951). 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.

As stated above, fluorescence response data for HPLC chromatograms is acquired and processed with the Waters Millenium™ 2010 chromatography software package. Each tray of sample vials (bile samples, calibration standards and reference bile pools) analyzed by HPLC is assigned a unique Sample Set ID. After processing, the chromatograms and results of peak integration are printed for review to assure the quality of the results. After checking quality assurance criteria, an electronic copy of the data files is stored on disk and a hard copy of the data and results are printed for archival purposes. Specific details of the use of the acquisition software and workstation can be found in the operation manuals provided by Waters/Millipore Corporation.

CYP1A

Hepatic microsomes are prepared from frozen liver samples as described previously (Collier et al., 1995) except that microsomes are resuspended in 0.25 M sucrose made up in 80/20 v/v water/glycerol. The suspensions are frozen at -80°C until CYP1A assays are performed. Aryl hydrocarbon hydroxylase (AHH) activities are assayed in triplicate at 25° using ^{14}C-BaP as the primary substrate (Collier et al., 1995) .

DNA adducts Hepatic xenobiotic DNA adducts are measured according to the methods of Reichert and French (1994). (Appendix).

Toxicopathic Conditions in Flatfish (Table 2B)

Age determination Fish age is determined to the nearest year by counting the number of clearly defined opaque zones of whole otoliths under a binocular dissecting microscope (Chilton and Beamish, 1982).

Histology Livers, kidney and gonad are preserved in Deitrichs' fixative. Tissues are embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined microscopically. Hepatic lesions are classified according to the criteria outlined in Myers et al. (1987), then grouped into the following categories: neoplasms (hepatocellular carcinoma,

cholangiocellular carcinoma, adenoma, and cholangioma); foci of cellular alteration (eosinophilic foci, basophilic foci, clear cell foci), specific degeneration/ necrosis (nuclear pleomorphism and/or megalocytic hepatosis), hydropic vacuolation, and non-specific necrotic lesions. Ovarian lesions and developmental stage are examined and classified as described in the reproductive toxicology section.

Organic Chemical Analyses of liver tissue and stomach contents are conducted as described for the juvenile salmon study (see page 12).

Biliary FACs are measured as described for the juvenile salmon study (see pages 12-14).
Biochemical Analyses. CYP1A and hepatic xenobiotic-DNA adduct levels are measured in liver tissues as described for the juvenile salmon study (see page 14).

Reproductive Toxicology Sample Analysis Techniques (Table 2C)
Plasma 17-b estradiol concentration. A 1-3 mL blood sample was taken from each fish with a heparinized syringe. Blood samples were centrifuged at 800 x g, and the plasma stored at -80^{o}C until analyses are conducted. Plasma 17-b estradiol levels are determined by radioimmunoassay as described in Sower and Schreck (1982).

Histological Analyses. Histology of the liver, kidney and gonad are conducted as described above in the toxicological conditions study. The developmental stage of ovaries are determined, and classified according to criteria described in Johnson et al. (1991) into the following categories: regressed, late regressed, pre-vitellogenic, vitellogenic, hydrated, spawning, and spent. Ovaries are also examined for follicular atresia, hermaphroditism, ovarian macrophage aggregates, and other inflammatory lesions associated with oocyte resorption, including lymphoid or macrophage infiltrates, using criteria described in Johnson et al. (1991).

Determination of Somatic Indices. Fish are weighed (to the nearest gram) and measured (fork length, to the nearest mm), and liver and gonads are excised and weighed (to the nearest gram). All other internal organs are then removed, and the animal is weighed (to the nearest gram) to determine gutted body weight.

Gonadosomatic index (GSI) (Nikolsky 1963, Shul-man 1974) is calculated according to the formula:

GSI = (ovary weight (g)/gutted body weight (g)) x 100

Hepatosomatic index (HSI) (Nikolsky 1963, Shul-man 1974) is calculated according to the formula:

HSI = (liver weight (g)/gutted body weight (g)) x 100

Condition factor. Because low body weight may be associated with suppressed ovarian development in adult female fish (Burton and Idler, 1987), a condition factor is determined for all sampled animals so the influence of emaciation on ovarian development can be

distinguished from any potential effects of contaminant exposure. Condition factor (Ricker, 1975) is calculated using the formula:

$$\text{Condition factor} = \text{gutted body weight (g)}/\text{length}^3 \text{ (cm)}$$

Age determination. Otoliths are examined to determine age of the fish as described for the toxicopathic condition study (see page 14).

Rapid PCB Analysis by HPLC with photodiode array (PDA) detection

Tissue Extraction and Cleanup. Selected individual samples are analyzed using rapid HPLC analyses for PCB congeners, DDTs and hexachlorobenzene (Specific analytes listed in Table 3.). Fish livers are analyzed for PCBs using the extraction and cleanup method of Krahn et al. (1994). Briefly, 0.3 - 2.0 g tissue, 20-mL hexane/pentane (1:1 v/v), 5 g sodium sulfate and the surrogate standard (1,7,8-TriCDD; 250 ng) is homogenized, centrifuged and the extract is decanted into a concentrator tube. The extraction process is repeated, and the extracts are combined and evaporated to ~1 mL. The sample extract is loaded onto a gravity-flow cleanup column that contains a glass wool plug, silica gel, basic silica gel and acidic silica gel to remove lipids and other interfering compounds. The PCBs are eluted from the cleanup column with 14 mL hexane/methylene chloride (1:1 v/v) and collected into a concentrator tube. The HPLC internal standard is added to each sample (1,2,3,4-TCDD; 250 ng) and the solvent volume is reduced to ~ 150 µL.

HPLC/PDA Analyses. Eleven dioxin-like PCB congeners (PCBs 77, 81, 105, 118, 126, 156, 157, 169, 170, 180 and 189), other selected PCBs (PCBs 101, 128, 138 and 153) and chlorinated pesticides (e.g., DDTs, hexachlorobenzene) were resolved by HPLC on two (1-pyrenyl) ethyldimethylsilylated silica (PYE) analytical columns cooled to 9°C and are monitored with a PDA detector (Krahn et al., 1994). The chlorinated hydrocarbons are identified by comparing their UV spectra (200-310 nm) and retention times to those of reference standards in a library. Analyte purity is confirmed by comparing UV spectra collected for a peak to the apex spectrum.

Biliary fluorescent aromatic compounds are measured for selected individuals as described for the juvenile salmon study (see pages 12-14).

Biochemical Analyses. Hepatic xenobiotic-DNA adduct levels are measured in liver tissues of selected individuals as described for the juvenile salmon study (see page 14).

Chain of Custody Procedures

all samples collected and stored are accompanied by a chain of custody form. Each box of samples is sealed with security tape, and transferred to the laboratory in secured containers. Samples are stored in locked freezers. Chain of custody is maintained throughout storage, distribution and analyses of samples. Samples will be stored by the QA officer for one year after the data are accepted.

Quality Assurance Procedures

Hylebos Waterway Fish Injury Studies 1994: Survey of Contaminant Exposure in Juvenile Salmon, Toxicopathic Conditions in Flatfish, and Reproductive Toxicology of Flatfish

Quality Assurance procedures will be followed to monitor (1) the performance of the measurement systems 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. Procedures for QA are summarized in Table 4.

Organic Chemical Analyses of Liver and Stomach Contents

Quality Assurance Procedures are described in detail for the detailed Organic Chemical analyses of liver and stomach contents in the Commencement Bay Damage Assessment Quality Assurance Plan (December 1995).

Rapid PCB Analysis by HPLC/PDA

The HPLC/PDA is calibrated daily (see Table 4, continuing calibration). Each sample set consists of 11-14 samples and the following quality assurance samples: a whale blubber control material, a method blank and a duplicate.

CYP1A

Quality assurance procedures include duplicate zero-time and boiled enzyme blanks for each set of assays. Each sample is run in triplicate and if samples show > 30% absolute difference between triplicates and >20 units (pmoles benzo[a]pyrene metabolized/mg microsomal protein/minute) difference between triplicates the outlying replicate will be omitted from the calculations. If the coefficient of variation (CV) still exceeds 30%, the analysis of that sample will be repeated.

DNA Adducts

Salmon testes DNA prepared from coho (*Oncorhynchus kisutch*), is used for measuring the efficiency of DNA hydrolysis and as a sample blank throughout the assay to assess background. The compound 7R,8S,9S,10R-(N^2-deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo[a]pyrene (BaPDE-dG-3'p) is used as an internal standard to monitor the efficiency of enzyme-mediated transfer of the ^{32}P-phosphate from [g-^{32}P]ATP to DNA adducts and as an alternate means of determining the specific activity of the [g-^{32}P]ATP used in the assay. In addition, the postlabeled BaPDE-dG-3'p is used as a chromatography standard. To assess reproducibility, every 10th tissue sample is analyzed in duplicate. The storage phosphor screens used to image the chromatograms in the assay are calibrated with dilutions of ^{32}P at each use.

Histopathology

Tissue sections of liver, kidney and gonad are examined for lesions by fish histopathologists with 3 or more years of experience. Standard diagnostic criteria are used to identify lesions (Myers et al. 1987, Johnson et al. 1991). Each individual specimen is coded so that the location of the sample collection is unknown to the histopathologists reading the slides. To maintain consistency and quality of interpretation, all pathologists routinely consult with the chief histopathologist on any questionable lesions. If cases remain problematic, the chief histopathologist will consult with colleagues at other agencies. In addition, any new lesion types are sent to the Registry of Tumors for lower vertebrates in Washington D.C. The chief histopathologist re-examines 10% of the slides read by the other histopathologists for quality control.

Bile FACs

The quality assurance procedures include analyses of HPLC calibration standards (CS), method blanks, replicates, and the bile reference pool materials. Before analyzing samples, the stability of the HPLC performance is verified by replicate analysis of an HPLC CS (3 or more times, as necessary), then comparing the area of each compound in the calibration standard from analysis to analysis. Before analyzing sample sets, the HPLC performance is considered stable when the mean response for each compound in the HPLC CS has a relative standard deviation (RSD) $\leq$ 15%. If the RSD for any analyte 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. If the fluorescence response of the blank exceeds the usual background noise, appropriate column maintenance is performed before proceeding with analyses of samples.

A bile reference material (BRM) is also analyzed in duplicate with each set of samples. The BRM is a pooled fish bile that was analyzed as part of an interlaboratory quality assurance exercise, which included two laboratories from the Environmental Conservation Division and one laboratory from Texas A & M (Krahn et al. 1991). The concentrations of FACs from each analysis of the BRM are then compared to the results from previous analyses of the material. 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 10 field samples.

Reproductive Steroid RIAs- Plasma 17-b Estradiol Concentrations

Steroid RIAs are competitive binding assays which require the use of radiolabeled standards and steroid specific antibodies. In this type of assay, a standard curve is generated from known concentrations of 17-b estradiol, based on the binding characteristics of the standards in the presence of antibody and radiolabeled hormone. The steroid concentrations

in plasma are then predicted by comparison with the standard curve using logistic regression analyses. In order to assure that reproductive steroid RIAs are conducted properly, a number of criteria are examined:

1) Standard curve: Standards typically range from 0 - 2000 pg estradiol 17-b/ml; if necessary the range of standards may be modified somewhat to accommodate the concentration range in the test samples. The regression coefficient, slope, Y-intercept, RSD of the triplicates, and the binding efficiency and binding range of the standard curve are calculated. Binding efficiency of antibody to labeled antigen ($0_{standard}$ (cpm) - NSB (cpm)/ Total (cpm) -NSB (cpm) x 100] should be approximately 30-35%, and the range of binding of the standard curve should be from approximately 20-80% of B_0; this constitutes the effective concentration range of the assay. These are typical values used in most radio- and enzyme-linked immunoassay procedures, and are documented in the literature (cf. see Sower and Schreck, 1982 and Chard, 1982). If binding efficiency falls outside the 25-40% range, or if the regression coefficient of the standard curve is < 0.95, the quality of the reagents is checked and the assay is re-run with appropriate modifications.

2) Samples: Samples are also tested in triplicate. Samples that do not fall in 20-80% binding range of the standard curve are retested by adjusting sample volume; additionally, if the within-assay RSD of the triplicates is > 20%, the sample is re-run.

3) Quality Control: Triplicates of pooled plasma from male and/or gravid female English sole collected in Puget Sound are tested with each assay as reference material for quality control. The pooled reference plasma from the male sole contains very low or undetectable concentrations of estradiol 17-b, while the pooled reference plasma from the female sole contains elevated concentrations of estradiol 17-b. The concentrations of estradiol from each analysis of the plasma reference pool material are then compared to the results from analysis of the material in the previous sample sets. If interassay (i.e., day to day) RSD of quality control samples exceeds 30%, the assay is re-run after making appropriate modifications to reagents or other assay conditions.

Chain of Custody Procedures

These are described in detail in the Commencement Bay Damage Assessment Quality Assurance Plan. Briefly, chain of custody procedures will be used for all samples and for all data and data documentation, whether in hard copy or electronic format. 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 and/or field 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.”

Budget Summary Table

Sampling and Analysis Plan
Hylebos Waterway Fish Injury Study

Juvenile Salmonid Assessment

# Analyses	Type of Analysis	Cost/sample	Total $
37	CYP1A	123	4551
36	DNA adducts	262	9432
37	bile FACs	124	4588
8	*Stomach organic Chem (1 set)	1003	8024
3	*Stomach organic Chem QA	1003	3009
37	**Liver organic Chem (4 sets)	779	28823
13	**Liver organic Chem QA	779	10127
20	Sampling days	3560	71200
Salmon Study Total =			**139754**

Toxicopathic Conditions in Flatfish

# Analyses	Type of Analysis	Cost/sample	Total $
36	CYP1A	123	4428
36	DNA adducts	262	9432
36	Bile FACs	124	4464
12	*Stomach organic Chem (2 sets)	1003	12036
7	*Stomach organic Chem QA	1003	7021
36	**Liver organic Chem. (4 sets)	779	28044
14	**Liver organic Chem. QA	779	10906
360	Fish histopathology	43	15480
10	Sampling days	3369	33690
Toxicopathic Study Total =			**125501**

* up to 10 stomach organics samples are analyzed in a set. For every two sets, 5 or more QA samples are needed (see Table 4). In some cases, there will not be enough samples to complete a set of 10, however QA is required for partial sets. Since stomach contents are analyzed for both AHs and CHs, these samples cannot be combined with sample sets of liver organics.

** up to 10 liver organics samples are analyzed in a set. For every two sets, 5 or more QA samples are needed (see Table 4).

Budget Summary Table (cont.)

Sampling and Analysis Plan
Hylebos Waterway Fish Injury Study

Reproductive Toxicology in Flatfish

# samples	Type of Analysis	Cost/sample	Total $
360	Estradiol	47	16920
120	DNA adducts	262	31440
120	Bile FACs	124	14880
12	*Stomach organic Chem. (2 sets)	1003	12036
7	*Stomach organic Chem QA	1003	7021
120	***Liver PCB screens (11 sets)	203	24360
33	***Liver PCB screens QA	203	6699
360	Fish histopathology	43	15480
20	Sampling days	3369	67380

Repro. Study Total = **198016**

1995 Hylebos Proj. Total = 463271

* up to 10 stomach organics samples are analyzed in a set. For every two sets, 5 or more QA samples are needed (see Table 4). In some cases, there will not be enough samples to complete a set of 10, however QA is required for partial sets.

*** up to 11 liver PCB samples are analyzed in a set. For every set, 3 QA samples are needed (see Table 4.)

References

Arkoosh, M.R., E. Casillas, E. Clemons, B.B. McCain, and U. Varanasi. 1991. Suppression of immunological memory in juvenile chinook salmon (*Oncorhynchus tshawytscha)* from an urban estuary. Fish and Shellfish Immunol. 1: 261-277.

Arkoosh, M.R., E. Clemons, M. Myers and E. Casillas. 1994. Suppression of B-cell mediated immunity in juvenile chinook salmon (*Oncorhynchus tshawytscha*) after exposure to either a polycyclic aromatic hydrocarbon or to polychlorinated biphenyls. Immunopharmacol. Immunotoxicol. 16(2): 293-314.

Burton, M.P. and D.R. Idler. 1987. An experimental investigation of the non-reproductive postmature state in winter flounder. J. Fish Biol. 30: 643-650

Casillas, E., D. Misitano, L. Johnson, L. Rhodes, T.K. Collier, J.E. Stein, B. McCain and U. Varanasi. 1991. Inducibility of spawning and reproductive success of female English sole (*Parophrys vetulus*) from urban and nonurban areas of Puget Sound, Washington. Mar. Environ. Res. 31: 99-122.

Chard, T. 1982. An introduction to radioimmunoassay and related techniques. Laboratory Techniques in Biochemistry and Molecular Biology, Volume 6, part 2 (Work, T.S. and E. Work, editors). Elsevier Biomedical Press, Amsterdam. 284 pp.

Chilton, D.E., and R.J. Beamish. 1982. Age determination methods for fishes studied by the Groundfish Program at the Pacific Biological Station. Can. Spec. Publ. Fish Aquat. Sci. 60: 1-54.

Collier, T.K. and U. Varanasi. 1991. Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in English sole *(Parophrys vetulus)* exposed to environmental contaminants. Arch. Environ. Contam. Toxicol. 20: 462-473.

Collier, T.K., J.E. Stein, H.R. Sanborn, T. Hom, M.S. Myers, and U. Varanasi. 1992. Field studies of reproductive success in English sole (*Parophrys vetulus*): Correlations with bioindicators of maternal contaminant exposure. Sci Total Environm. 116: 169-185.

Collier, T.K., J.E. Stein, A. Goksøyr, M.S. Myers, J.W. Gooch, R.J. Huggett, and U. Varanasi. 1993a. Biomarkers of PAH exposure and effects in oyster toadfish (*Opsanis tau*) from the Elizabeth River, Virginia. Environ. Sci. 2: 161-177.

Collier, T.K., M.M. Krahn, C.A. Krone, L.L. Johnson, M.S. Myers, S.-L. Chan, and U. Varanasi. 1993b. Oil exposure and effects in subtidal fish following the EXXON Valdez oil spill. In: Proceedings 1993 International Oil Spill Conference pp. 301-305.

Collier, T.K., B.F. Anulacion, J.E. Stein, A. Goksøyr, and U. Varanasi. 1995. A field evaluation of cytochrome P4501A as a biomarker of contaminant exposure in three species of flatfish. Environ. Toxicol. Chem. 14(1): 143-152.

Johnson, L.L., E. Casillas, T.K. Collier, B.B. McCain and U. Varanasi. 1988. Contaminant effects on ovarian development in English sole (*Parophrys vetulus)* from Puget Sound, Washington. Can. J. Fish. Aquat. Sci. 45: 2133-2146.

Johnson, L.L., E. Casillas, M.S. Myers, L.D. Rhodes and O.P. Olson. 1991. Patterns of oocyte development and related changes in plasma 17-b estradiol, vitellogenin and plasma chemistry in English sole (*Parophrys vetulus*). J. Experimental Mar. Biol. Ecol. 152:161-185.

Johnson, L.L., J.E. Stein, T.K. Collier, E. Casillas and U. Varanasi. 1994. Indicators of reproductive development in prespawning female winter flounder (*Pleuronectes americanus*) from urban and non-urban estuaries in the northeast United States. Sci. Tot. Environm. 141: 241-260.

Krahn, M.M., L.K. Moore and J.W.D. MacLeod. 1986. Standard Analytical Procedures of the NOAA National Analytical Facility, 1986: Metabolites of Aromatic Compounds in Fish Bile. NOAA Technical Memorandum NMFS F/NWC-102. 25.

Krahn, M.M., T. Hom, G. Ylitalo, S-L. Chan and U. Varanasi. 1991. Report on quality assurance for bile measurements: Alaska oil spill damage assessment program. NOAA/ NMFS/NWFSC/ECD.

Krahn, M.M., G.M. Ylitalo, J. Buzitis, C.A. Sloan, D.T. Boyd, S-L. Chan and U. Varanasi. 1994. Screening for planar chlorobiphenyl congeners in tissues of marine biota by high-performance liquid chromatography with photodiode array detection. Chemosphere. 29: 117-139.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Landahl, J.T., and L.L. Johnson. 1993. Contaminant exposure and population growth of English sole (*Pleuronectes vetulus*) in Puget Sound: the need for better early life history data. Am. Fish. Soc. Symp. 14: 117-123.

Malins, D.C., B.B. McCain, D.W. Brown, A.K. Sparks, H.O. Hodgins, and S-L. Chan. 1982. Chemical contaminants and abnormalities in fish and invertebrates from Puget Sound. NOAA Tech. Memo. OMPA-19, 168 p.

Malins, D.C., B.B. McCain, D.W. Brown, S-L. Chan, M.S. Myers, J.T. Landahl, P.G. Prohaska, A.J. Friedman, L.D. Rhodes, D.G. Burrows, W.D. Gronlund, and H.O. Hodgins. 1984. Chemical pollutants in sediments and diseases in bottom-dwelling fish in Puget Sound, Washington. Environ. Sci. Technol. 18: 705-713.

McCain, B.B., Malins, M.M. Krahn, D.W. Brown, W.D. Gronlund, L.K. Moore and S-L. Chan. 1990. Uptake of aromatic and chlorinated hydrocarbons by juvenile chinook salmon (*Oncorhynchus tshawytscha*) in an urban estuary. Arch. Environ. Contam. Toxicol. 19: 10-16.

Myers, M.S., L.D. Rhodes and B.B. McCain. 1987. Pathologic anatomy and patterns of occurrence of hepatic neoplasms, putative preneoplastic lesions, and other idiopathic hepatic conditions in English sole (*Parophrys vetulus*) from Puget Sound, Washington, U.S.A. J. Natl. Cancer Institute 78(2): 333-363.

Myers, M.S., J.T. Landahl, M.M. Krahn, and B.B. McCain. 1991. Relationships between hepatic neoplasms and related lesions and exposure to toxic chemicals in marine fish from the U.S. West Coast. Environ. Health Perspect. 990: 7-15.

Myers, M., C.M. Stehr, O.P. Olson, L.L. Johnson, B.B. McCain, S.-L. Chan, U. Varanasi. 1993. National Status and Trends Program, National Benthic Surveillance Project: Pacific Coast. Fish histopathology and relationships between toxicopathic lesions and exposure to chemical contaminants for cycles I to V (1984-1988). U.S. Dep. Commer., NOAA Tech. Memo NMFS-NWFSC-6, 160p.

Myers, M.S., C.M. Stehr, O.P. Olson, L.L. Johnson, B.B. McCain, S-L. Chan and U. Varanasi. 1994a. Relationships between toxicopathic hepatic lesions and exposure to chemical contaminants in English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*), and while croaker (*Genyonemus lineatus*) from selected marine sites on the Pacific Coast, U.S.A. Environ. Health Perspect. 102: 200-215.

Myers, M.S., L.L. Johnson, T. Hom, T.K. Collier, J.E. Stein, and U. Varanasi. 1996. Toxicopathic hepatic lesions in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, U.S.A.: relationships with other biomarkers of contaminant exposure. Mar. Environ. Res. (in press).

Nikolsky, G.V. 1963. The Ecology of Fishes. New York, Academic Press.

Payne J.F., L.L. Fancey, A.D. Rahimtula, E.L. Porter. 1987. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. Comp. Biochem. Physiol. 86C: 233-245.

PTI, 1990. Recommended Guidelines for Sampling Soft-Bottom Demersal Fishes by Beach Seine and Trawl in Puget Sound. Prepared for E.P.A.; Puget Sound Estuary Program. In: Recommended Protocols for Measuring Selected Environmental Variables in Puget Sound. Vol 2.

Reichert, W.L., and B. French. 1994. The ^{32}P-postlabeling protocols for assaying levels of hydrophobic DNA adducts in fish. U.S. Dep. Commer., NOAA Tech. Memo. NMFS-NWFSC-14, 89 p.

Ricker, W.E. 1975. Computation and Interpretation of Biological Statistics of Fish Populations. Can. Bull. of Fish. Aquatic Sci. No. 191. 382 pp.

Schiewe, M.H., D.D. Weber, M.S. Myers, F.J. Jacques, W.L. Reichert, C.A. Krone, D.C. Malins, B.B. McCain, S-L. Chan and U. Varanasi. 1991. Induction of foci of cellular alteration and other hepatic lesions in English sole (*Parophrys vetulus*) exposed to an extract of an urban marine sediment. Can. J. Fish. Aquat. Sci. 48: 1750-1760.

Shul-man G.E. 1974. Life Cycles of Fish. New York, John Wiley & Sons.

Sloan, C.A, N.G Adams, R.W. Pearce, D.W. Brown and S.-L. Chan. 1993. Northwest Fisheries Science Center Organic Analytical Procedures. In: Sampling and Analytical Methods of the National Status and Trends Program, National Benthic Surveillance and Mussel Watch Projects, 1984-1992. Volume IV. Comprehensive Descriptions of Complementary Measurements. NOAA Tech. Memo. NOS ORCA 71.

Sower, S.A., and C.B. Schreck. 1982. Steroid and thyroid hormone during sexual maturation of coho salmon *(Oncorhynchus kisutch)* in seawater or freshwater. Gen. Comp. Endocrinol. 47: 42-53.

Stehr, C.M., M.S. Myers, M.J. Willis. 1993. Collection of Fish Tissues for the National Benthic Surveillance Project. Necropsy Procedure, Tissue Processing, and Diagnostic Procedure for Histopathology. In: Sampling and Analytical Methods of the National Status and Trends Program, National Benthic Surveillance and Mussel Watch Projects, 1984-1992. Volume II. Comprehensive Descriptions of Complementary Measurements. NOAA Tech. Memo. NOS ORCA 71.

Stein, J.E., T. Hom, H.R. Sanborn and U. Varanasi. 1991. Effects of exposure to a contaminated-sediment extract on the metabolism and disposition of 17b-estradiol in English sole (*Parophrys vetulus*). Comp. Biochem. Physiol. 99C: 231-240.

Stein, J.E., T.K. Collier, W.L. Reichert, E. Casillas, T. Hom and U. Varanasi. 1992. Biomarkers of contaminant exposure and sublethal effects: Studies with benthic fish in Puget Sound, WA. Environ. Toxicol. Chem. 11: 701-14.

Stein, J.E., W.L. Reichert, B. French and U. Varanasi. 1993. ^{32}Postlabeling analysis of DNA adduct formation and persistence in English sole (*Pleuronectes vetulus*) exposed to benzo[a]pyrene and 7H-dibenzo[c,g]carbazole. Chem.-Biol. Interact. 88: 55-69.

Stein, J.E., W.L. Reichert and U. Varanasi. 1994. Molecular epizootiology: assessment of exposure to genotoxic compounds in teleosts. Environ. Health Perspect. 102(Suppl 12): 19-23.

Stein, J.E., T. Hom, T.K. Collier, D.W. Brown, and U. Varanasi. 1995. Contaminant exposure and biochemical effects in outmigrant juvenile Chinook salmon from urban and non-urban estuaries of Puget Sound, WA. Environ. Toxicol. Chem. 14(6): 1019-1029.

Varanasi, U., W.L. Reichert and J.E. Stein. 1989. ^{32}Postlabeling analysis of DNA adducts in liver of wild English sole (*Parophrys vetulus*) and winter flounder (*Pseudopleuronectes americanus*). Cancer Res. 49:1171-1177.

Varanasi, U., E. Casillas, M.R. Arkoosh, T. Hom, D.A. Misitano, D.W. Brown, S-L. Chan, T.K. Collier, B.B. McCain, and J.E. Stein. 1993. Contaminant exposure and associated biological effects in juvenile chinook salmon (*Oncorhynchus tshawytscha*) from urban and nonurban estuaries of Puget Sound. U. S. Dept. of Commerce, NOAA Tech. Memo. NMFS-NWFSC-8, 112 p.

Table 2A. Summary of Analytic Methods for Assessment of Injury to Juvenile Salmon.

Sample type	Analysis	Reference
liver	CHs, pesticides CYP1A DNA adducts	Sloan et al. 1993 Collier et al. 1995 Reichert et al. 1994
stomach contents	AHs, CHs, pesticides	Sloan et al. 1993
bile	AH metabolites	Krahn et al. 1986

Table 2B. Summary of Analytic Methods for Toxicological Conditions in Flatfish.

Sample type	Analysis	Reference
liver	CHs, pesticides (detailed) CYP1A DNA adducts	Sloan et al. 1993 Collier et al. 1995 Reichert et al. 1994
stomach contents	AHs, CHs, pesticides	Sloan et al. 1993
bile	AH metabolites	Krahn et al. 1986
liver, kidney, gonad	Histopathology	Liver lesions classified after Myers et al. (1987). Ovarian stages and lesions classified after Johnson et al. (1991)
Otoliths	Age determination	Chilton and Beamish 1982
Sediment	AHs, CHs, pesticides	Sloan et al. 1993

Table 2C. Summary of Analytic Methods for Reproductive Toxicology in Flatfish.

Sample type	Analysis	Reference
liver	CHs, pesticides (screening) DNA adducts	Krahn et al. 1994 Reichert et al. 1994
stomach contents	AHs, CHs, pesticides	Sloan et al. 1993
bile	AH Metabolites	Krahn et al. 1986
Liver, kidney	Histopathology	Liver lesions classified after Myers et al. (1987)
Ovary	determine maturation stage	Ovarian stages and lesions classified after Johnson et al. (1991)
otoliths	Age determination	Chilton and Beamish 1982
Ovary, liver and gutted body weight	Gonadosomatic index Hepatosomatic index Condition index	Johnson et al. 1991
Blood plasma	plasma E2 levels	Sower and Schreck (1982).

Table 3. List of Organic Analytes

Detailed Organic Analyses (liver and stomach content tissues for juvenile salmon and toxicopathological studies, and for stomach contents for the reproductive toxicology study).

Low molecular weight AHs (stomach contents)
Naphthalene
Acenaphthylene
Fluorene
Anthracene
Acenaphthene
Phenanthrene
2-Methylnaphthalene
LAH [sum of low molecular weight AHs]

High molecular weight AHs (stomach contents)
Fluoranthene
Pyrene
Chrysene
Benzo(a)pyrene
Dibenz(ah)anthracene
Benz(a)anthracene
Benzo(b+k)fluoranthenes
ldeno(1,2,3-cd)pyrene
Benzo(ghi)perylene
HAHs [sum of high molecular weight AHs]

PCBs (stomach contents, liver)
PCB congeners (Nos. 18, 28, 44, 52, 66, 101, 118, 128, 138, 153, 170, 180, 187, 195, 206,209)

DDTs (stomach contents.. liver)
p,p'-DDE
p,p'-DDT
p,p'-DDD

Pesticides (stomach contents, liver)
Aldrin
Chlordane [sum of a andy]
Heptachlor
Dieldrin
Lindane
Hexachlorobenzene

Table 3. cont.

Hexachlorobutadiene (stomach contents, liver); see p 19 of December, 1995 Commencement Bay Damage Assessment Quality Assurance Plan

Analytes for Rapid CB analysis by HPLC/PDA
(liver tissues for reproductive toxicology study)

Dioxin-like PCB Congeners
77,105,118,126,156,157,169,170,180,189

Other PCB Congeners
101,128,138,153

DDTs
pp'-DDE, op'-DDT, op'-DDT, op'-DDD, pp' DDD

Hexachlorobenzene (HCB)

Table 4. Minimum Analytical Quality Control Criteria

Sample Type	Minimum Frequency	Acceptance Criteria
Organic chemicals in tissues		
Performance Evaluation	Initial	Majority of values for SRM within ± 50% of reference value (except when < 10 x MDL). RSD not to exceed ± 30%.
Calibration[1]	Initial/weekly	At least a four point curve. Standard curve correlation (r) > 0.9900 for all analytes.
Continuing calibration	Must start and end analytical sequence and every 10 field samples	For AHs, pesticides, and PCBs, the relative standard deviation (RSD) of the analyte responses relative to the internal standard will be ≤ 25%.
Reference material	Every 10 field samples	Concentrations of > 70% of individual analytes (AHs, PCBs, chlorinated pesticides) will be within 35% of either end of the 95% confidence interval range of the reference values. Does not apply to analytes with concentrations < 10 x MDL.
Method blank	Every 10 field samples	No more than 5 analytes in tissues are to exceed 3 x MDL, unless analyte not detected in associated sample(s) or analyte concentration > 10X blank value.
Sample duplicate	Every 20 field samples	RSD ≤ 50% or within 5 x MDL if less than 10 x MDL.
Internal standards/surrogates	Every sample	50-125 % recovery.
Mass spectral confirmation	About 10% of field samples	Confirmation based on acceptable match of 2 ions (PCBs and pesticides).
Interlab comparisons	Once per year	As defined by NIST.

1 Calibration standards will be supplied by or traceable to the National Institute of Standards and Technology (NIST), whenever available.

Table 4. Minimum Analytical Quality Control Criteria cont.

Sample Type	Minimum Frequency	Acceptance Criteria
Fluorescent aromatic compounds (bile)		
Performance Evaluation	Initial	Values for BaP, PHN and NPH should be within ±2 standard deviations of the interlaboratory mean that was established for the bile reference material (BRM) in the intercomparison exercise for the Alaska Oil Spill Damage Assessment Program.
Calibration	Initial/before each set	For BaP, PHN and NPH, the relative standard deviation of the analyte responses for two or more analyses of the calibration solution will be ≤5% before proceeding to analysis step.
Continuing calibration	Must start and end each analytical sequence and every 10 field samples	RSD ≤ 25% for each analyte (BaP, phenanthrene or naphthalene).
Reference material	2 BRMs for each sample set (up to 24 field samples)	Values should be within ±2 standard deviations of the interlaboratory mean for the bile reference material (BRM) as specified above in the initial performance evaluation.
Replicates	One for every 10 field samples	RSD ≤50%.
Method blank	Every sample set (up to 24 field samples)	HPLC analysis of solvent blank (water).

Table 4. Minimum Analytical Quality Control Criteria cont.

Sample Type	Minimum Frequency	Acceptance Criteria
Cytochrome P450 1A (measured as aryl hydrocarbon hydroxylase)		
Performance Evaluation	Each sample run in triplicate	RSD not to exceed ± 30% on any individual sample with activity > 50 pmol/mg microsomal protein/minute. For samples with activity < 50 pmol/mg microsomal protein/minute, allowable standard deviation is 20 pmol/mg microsomal protein/minute.
Microsomal Protein Standard Calibration	Every sample set (5-20 samples)	Standards run in triplicate. RSD not to exceed ±20%. Regression coefficient (r^2) of protein standard curve generated ≥0.990.
Method blank	Every sample set	1)boiled microsomal blanks run in duplicate 2)microsomal blank minus substrate run in duplicate Average value for 2 sets of duplicates (i.e. 4 blanks) not to exceed 50 dpm ^{14}C, for 150 µl aliqout of extracted reaction mixture.
Sample Type	**Minimum Frequency**	**Acceptance Criteria**
DNA adducts		
Performance evaluation	Duplicate every sample set	Based on confirmation of analysis of standard available from Chemsyn.
Calibration		
a) storage phosphor screens	Every sample set	2-point line; 3 dilutions of a solution containing ^{32}P to ensure that at least one dilution is less than 80,000 volume units per pixel.
b) liquid scintillation counter	Weekly	Less than ± 5% RSD for ^{14}C standards.
Method blank	Every sample set	Less than 10 nmol adducts/mol DNA.
Sample duplicate	Every 10 field samples	RSD ≤ 50% or within 5 x MDL if less than 10 x MDL.

Table 4. Minimum Analytical Quality Control Criteria, continued.

Sample Type	Minimum Frequency	Acceptance Criteria
Blood Plasma - Estradiol		
Performance evaluation	Each sample including standards, and quality control samples are tested in triplicate	RSD not to exceed ±20% on any individual sample.
Calibration	Each standard tested in triplicate	Serial dilution of the standards are run in triplicate including method blank. RSD not to exceed ±20% on any individual standard. Regression coefficient (r^2) must be >0.95. Binding efficiency of antibody to labelled antigen should be within the 25-40% range.
Sample triplicate	Each sample tested in triplicate	RSD not to exceed ±20% on any individual sample. The percent binding of the sample, before correction for dilution factors, must be within the effective range of the assay (effective range is the linear range of the standard curve where % binding = 20-80%). Samples which are below the detection limit of the assay are given a value equal to half of lower detection limit (EC 80).
Quality control sample	Tested in triplicate	Within run RSD not to exceed ±20%. Run to run RSD not to exceed ±30%.

Table 4. Minimum Analytical Quality Control Criteria, continued.

Sample Type	Minimum Frequency	Acceptance Criteria
Histopathology (Liver, Kidney and Gonad)		
Performance Evaluation	10% of samples re-read by chief histopathologist	If differences or errors in diagnoses are detected, the cause will be identified and appropriate cases re-evaluated.
Reference Material	Every sample	Meets standard processing techniques, diagnoses based on published criteria for identifying lesion types, problematic cases will be reviewed by Mark Myers, the chief histopathologist. Mark will consult with additional experienced fish histopathologists at other agencies if the need arises.

Sample Type	Minimum Frequency	Acceptance Criteria
Rapid PCB Analysis of Livers		
Performance Evaluation (NIST whale blubber control material)	Initial	Only a few CH analytes are resolved by HPLC method, so selected CHs are quantitated. Concentrations of ≥ 70% of these selected analytes [PCBs 105, 118, 138, 153, 156, 180, p,p'-DDE (at 266 nm), o,p-DDD, p,p'-DDD, p,p'-DDT, hexachlorobenzene] will be within ±50% of the published reference values. Does not apply to analytes with concentrations < 10 x MDL.
Calibration	Must start and end each analytical sequence and every 10 field samples	RSD ≤ 25% for each analyte (for the 15 PCBs and 5 DDTs in the calibration solution).
Control material (NIST whale blubber control material).	Every sample set (up to 14 field samples in set)	Same criteria as in performance evaluation (above).
Method blank	Every sample set (up to 14 field samples in set)	No more than 4 analytes in tissues are to exceed 4 x MDL, unless analyte not detected in associated sample(s).

Table 4. Minimum Analytical Quality Control Criteria, continued.

Sample Type	Minimum Frequency	Acceptance Criteria
Rapid PCB Analysis of Livers, continued.		
Sample duplicate	Every sample set (up to 14 field samples in set)	RSD ≤ 50% or within 5 x MDL if less than 10 x MDL.
Internal standards/ surrogates	Every sample	60-125% recovery.
MDL calculations	"Smallest analyte area" is determined for each analyte as the smallest peak that can be reliably integrated.	$\text{conc (ng/g)} = \dfrac{\dfrac{\text{ng surrogate std}}{\text{area surrogate std}} \times \text{smallest analyte area} \times \text{Rr}}{\text{sample weight (g, wet weight)}}$ $\text{Rrf} = \dfrac{\text{ng/µL of analyte in cal std}}{\text{area analyte in cal std}} \times \dfrac{\text{area surrogate in cal std}}{\text{ng/µL surrogate in cal std}}$

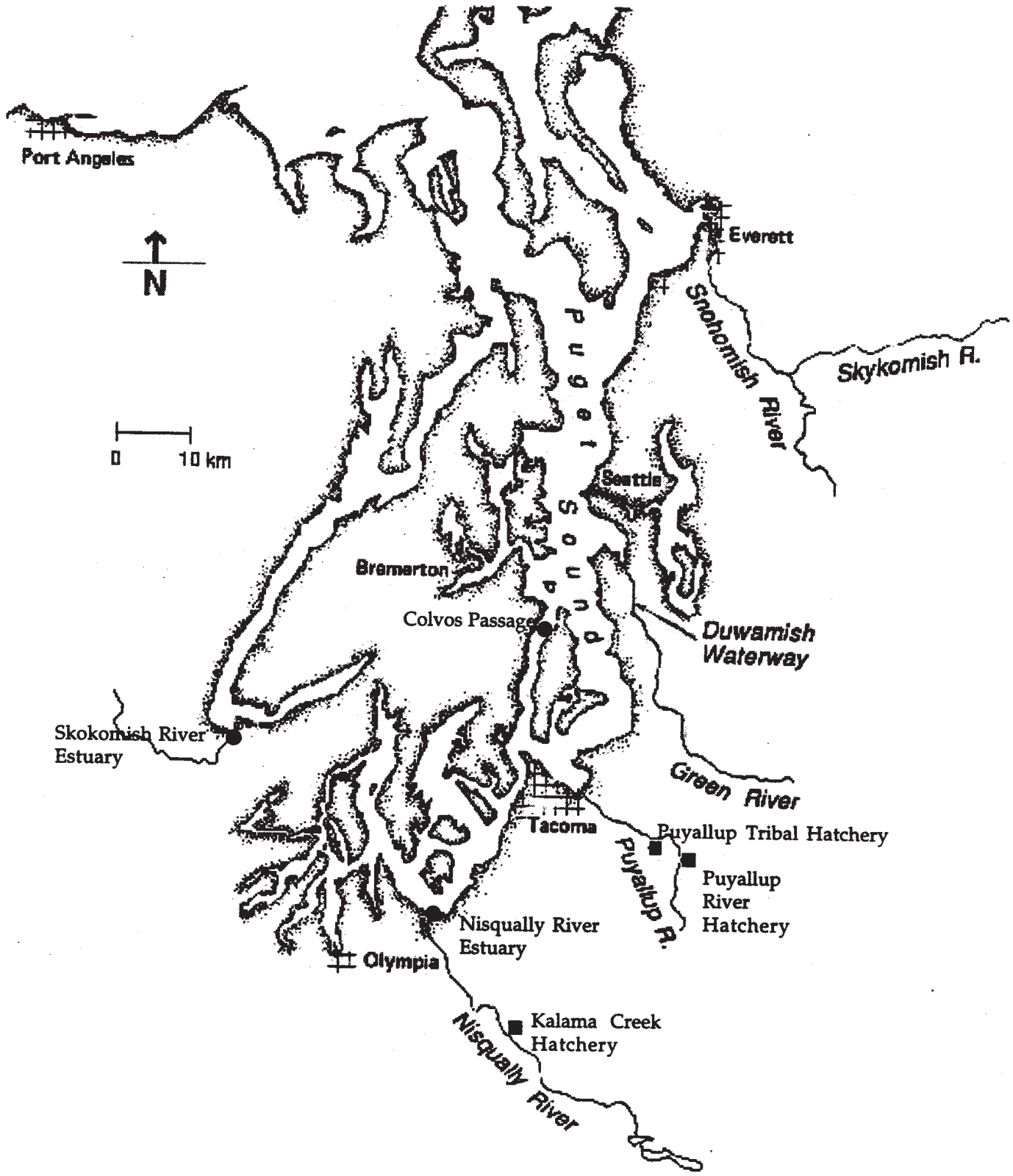

Figure 1. Reference sites for Hylebos Waterway fish injury studies

● = Reference sites: Colvos Passage is the reference site for flatfish investigations; Nisqually and Skokomish River Estuaries are reference sites for the juvenile salmon investigation.

■ = Hatcheries for reference juvenile salmon

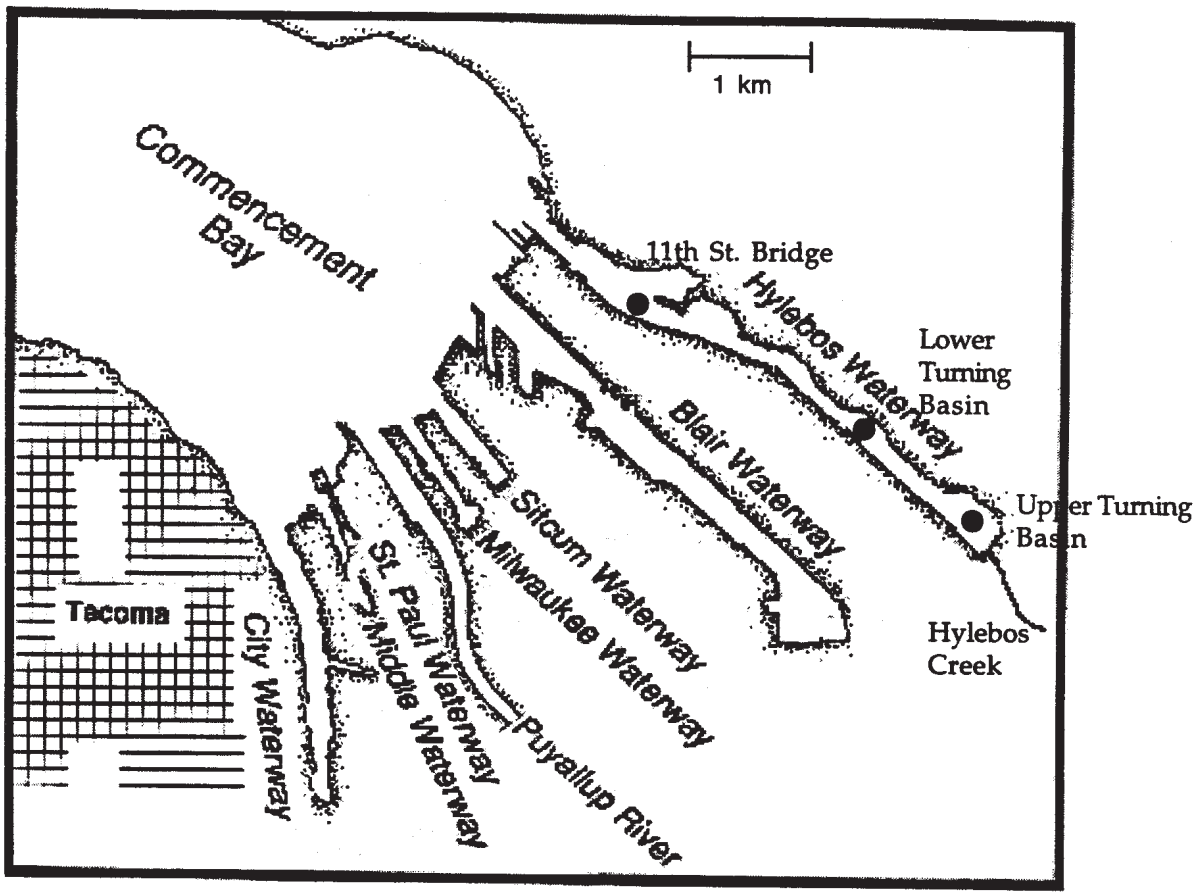

Figure 2. Sites located in Hylebos Waterway.

● = Site locations.

All three sites were used for the flatfish contaminant study. English sole captured for the reproductive toxicology study were primarily located near the 11th Street Bridge site. Juvenile salmon were captured from beaches and docks around the 11th Street Bridge Site.

Addendum for the
Hylebos Fish Injury Sampling and Analysis Plan (SAP)
1/28/97

This addendum contains changes to the SAP, primarily to, reflect differences between the work as originally proposed and the work as conducted. It also corrects minor errors noted in the SAP as originally submitted.

1) **Standard length vs Fork length.** Correction to the SAP: Standard length of flatfish was measured for the Toxicopathic Conditions Study, and the Reproductive Toxicology Study. Fork length of juvenile salmon was measured for the Juvenile Salmon, Injury Study.

2) **Number of flatfish collected.** The SAP originally stated that approximately 360 fish would be sampled for each of the flatfish studies (Toxicopathic Conditions Study, and the Reproductive Toxicology Study). Although 60 fish/site was the goal for the Toxicopathic, Conditions Study, and 30 fish/site/sampling period was the goal, for the, Reproductive Toxicology Study, the number of fish available at any given sampling period was sometimes fewer than anticipated. The most significant -change from the expected number of fish collected occurred in the March, 1995 Reproductive Toxicology sampling. No English sole were present at the Hylebos site during this sampling period, most likely due to normal migration patterns that occur during the spawning season. As a result, the reference site was also not sampled for that month.

The actual number of fish collected for the Toxicopathic; Conditions Study was 120 Rock sole and 233 English. sole for a total of 353. An additional 30 English sole were also collected at Case Inlet, which was the original choice for a reference site, but these fish were too small to be comparable with Hylebos fish. Therefore, these fish were not analyzed, and a new reference site (Colvos Passage) was identified and successfully sampled. The actual number of female English sole caught during the Reproductive Toxicology Study was 286. Of those, 275 were used in the Reproductive Toxicology Interpretive Report (11 fish were not used due to missing or questionable data).

In addition, extra fish not included in the SAP were collected during the Reproductive Toxicology Study, including male English sole, and female and male rock sole. These fish were not originally intended to be part of the Fish Injury Study, however the histopathology samples were processed and analyzed as outlined in the SAP, and these samples were also maintained under chain of custody. Histopathology data from these extra fish were ultimately included in the Toxicopathic Conditions Interpretive report to increase the size of the data set used to evaluate lesion prevalences and risk assessment. The number of extra fish added to the histopathology data was 7 male rock sole, 100 female rock sole and 110 male English sole. These additional fish are included in the histopathology case narrative. However, they are not included in the Summary reports of average site values; the Toxicopathic Conditions Summary Report includes only data specific to that study, while the Reproductive Toxicology Summary Report includes only female English sole. The Toxicopathic Conditions Study Interpretive Report (Section 2 of the Final Report) takes this data one step further by combining histopathology data from both

studies, as well as data from the extra fish described above, and includes additional bile FAC data described below.

3) **Additional individual bile analyses:** In addition to the bile analyses of individual fish collected from the December and January samplings for the Reproductive Toxicology Study as described in the SAP, a miscommunication resulted in the analysis of bile from an extra 106 individual fish. These additional bile samples were from some of the extra male English sole that were collected during the December and January samplings for the Reproductive Toxicology Study, and from both male and female English sole collected during months other than December and January. These additional bile samples were processed as described in the SAP, handled under chain of custody and met QA criteria. We therefore took advantage of this opportunity to increase the size of the data set, and included the extra bile FAC data in the lesion risk assessment portion of the Toxicopathic Conditions Interpretive Report (Section 2 of the Final Report). These additional bile samples are included in the revised case narrative (Appendix 3 to the Final Report).

4) **Correction of salmon bile FAC data for protein content of bile:** During collection of the bile from juvenile salmon, it was noted that it was difficult to ensure that there was no contamination of the collected bile with other fluids, such as blood or mucus. This was due to the very small sizes of the gall bladders, especially from the chum salmon. Because of this, protein correction was not used for the salmon bile data, as contamination by blood or mucus could substantially affect this value. For purposes of comparisons to data from other sites (Figure 1.13 in Final Report), only non-protein corrected values were used. For other portions of the fish injury studies [Toxicopathic and Reproductive Injury in Flatfish (Sections 2 and 3, respectively)], protein-corrected bile data were used for interpretation of results.