EVALUATION OF SEDIMENT TOXICITY USING A SUITE OF ASSESSMENT TOOLS

A Dissertation

by

MATTHEW ALLEN KELLEY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Toxicology

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Approved by:

Chair of Committee,	Thomas McDonald
Committee Members,	Bruce Duncan
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ABSTRACT

Evaluation of Sediment Toxicity Using a Suite of Assessment Tools.

(May 2010)

Matthew Allen Kelley, B.S., Texas A&M University;

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Chair of Advisory Committee: Dr. Thomas McDonald

Accurate characterization of risk of adverse ecological effects related to contaminated sediment presents a particularly difficult challenge. A series of studies has been conducted to investigate the utility of various tools for assessment of sediment toxicity. The goal of this research was to provide information which could help increase the accuracy with which predictions of toxicity could be made at hazardous sites.

A calibration study was conducted using model PAHs, PCBs, a binary PAH mixture and a coal-tar mixture. This study was a collaborative effort among five university-based Superfund Research Programs (SRPs). Each program, with the help of funding through the NIEHS Superfund Research Program, has developed a chemical-class specific assay to estimate toxicity of contaminants in sediment. This suite of bioassays expands the range of data typically obtained through the use of standard aquatic toxicity assays.

A series of caged *in situ* exposure studies has been conducted using juvenile Chinook salmon and Pacific staghorn sculpin in the Lower Duwamish Waterway. The study aimed to investigate the utility of selected biomarkers in evaluating the relationship between contaminants present in environmental samples and response in receptors following an *in situ* caged exposure. Results found that DNA adducts detected in exposed fish were significantly higher than controls in 2004 and 2006, and DNA adducts appear to be a reliable indicator of exposure, although no dose-response

relationship was present. Western blot analysis of CYP1A1 was not indicative of exposure levels.

The final study conducted was concerned with evaluating the utility of using solid phase microextraction (SPME) fibers *in situ* to evaluate contaminated sediment. Levels of PAHs and PCBs in sediment often exceeded sediment quality guidelines; however, results from aquatic toxicity bioassays using *Hyalella azteca* were mostly negative, thus levels of contaminants detected on SPME fibers could not be associated with adverse effects in *Hyalella*. However, regression analysis of total PAHs present in sediment and levels of PAHs detected in porewater SPME fiber samplers, which were placed 5 cm into the sediment for 30 days, revealed a strongly correlated linear relationship (R^2 = .779). Normalization of the sediment data to total organic carbon was performed to determine if the trend would remain present, and the linear relationship was again confirmed (R^2 =.709).

DEDICATION

To my family, especially my parents, Steve and Judy Kelley, my grandmother, Jane Butler, and to my mentor, the late Dr. K.C. Donnelly, who succumbed to esophageal cancer during preparation of this dissertation.

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NOMENCLATURE

1-OHP	1-hydroxypyrene
PCB 125	2,2’,4,4’,5,5’-hexachlorobiphenyl
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
DMN	2,6-dimethylnaphthalene
PCB 126	3,3’,4,4’,5-pentachlorobiphenyl
7-ER	7-ethoxyresorufin
ASE	Accelerated solvent extractor
AVS	Acid volatile sulfide
ARNT	AhR nuclear translocator
AET	Apparent effects threshold
AETA	Apparent effects threshold approach
AhH	Aryl hydrocarbon hydroxylases
AhR	Aryl hydrocarbon receptor
BPDE	BaP 7,8-diol-9,10-epoxide
BaPEq	BaP equivalents
BaP	benzo[a]pyrene
BCF	Bioconcentration factor
BEDS	Biological effects database for sediments
BED	Biologically effective dose
BSTT	Bulk sediment toxicity testing
CaMKIV	Calcium calmodulin-dependent kinase IV
CALUS	*Chemical-activated luciferase expression*
PCDD	Chlorinated dioxins
CSO	Combined sewer overflows
COC	Contaminants of concern
iCtrl	Control cells
CYP450	Cytochrome P450
M169	Dark mutant strain
DMSO	Dimethyl sulfoxide
DRE	Dioxin responsive elements

DL-PCB	Dioxin-like PCB
DO	Dissolved oxygen
ERA	Ecological risk assessment
EC_{50}	Effective concentration-50
ERL	Effects range-low
ERM	Effects range-medium
ETS	Environmental tobacco smoke
EH	Epoxide hydrolase
EqP	Equilibrium partitioning
ERE	Estrogen responsive elements
EROD	Ethoxyresorufin-O-deethylase
EEC	Extreme effects concentration
FCV	Final chronic values
Flu	Fluoranthene
FL/PY	Fluoranthene to pyrene
FAC	Fluorescent aromatic compounds
GJIC	Gap junctional intercellular communication
GST	Glutathione S-transferase
HAH	Halogenated aromatic hydrocarbons
HMW	High molecular weight
HPLC	High pressure liquid chromatography
hpf	Hours post fertilization
iAhR	Inhibited aryl hydrocarbon receptor
IACUC	Institutional animal care and use committees
IJC	International Joint Commission
IP	Intraperitoneal
LC50	Lethal concentration-50
LEL	Low effect level
LMW	Low molecular weight
LDW	Lower Duwamish Waterway
MPTC	Maximum permissible tissue concentration
mPECQ	Mean probable effect concentrations

mSQGQ	Mean sediment quality guideline quotients
MN	Micronucleus
MET	Minimal effect threshold
MAPK	Mitogen-activated protein kinase
MFO	Mixed function oxidases
NOAA	National Oceanic and Atmospheric Administration
NPL	National Priority List
NSTP	National Status and Trends Program
NTD	Neural tube defects
NEC	No effect concentration
NEL	No effect level
NET	No effect threshold
NDL-PCB	Non-dioxin-like PCBs
NWW	North Wind's Weir
OR	Odds ratio
OC	Organic carbon
K_{oc}	Organic carbon partition coefficient
OCP	Organochlorine pesticides
P450 RGS	P450 reporter gene system
CYP1A	P4501A
K_p	Partitioning coefficient
ppb	Parts per billion
ppm	Parts per million
CaMgPBS	PBS supplemented with 0.46 mM calcium chloride and 0.49 mM magnesium chloride
PH/AN	Phenanthrene to anthracene
PI3-K	Phosphatidylinositol-3-kinase
PCB	Polychlorinated biphenyls
PCDF	Polychlorinated dibenzofurans
PAH	Polycyclic aromatic hydrocarbons
PDMS	Polydimethylsiloxane
PEI	Polyethyleneimine

PEC	Probable effect concentration
PEL	Probable effects level
PKC	Protein kinase C
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
QSAR	Quantitative structure activity relationships
RAL	Relative adduct labeling
RLU	Relative light units
RM	River mile
SL/DT	Scrape loading/dye transfer
SLC	Screening level concentration
SMS	Sediment management standards
SQAL	Sediment quality advisory level
SQC	Sediment quality criteria
SQGQ	Sediment quality guideline quotients
SQG	Sediment quality guidelines
SQTA	Sediment quality triad approach
SPMD	Semi-permeable membrane devices
SVOC	Semivolatile organic compounds
SEL	Severe effect level
SPME	Solid phase microextraction
SCCWRP	Southern California Coastal Water Research Project
SSLC	Species screening level concentration
SSTT	Spiked-sediment toxicity test
SEM	Standard error of the mean
SRP	Superfund Research Programs
TLC	Thin-layer chromatography
TEC	Threshold effect concentration
TEL	Threshold effects level
TRA	Tissue residue approach
TDS	Total dissolved solids
TOC	Total organic carbon
TET	Toxic effect threshold

TIE	Toxicity identification evaluation
USEPA	United States Environmental Protection Agency
UDW	Upper Duwamish Waterway
VOC	Volatile organic compounds
WDOE	Washington Department of Ecology
WQC	Water quality criteria
WOE	Weight of evidence

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CHAPTER I

INTRODUCTION AND RESEARCH OBJECTIVES

Contaminated sediments represent a threat to the health of human or ecological receptors when the toxic or hazardous materials are available for absorption. Adams et al. (1992) define aquatic sediments loosely as “a collection of fine-, medium-, and coarse-grain minerals and organic particles that are found at the bottom of lakes, rivers, bays, estuaries and oceans”. These locations where contaminated sediments may be found include freshwater bodies of water, as well as marine and estuary locations. The U.S. Environmental Protection Agency (USEPA) defines contaminated sediments as “soils, sand and organic matter or minerals that accumulate on the bottom of a water body and contain toxic or hazardous materials that may adversely affect human health or the environment” (USEPA 1998).

These toxic or hazardous materials may include both organic and inorganic substances that adhere to the sediment particles with different strengths or affinities. The extent of absorption of the contaminants into target organs of ecological receptors is dependent on a range of factors including sediment characteristics, as well as the concentration of contaminant(s), route of exposure and the solubility of the contaminant(s). In an attempt to help site managers and risk assessors evaluate potential toxicity of contaminated sediments, various sediment screening levels have been created. These sediment screening levels typically compiled large amounts of toxicity data in order to estimate three different ranges of contaminant concentrations. The ranges often represent the concentration ranges below which toxicity would not be expected, middle range where toxicity would sometimes occur, or high ranges where toxicity would frequently be expected. Site managers can then compare concentrations of contaminants that have been measured in samples from their site and infer risk of toxicity.

This dissertation follows the style of Environmental Health Perspectives.

For many inorganic chemicals (e.g., cadmium, selenium), sediment screening levels are available. However, acceptable levels for most organics in sediment do not exist. Organic mixtures common in sediment include the polycyclic aromatic hydrocarbons (PAHs), polychlorinated organics such as chlorinated pesticides, polychlorinated biphenyls (PCBs) and chlorinated dioxins (PCDDs), and plasticizers including the phthalates and bisphenol. Sediments are usually contaminated with complex mixtures of chemicals rather than a single compound (Long et al. 2006). As most of these compounds are relatively insoluble in water, contaminants are often transpoted through partitioning to particulate organic carbon (OC) found in both sediment and the water column, as well as partitioning to OC which is dissolved in the water column, and inorganic particles in sediments.

Sediments are of concern as they may serve as a sink for contaminants in surface waters. Contaminants from groundwater may also reach ecological receptors in certain exposure pathways. Over time, inorganic and organic chemicals partition among various environmental compartments, including particulate matter, interstitial porewater and biological tissues (Reynoldson and Day 1993). Partitioning behavior and subsequent differences in bioavailability among contaminants have been explained for some divalent cations by equilibrium partitioning (EqP) theory for metals (Di Toro et al. 1990) using acid volatile sulfides. For organics, the EqP theory has been used to characterize partitioning behavior using organic carbon (Di Toro et al. 1991). Contamination of freshwater sediments is common in the United States. Ecological receptors may experience irreversible adverse effects if elevated contaminant concentrations are present in sediment or adjacent habitat (Oberholster et al. 2005). Contamination of freshwater sediments prompted the International Joint Commission (IJC) to list forty-three areas of concern in the Great Lakes (Crane and MacDonald 2003; IJC 1989). Other freshwater sites where sediments have been found to be impacted by environmental contaminants include the lower Columbia River in Washington, the St. Louis River in Minnesota, and the Hudson River in New York (Long et al. 2006).

In addition to their presence in freshwater sediment, complex chemical mixtures have also been detected in a broad range of marine and estuary sediments in the United

States. As with freshwater sediments, contaminants in marine sediments may also be transferred to human or ecological receptors (National Research Council 1997). The National Academy of Science also recommends that ranking and remediation of marine sediments be based on formal assessments of the risk (National Research Council 1997). Contaminated marine and estuary sediment sites are prevalent across the United States. Extensive data exist to characterize sediment contamination in the Puget Sound in Washington (Malins et al. 1987; McCain et al. 1990; Stein et al. 1995). Other contaminated estuary or marine sediments reported in literature include the Hudson River Estuary in New Jersey and New York, Biscayne Bay in Florida, San Pedro Bay in California, New Bedford Harbor in Massachusetts, Baltimore Harbor in Maryland, and Delaware Bay, among others (Ford et al. 2005; Hartwell and Hameedi 2006; Leblanc et al. 2006; Long et al. 2001; Manyin and Rowe 2006). Once areas of concern are identified, assessment of potential toxicity in both ecological receptors and humans from exposure to contaminated sediments can prove to be a difficult task.

Although it has been clearly established that sediment contamination can have many detrimental effects on aquatic organisms, methods to quantify the potential of contaminated sediment to effect an ecosystem are less well-defined (Apitz et al. 2004). The presence of organic and inorganic contaminants in sediments may affect ecosystems, resources, and human health (Chapman et al. 2002). Crane and McDonald (2003) indicate that the contamination of sediments may lead to restrictions on the use of water systems including fish advisories and restrictions on disposal of dredged material. The EPA has estimated that there are 1 billion cubic meters of contaminated sediments in the United States that may represent a risk to human health (USEPA 1997). Birch and Taylor (2002) have suggested that sediments may represent a significant risk to human and ecological health because they are a major carrier of pollutants and have high sorptive capacities for both organic and inorganic compounds. A potential adverse outcome of contaminated sediments is reduced survival in the benthic community that is in intimate contact with sediments (Birch and Taylor 2002). In addition, transfer of sediment contaminants to the benthic community may also affect other wildlife and humans as the contaminants are transferred through the food chain (Ankley et al. 1992; Kannan et al. 2005). Bridges et al. (2006) observe that the level of risk associated with contamination of sediments is influenced by a complex array of

physical, chemical, and biological processes. The report on Great Lakes water quality by the International Joint Commission (2002) describes sediment as the largest source of contaminants in the food chain. Complicating matters, a variety of contaminants may be present in contaminated sediment.

Types of contaminants

Surface waters and sediments may receive contaminants from a variety of sources, including air deposition, surface runoff and direct discharge. Because of the diverse sources of contaminants, sediments most often contain both organic and inorganic contaminants. For example, analysis of sediments from the St. Louis River in Wisconsin detected metals, PAHs, PCBs and chlorinated pesticides (Crane and MacDonald 2003). Mercury, which is a concern for biomagnification in the food chain, has also been detected in sediments at numerous locations (Chapman and Anderson 2005). PAHs and PCBs are two of the most common classes of organic contaminants detected in sediments and are thus a major focus sediment-related research.

PAHs

The term Polycyclic Aromatic Hydrocarbons **(**PAHs) is used to describe a group of over 100 chemicals which can be formed during the incomplete burning of fossil fuels such as coal, oil and gas, as well as garbage, or other organic substances like tobacco and charbroiled meats (ATSDR 1995). PAHs first became of concern in 1775, when soot was implicated in causing scrotum cancer among chimney sweeps (Pott 1775). Exposure to PAHs often occurs in the form of complex mixtures of PAHs instead of individual chemicals (Ates et al. 2004). In addition, studies have shown that PAHs cause cancer in animals and PAHs have been classified as human carcinogens due to associations with cancers such as bladder, skin, breast, lymphoma, and testicular cancer (Ates et al. 2004). Following exposure, PAHs have the ability to readily cross the placenta, thus PAHs can also be classified as developmental toxicants (Barbieri et al. 1986; Bui et al. 1986; Neubert and Tapken 1988; Srivastava et al. 1986). Many PAHs have no known use except as research chemicals (Hawley 1987; HSDB 1994), while other PAHs are often formed as a byproduct or a chemical intermediate of manufacturing processes. For example, anthracene serves as an intermediate during

dye production and manufacturing of synthetic fibers, as well as serving as a diluent for wood preservatives (Hawley 1987; HSDB 1994). Anthracene provides an additional example, as the PAH is used during synthesis of the chemotherapeutic agent amsacrine (Wadler et al. 1986). Other uses for selected PAHs include manufacturing of pharmaceuticals and plastics, insecticide and fungicide, explosives, polyradicals for resins, and as lining material in steel and iron drinking water pipes and storage tanks (Hawley 1987; HSDB 1994; NRC 1983; Windholz 1983).

Due to this abundance of historical uses of PAHs, these compounds are now widespread environmental contaminants. Further, sources of emission of these compounds mainly stem from human activities (Schoket 1999; WHO 1987). At least 54 PAHs have been found at one or more National Priority List (NPL) sites (ATSDR 1995). Figure 1 lists the 16 PAHs that the United States Environmental Protection Agency has included on the list of priority pollutants. Measurements of these contaminants are often presented as total PAHs, as PAHs rarely occur as a single compound. Thus exposure to PAHs typically involves complex mixtures of multiple contaminants (Gladen et al. 2000).

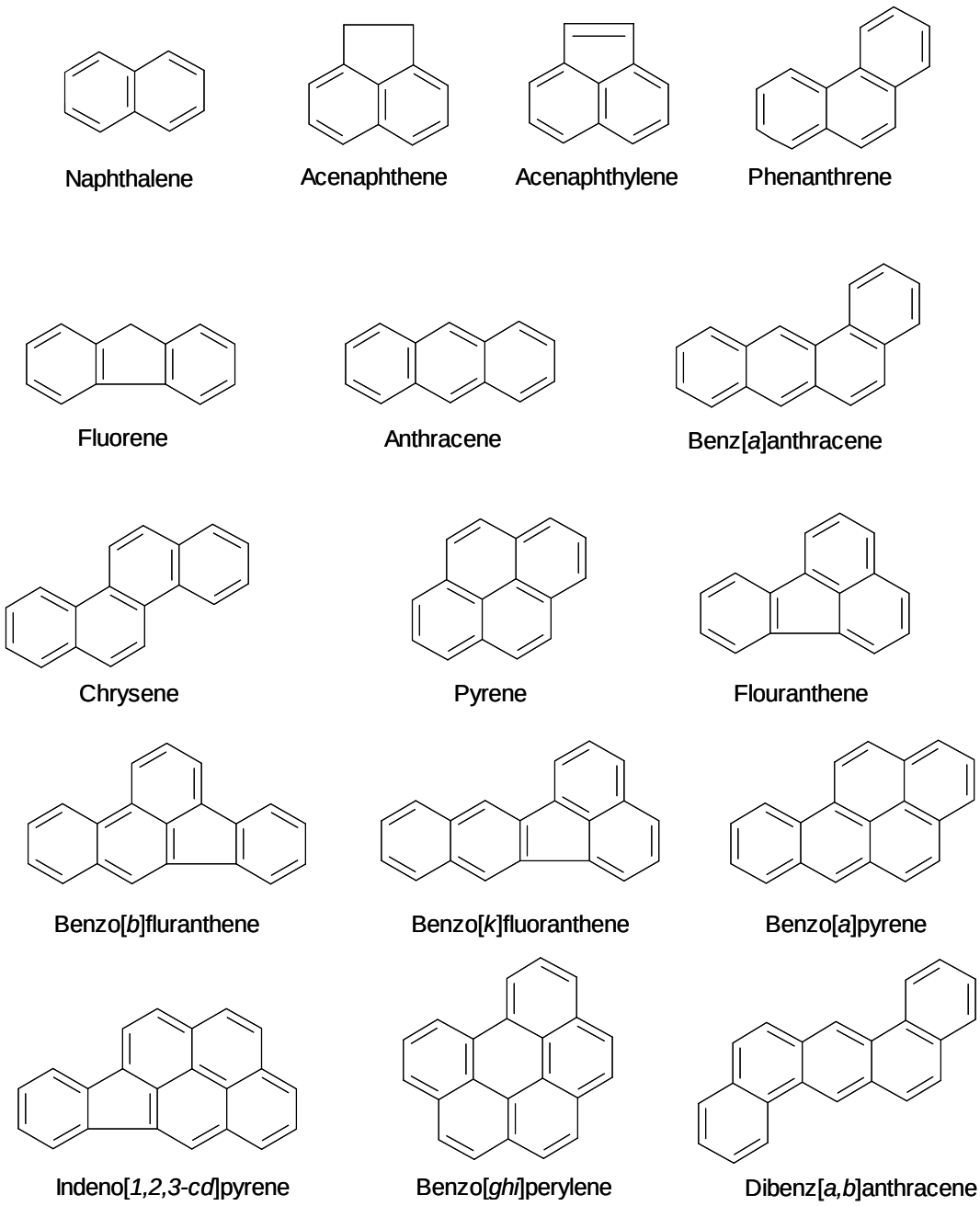

Figure 1. Structures of the 16 EPA Priority Pollutant PAHs (Office of the Federal Registration 1982).

PAHs are usually formed during processes such as incomplete combustion or pyrolysis of organic materials used in energy production such as fossil fuels (Jedrychowski et al. 2003). While PAHs are naturally occurring chemicals which are ubiquitous in the environment (Ates et al. 2004), the dependence of industrial nations on fossil fuels has exacerbated the situation, making exposure to PAHs unavoidable.

The main sources of human exposure to PAHs include occupational exposures, passive and active smoking, and consumption of PAH-contaminated food or water (Jongeneelen 1997). In non-smokers, occupation followed by diet are the two most important sources of exposure (Castano-Vinyals et al. 2004). One example of PAH exposure in foods would be char-broiled meats (Jedrychowski et al. 2003). Inhalation of contaminated air is also of concern as PAHs are also often found in the atmosphere sorbed to particulates or as gases (ATSDR 1995). The most prominent sources of PAHs in air pollution include incomplete combustion of fuel used in residential heating as well as industrial and motor vehicle exhaust (Peluso et al. 1998). In addition, PAH occurrence is not limited to the outdoor environment, as PAHs have also been detected in house dust in various regions around the world (Naspinski et al. 2008; Naufal et al. 2007). Other sources of PAHs in air pollution include coal-fired power plants, kerosene heaters, waste incinerators, and volcanoes (Ates et al. 2004; Gladen et al. 2000; Harris et al. 1984; Tokiwa et al. 1985). Non-combustion sources of PAHs include production of coal tar and creosote among others (Pozzoli et al. 2004).

The potential of PAHs to cause adverse effects in both ecological and human receptors is related to the chemical and physical properties of the compounds. PAHs are a group of organic chemicals characterized by chemical stability, low volatility, and low solubility in water (Castano-Vinyals et al. 2004). In addition, PAHs are considered to be potentially hazardous to humans due to their lipophilicity and biological activity (Tang et al. 2003). It is also important to note that the biological properties of PAHs are not yet fully known (Jedrychowski et al. 2003). Environmental PAHs are mainly particle-bound and exposure may be the result of ingestion, skin absorption, or inhalation (Marafie et al. 2000). Molecularly, PAHs are large flat molecules consisting of a collection of fused benzene-like rings (Goodsell 2004). This structure allows PAHs to pass easily through cell membranes and enter cells rapidly (Goodsell 2004). PAHs are also thermally

stable and demonstrate a high melting point, a high boiling point, and low vapor pressure due to their molecular weight and structure (Pozzoli et al. 2004). Also, it should be noted that as the molecular weight or ring number of PAHs increases, the vapor pressure decreases (Pozzoli et al. 2004).

PAHs have also been the subject of a substantial amount of toxicity research. This research has shown that many PAHs are known human mutagens, carcinogens, and/or developmental toxicants (Perera et al. 2005). Much of the available literature focuses on chronic effects of PAHs such as cancer. In fact, ATSDR indicates a data gap by stating that more information is needed regarding potential adverse health effects associated with acute-duration exposure to PAHs in humans and animals (ATSDR 1995). Though still in small quantities, some data is available concerning other exposure routes. ATSDR states that studies have identified the skin and liver as target organs for acute duration oral and dermal exposures to PAHs (Iwata et al. 1981; Nousiainen et al. 1984). The respiratory system is also a target organ of acute exposure to PAHs. This is because PAHs often reside on the particulate fraction of combustion products (Talaska et al. 1996) and have been associated with adverse respiratory effects in literature. Acute effects in the respiratory system that have been associated with exposure to PAHs include problems such as vomiting with blood, difficulty breathing, chest pains and irritation, coughing and throat irritation (ATSDR 1995).

The most prominent outcome from chronic exposure to PAHs is cancer, as seven PAHs have been classified as human carcinogens (Castano-Vinyals et al. 2004). Several target organs may show effects of chronic exposure to PAHs, as associations have been found between PAHs and bladder, skin, breast, lymphoma, and testicular cancer (Ates et al. 2004). Other chronic or sub-chronic effects include reproductive and developmental effects (Gladen et al. 2000). The endocrine system is another target organ of chronic exposure as chronic PAH exposure has been associated with disruption of the endocrine system (Jedrychowski et al. 2003). Finally, the brain may also be a target organ of chronic exposure to PAHs. This is because some PAHs may be distributed to the brain and have been associated with various effects such as

tumorigenesis, inhibition of enzymes that help with metabolism of neurotransmitters, as well as impairment of various nervous system functions (Tang et al. 2003).

Biomarkers are often used in assessment of risk for adverse effects, such as those mentioned above, from PAH-related exposures or as early indicators of exposure to PAHs. According to ATSDR, biomarkers are indicators signaling events in biologic systems (1995). Biomarkers are very important because they can give an estimation of the total internal dose from all routes of exposure (Talaska et al. 1996). This eliminates some of the limitations involved in environmental sampling. Biomarkers are often classified into various categories including biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. Urinary 1-hydroxypyrene (1-OHP) can be used as a biomarker of exposure as it is a biomarker which demonstrates the absorbed dose of aromatic hydrocarbons (Ates et al. 2004). DNA adducts are another biomarker of exposure, though some also call it a biomarker of effect as well. DNA adducts are used in multiple forms of exposure monitoring. Exposure to PAHs or PAH-containing mixtures results in the formation of DNA adducts, which can measured in tissues or blood in both humans and animals (ATSDR 1995). Biomarkers such as DNA adducts are particularly useful as it is generally considered that DNA adduct formation represents the biologically effective dose, or in other words, the dose which actually reaches the target tissues (Poirier and Weston 1996).

Much of what is known about the toxicigty of PAHs has been discovered based on research conducted using animals. Results have demonstrated that PAHs have been linked to several adverse health effects in animal studies. Among these effects of exposure in animals are immunotoxicity, genotoxicity, carcinogenicity, and reproductive toxicity such as stillbirths, reabsoprtion, and congenital abnormalities (Jedrychowski et al. 2003). More specifically, Benzo[a]pyrene (BaP), one of the most studied PAHs, has been associated with various types of tumors when administered to mice including lung, liver, lymphoreticular, and stomach (Vesselinovitch et al. 1975). In addition, an animal experiment conducted by Mukhtar et al. (1986) exposed mice to crude coal tar, a complex PAH mixture, and individual PAHs, such as BaP. Results of this study indicated that while crude coal tar was found to induce skin papillomas, it was a weaker

tumor initiator than many individual PAHs. This may be indicative of antagonistic interaction among PAHs in complex mixtures such as coal tar.

In an attempt to evaluate a potential relationship between PAH exposure and breast cancer, Rudel et al. (2007) searched literature and databases to identify published works in which PAHs have been associated with mammary gland tumors in animal studies. It should be noted that results of the search produced a list of PAHs such as benzo[a]pyrene (BaP) and dibenz[a,h]anthracene. Shum et al. (1979) demonstrated that BaP administered by ip injection in doses ranging from 50 to 300 mg/Kg body weight to mice at day 7 or day 10 of gestation caused in utero toxicity and teratogenicity in responsive mice strains. Some of the most common malformations observed in the study include club foot, red neves of skin, cleft palate or lip, curly tail, and abnormal pigmentation, among others. In addition, Shum et al. (1979) also noted that allelic differences at a single genetic locus (the Ah locus) can be correlated with dysmorphogenesis.

While animal research which has demonstrated that PAHs have long been known to cause cancer in animals and the belief that PAHs contribute to cancer in humans as well (Ates et al. 2004), epidemiologic studies have been used to further illustrate the relationship between exposure to PAHs and adverse effects in humans. In a review of epidemiologic studies involving occupational and environmental exposures to PAHs, Boffetta et al. (1997) concluded that heavy occupational exposure to PAHs substantially increased risk of lung, skin and bladder cancer. Among these results, lung cancer was the most consistent association found, while skin cancer was typically only associated when dermal exposure was present, and bladder cancer associations were only present in certain industries, such as aluminum production and coal and tar related industrial processes (Boffetta et al. 1997). Further evidence of an association between PAHs and lung cancer was provided by a case-control study, conducted by Tang at al. (1995), which found that cases had a constitutional susceptibility to lung cancer, as there were several distinct differences between cases and controls. For example, among cases, DNA adducts increased with cigarettes smoked. Still another epidemiologic study, conducted by Bennett et al. (1999), noted a dose-response relationship between environmental tobacco smoke and lung cancer risk among never-smokers with a

glutathione S-transferase (GST) variant, which results in a deficiency in GSTM1 activity. In addition, Peluso et al. (2005) observed that adducts were associated with increased lung cancer risk (OR = 1.86) and interestingly enough, the association was much stronger among never-smokers (OR = 4.04).

While associations between PAH exposure and lung cancer have frequently been investigated, other types of cancer have also been examined with epidemiologic studies. Interpretation of results from studies in which adverse effects are weakly or mildly associated with exposure to contaminants has often been challenging. For example, Castano-Vinyals et al. (2007) noted that while occupational studies in which there was exposure to PAHs have shown excess risk of bladder cancer, the exposures used in these studies also included other chemicals such as nitro-PAHs and aromatic amines, thus it is difficult to determine what portion of the elevated risk may be due to PAH exposure. In an effort to control for confounding, Castano-Vinyals et al. (2007) conducted a study using only nonsmokers and found no association between levels of DNA adducts and bladder cancer risk. Interpretation of results from studies examining the relationship between PAH exposure and breast cancer has also proved difficult. For example, McCarty et al. (2009) observed a moderate increased risk of breast cancer (OR = 1.56) among women with detectable levels of DNA adducts and three or more GST variants.

Adverse effects that have been associated with PAH exposure are not limited to various types of cancer. In addition to being human mutagens, PAHs have also been shown to be developmental toxicants (Perera et al. 2005). Among those developmental effects that have been associated with PAH exposure, Wang et al. (2008) noted that PAH-DNA adducts have also been implicated in neuro-developmental deficits in exposed children. Another recent study found that PAH-DNA adducts, along with Environmental Tobacco Smoke (ETS) exposure, were associated with developmental adverse effects such as reduced birth weight and smaller head circumference (Perera et al. 2005). Further supporting the asoociation between PAH exposure and developmental effects, a case-control studyconducted by Naufal et al. (2010), noted that PAH exposure may be linked to adverse birth outcomes such as neural tube defects (NTDs) in a heavily exposed population in Shanxi, China. The study found that mothers whose concentrations of total PAHs in venous blood were above the median were found to have an age-adjusted odds ratio (OR) of 8.7 for having a child with an NTD. Recently, research has been conducted examining potential links between PAHs and breast cancer.

Much attention has also been devoted to the mechanism by which PAHs exert their toxicity. PAHs require metabolic activation prior to exertion of genotoxicity (Pisoni et al. 2004). Cancer is thought to be a multistage process, whose mechanism includes an initiating event and a series of events in which growth of the initiated cell is promoted, followed by progression of the cell to a rapidly growing malignant stage (Reeves et al. 2001).

One potential mechanism of toxicity for PAH-related cancer through inhalation is described by Castano-Vinyals et al. (2004). In this mechanism, PAHs are mainly inhaled through the bronchial epithelium and then distributed to tissues. Once in tissues, the PAHs are biotransformed to chemically reactive intermediates which bind to DNA forming DNA adducts. DNA adducts, with bulky aromatic rings attached at the base blocking replication and transcription, prevent the action of helicases and topiosomerases (Goodsell 2004). Cells are capable of repairing these molecules, however the enzymes responsible for this task sometimes fail, creating mutations which if occurring at an essential part of the genome can cause tumor formation and cancer (Goodsell 2004). It is also of note that the amount of ultimate carcinogen produced can vary greatly as it is a function of competing activation and detoxification pathways (Pisoni et al. 2004).

While PAHs are ubiquitous in the environment, concentrations of PAHs in sediment are of particular interest. In areas of industrial activity, sediments act as an ultimate sink for contaminants which have been released into the environment (Jarvis et al. 1996). This is in part because PAHs partition preferentially from water to sediments due to their hydrophobicity (Kannan et al. 2005). Largely due the primary source of PAHs (e.g. combustion by-products), these compounds are typically found as mixtures, and most often are present in sediments in association with other classes of chemicals (Neff et al. 2005).

A large number of investigations have charcterized PAHs in United States sediments. Selected concentrations of PAHs that have been reported in literature are listed in table 1. The reported values emphasize the variation in regard to concentrations of contaminants present from one site to another as values range from less than 1 ppm to over 17000 ppm.

Table 1. Selected concentrations of total PAHs in sediment reported in literature are displayed.

Reference	Location	PAH Concentration
Manyin and Rowe 2006	Baltimore Harbor	10.8 ppm
Neff et al. 2005	Wycoff/Eagle Harbor, WA	17,283 ppm
Gu et al. 2003	Black River, OH	250 ppm
Kannan et al. 2005	Various Inland Lakes, MI	0.25 – 17 ppm
Su et al. 1998	Green Bay, WI	0.8 – 8 ppm

In Wycoff/Eagle Harbor in Washington, the release of creosote from a wood preserving plant resulted in total PAH concentrations in sediment as high as 17,283 mg/kg (Neff et al. 2005). Fish and Principe (1994) found total PAH in sediment from the Hudson River in New York to be approximately 25 mg/kg, while Gu et al. (2003) detected 250 mg/kg total PAH in the Black River in Ohio. Total PAH concentrations in sediment from inland lakes in Michigan were found to range from less than 0.25 to 16.8 mg/kg dry weight (Kannan et al. 2005). Sediments from Green Bay were found to contain total PAH concentrations ranging from 0.84 to 8 mg/kg (Su et al. 1998). Once contaminants such as PAHs are present in sediment, they have the potential to biomagnify through the food web. This is of concern as, in addition to non-carcinogenic adverse effects, seven individual PAHs have been listed by the US EPA as Class B2 carcinogens (USEPA 2005). In addition, many of the lower molecular weight PAHs may induce enzyme activity and potentially increase the toxicity of the carcinogenic PAHs. Currently, limited data exist from which to characterize the carcinogenicity or bioavailability of PAH mixtures. Among the data that are available, the narcotic effects of PAHs are generally associated with relatively high dose acute exposures. The proposed research will focus on effects that are more likely to be associated with chronic low dose exposures.

Much of the metabolism research for PAHs in aquatic receptors has been conducted using one or two contaminants, typically naphthalene and BaP, as a model for PAHs. For instance, Varanasi et al. (1979) conducted a study investigating the metabolism of naphthalene by exposing juvenile starry flounder (*Platichthys stellatus*) and rock sole (*Lepidopsetta bilineata*) to radiolabled napthalene. The finding revealed that these species were in fact able to extensively metabolize naphthalene and within 168 hours, more than half of the radioactivity was associated with metabolites, rather than the parent naphthalene. In addition, the authors noted that naphthalene and its metabolites were broadly distributed throughout various tissues and body fluids of the test species. Exposures were conducted through both dietary and intraperitoneal (ip) injection. The brain was among those tissues where naphthalene and metabolites were found and the authors speculate that it may have occurred because the blood-brain barrier is not fully developed yet in juveniles. Consistent with previous literature, large amounts of naphthalene metabolites accumulated in bile. Results also demonstrated that detectable levels of naphthalene and metabolites were present in epidermal mucas and

gills, which suggest that in addition to biliary and renal excretion, PAHs such as naphthalene may be eliminated via the epidermal mucas and gills.

In another PAH metabolism study, Collier et al. (1978) investigated the effect of varying temperature affect uptake and metabolism of naphthalene. The results indicated that lower temperatures resulted in higher retention of naphthalene and metabolites. Roubel et al. (1977) also conducted a metabolism and feeding study using naphthalene and anthracene administered to coho salmon (*Oncorhynchus kisutch*). Results indicated that anthracene and metabolites were retained to a greater extent than naphthalene and metabolites in liver brain and flesh. In addition, the authors administered radiolabeled benzene-U-^{14}C, naphthalene-1-^{14}C, and anthracene-9-^{14}C by ip injection and noted that accumulation of radio activity was greatest for anthracene, followed by naphthalene, and then benzene.

Melancon and Lech (1978) conducted a study examining both short- and long-term water exposures with rainbow trout (*Salmo* gairdneri) using ^{14}C-napthalene and ^{14}C-2-methylnapthalene. For the short-term exposure, naphthalene was found to be present in fish tissue at levels 22 to 340 times the initial water concentration, while average biliary concentration of naphthalene and metabolites was 300 times the initial exposure level during an 8-32 hour exposure period. For the long-term exposure study, 2-methylnapthalene was taken up faster than naphthalene and accumulated more in tissue initially, however over time 2-methylnapthalene was eliminated more quickly from the liver, blood and muscle. The authors noted that this may result in greater accumulation of naphthalene than 2-methylnapthalene over time given equal exposures.

Concerned about the effects of pollution related to transport of petroleum, Roubal et al. (1978) conducted a water exposure study in which coho salmon and starry flounder were exposed to a water soluble fraction of crude oil. Exposed fish were evaluated over two and six week exposure periods. Important findings of the study include that alkyl-substituted aromatic hydrocarbons accumulated preferentially compared to parent compounds and accumulation of water-soluble hydrocarbons increased with extent of ring substitution. In addition, important differences in uptake and elimination between the fish species were noted. Starry flounder accumulated hydrocarbons to a much

greater extent than coho salmon and retained those hydrocarbons longer when fish were transferred to clean water.

In another study related to petroleum pollution, Grunger et al. (1977) examined aryl hydrocarbon hydroxylases (AHH) induction following a water exposure study dosing coho salmon with15 parts per billion (ppb) and 150 ppb water soluble crude oil extracts. In addition, the authors conducted two feeding experiments in which food pellets were impregnated with 1 part per million (ppm) crude oil, PCBs alone, or a mixture of crude oil and PCBs. The water exposure period was six days. At the 15 ppb exposure level no significant difference in AHH activity between controls and exposed groups was noted, however AHH activity in the 150 ppb was significantly increased. In the two feeding studies, exposure time was 60 and 68 days respectively. In the two diet exposure study, fish fed mixtures of crude oil and PCBs had significantly higher AHH activity than fish fed only crude. In the 68 day exposure, crude oil and PCB mixture fish and PCB alone exposed fish were also significantly higher than control fish in regard to AHH activity.

Malins et al. (1979) conducted a study on metabolic fate of aromatic hydrocarbons using rainbow trout and coho salmon which were dosed with radiolabeled naphthalene and 2,6-dimethylnaphthalene. The authors compared naphthalene metabolites in the brain, liver and urine and found different proportions of metabolites at each location. In adult fish, primarily non-conjugated derivatives were found in the brain, likely due to blood-brain barrier, while a more complex mixture of conjugated and nonconjugated derivatives were found in the liver.

Collier et al. (1980) examined the disposition of orally administered naphthalene in the brain of rainbow trout. The author did note accumulation of naphthalene in the liver, blood and brain, with the maximum level occurring at 16 hours following exposure. Levels of the parent compound in the brain were similar to that found in the liver. In the liver and blood, the dihydrodiol was the major non-conjugated metabolite, while the sulfate was the major conjugated metabolite of naphthalene. While the dihydrodiol was also the major metabolite in the brain, evidence of conjugated metabolites in the brain was not found.

Reichert and Varanasi (1982) orally administered naphthalene to spawning English sole (*Parophrys vetulus*) and examined metabolism and sex differences. Results indicated that after 24 hours, 5% of the total dose of naphthalene was present in ovaries, while 1-2% was present in liver and muscle. Twenty-four hours following exposure, concentration of naphthalene in tissue declined rapidly. In relation to sex differences, consistent with other literature on cytochrome P-450, the results demonstrated that males exhibited higher naphthalene hydroxylase than females. In addition, English sole, during each exposure period, ovaries were found to have concentrations three times higher than that of testes. The authors noted that if fish were sedentary, it would be possible to accumulate enough aromatic hydrocarbons in the ovaries to potentially prevent successful reproduction.

Sanborn and Malins (1977) conducted a water based naphthalene exposure study using marine larval invertebrates. The test species were exposed to 8-12 ppb of radiolabeled naphthalene. Results revealed that all test organisms dies within 24-36 hours, thus the naphthalene was acutely toxic to stage I and stage V spot shrimp (*Pandalus platyceros*), as well as newly hatched Dungeness crab (*Cancer magister*). The authors note that these results were in contrast to other published reports which detailed resistance among invertebrate larvae to higher concentrations of petroleum hydrocarbons.

In addition to the naphthalene research discussed above, several studies have been carried out investigating the metabolism, uptake and elimination of BaP in fish. Gmur and Varanasi (1982) force fed English sole BaP and the after 24 hours examined bile, liver and muscle for metabolic products. Consistent with many of the studies mentioned above, thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) were used to conduct the analysis. The most important finding of the study was the presence of BaP 7,8-dihydrodiol in English sole. This is important because BaP 7,8-dihydrodiol could possibly be metabolized further to BaP 7,8-diol-9,10-epoxide (BPDE), which has been shown to covalently bind with DNA and is implicated as the ultimate carcinogenic metabolite of BaP (Kapitulnik et al. 1978; Koreeda et al. 1978; Sims et al. 1974; Slaga et al. 1977; Thakker et al. 1976). The authors also note that Varanasi et al. (1981) measured covalent binding of BaP to DNA in English sole liver

and found levels of binding that were comparable to the BaP covalent binding levels that Lutz (1979) reported as carcinogenic in mouse skin and mammary glands.

Varanasi et al. (1982) conducted an exposure study in which BaP was administered orally a few days prior to spawning. The purpose of this study was to determine if radiolabeled BaP and metabolites would be transferred to the gonads. Results revealed that after 24 hours, liver gonads, blood and bile all had detectable levels of BaP, as well as metabolites. Another important finding is that following the single exposure, BaP was retained in the gonads for a minimum of one week, thus indicating that if exposure occurs prior to spawning, BaP and metabolites may be present in eggs and sperm during fertilization. The authors emphasized the importance of this finding as they noted that studies in which other species of fish were force-fed BaP hours before spawning resulted in adverse reproductive outcomes. In addition, the extensive metabolism of BaP in the liver and the presences of large concentrations of multiple BaP metabolites in bile demonstrated that BaP is indeed extensively metabolized and excreted by ripe English sole when administered orally.

Egaas and Varanasi (1982) conducted a study in which rainbow trout were held in water at two different temperatures and exposed to the PCB aroclor 1254. Following this water exposure, the livers were excised and binding and metabolism studies were conducted *in vitro* by exposing the samples to radiolabeled BaP. Key findings included that induction with aroclor 1254 increased the ability of liver enzymes to metabolize BaP into toxic metabolites which are capable of binding with DNA. In addition, decreased temperature resulted in greater basal metabolism and increased time before the effect of the inducing PCB was evident.

Nishimota and Varanasi (1985) conducted a study using liver microsomes from wild caught English Sole. The experiment exposed these microsomes to radiolabeled BaP and BaP 7,8-dihydrodiol in order to determine if both of these compounds were metabolized to the *anti*-BPDE and also examined DNA adduct formation when BaP and BaP 7,8-dihyrodiol-modified DNA was added to the incubation mixture. Results confirmed that both test compounds were metabolized extensively and the *anti*-BPDE-DNA adduct was formed in much greater proportion than the *syn*-BPDE-DNA adducts, even when the precursors to the formation of these adducts were present in equivalent

amounts. The authors suggest that their findings, when combined with previous research, indicate that liver may be the target organ for carcinogenesis induced by BaP exposure. In addition, Nishimota and Varanasi (1985) note that due the lack of K-region metabolism in English sole, as demonstrated by Gmur and Varanasi (1982), binding of BaP metabolites to liver DNA may be the result of bay region-diol epoxides alone.

PCBs

Polychlorinated Biphenyls (PCBs) are another important contaminant of concern. PCBs are synthetic organic molecule with 209 possible congeners (ASTDR 2000). Structure of these congeners consists of two connected benzene rings, which have between 1-10 chlorine atoms attached (Ross 2004). Often, following chemical analysis, concentrations of PCBs measured are reported as sums for each chlorination level. For example, all PCB congeners which have 3 chlorines present may be referred to as Cl3-Homologues.

Typical uses for PCBs include applications in capacitors, transformers, flame retardants, adhesives, inks, plasticizers, paint additives, sealing liquids, and emersion oils among others (Kimbrough and Krouskas 2003; Safe 1990). Trade names used for PCBs in the United States, such as Aroclor 1254, were named for the amount of chlorine in each of the mixtures, which is denoted by the last two digits (Stalling and Mayer 1972). PCBs were produced commercially in the Unites States from 1929 and 1977, when health concerns promoted the manufacturer to voluntarily suspend production, with the exception of electrical equipment for closed uses (Ross 2004). Later in 1979, a former federal ban was placed on production and sale of PCBs (Davis et al. 2007). Production continued in Eastern Europe until the 1990's while little is known regarding potential use of PCBs in undeveloped countries (Brent and Vale 2003). Foster and Cui (2008) assert that it has been estimated that 1.5 million tons of PCBs have been produced globally, while Abramowicz (1995) estimated that several hundred million pounds of PCBs have been released into the environment. Limited amounts of PCBs are currently produced for use on research projects studying the effects of PCBs (Kimbrough and Krouskas 2003).

Much of the health concern related to PCBs is focused on accumulation of PCBs in the body. PCBs are lipophilic and thus accumulate in biological tissue and in environments rich in organic matter such as sediments (Ganey and Boyd 2005). This allows PCBs to move up the food chain as PCBs can be taken up by bottom-dwelling aquatic organisms and magnified as one moved up the food chain (Ross 2004). Fish are able to metabolize and excrete some PCBs, others simply accumulate in fatty tissue (Ross 2004). The PCBs with fewer chlorine atoms are typically metabolized more easily than PCB with a larger number of chlorine atoms, likely due to the presence of more unsubstituted positions on the biphenyl ring where metabolic activities are likely to take place (Pinto et al. 2008). Among those PCBs that are excreted, the process generally involves transformation into more polar metabolites and conjugation (Kimbrough and Krouskas 2003).

The USEPA classifies PCBs as B_2 carcinogens, also known as probable human carcinogen based on animal studies which have reported adverse effects on the liver, immune system, reproductive system, and the thyroid, in addition to causation of behavior problems (ATSDR 2000; Brent and Vale 2003; Kimbrough and Krouskas 2003; Ross 2004; Safe 1994). However, inconsistent results in studies of highly exposed occupational cohorts have thus far failed to establish carcinogenicity in humans (Safe 1994). Furthermore, based on minimal adverse effects in occupational studies, it is unlikely that low environmental exposures to PAHs would cause any adverse health effects in humans (Safe 1994).

Since most PCBs are negative for genotoxicity when tested, carcinogenicity of PCBs is thought to most likely occur indirectly through tumor promoting activity (Knerr and Schrenk 2006). In addition, although the entire group of PCB congeners was classified as probable human carcinogens by the USEPA, research suggest that some PCBs likely exert toxicity through a different mechanism than other PCBs. PCBs are often separated into two categories by researchers including dioxin-like PCBs (DL-PCBs) and non-dioxin-like PCBs (NDL-PCBs). Safe (1994) summarizes the toxicity mechanism for DL-PCBs in his review. The DL-PCBs are non-ortho-substituted coplanar PCBs which typically exert toxicity though activation of the aryl hydrocarbon receptor (AhR). Binding of the AhR takes place in cytosol, after which the complex binds the AhR nuclear

translocator (ARNT), translocates to the nucleus and binds dioxin responsive elements (DRE). Binding of the DRE is followed by the eventual induction of gene expression. Genes induced by DL-PCBs include CYP1A1, CYP1A2, and CYP1B1 (Knerr and Schrenk 2006). Adverse effects which appear to be mediated by the AhR include tumor promotion, hepatotoxicity, neurotoxicity, immune system suppression, and reproductive toxicity (Safe 1994).

In contrast to DL-PCBs, the ortho substituted NDL-PCBs tend to form non-coplanar structures and do not significantly induce the AhR (Umannova et al. 2008). In addition, these compounds are commonly referred to as phenobarbital-type inducers (Knerr and Schrenk 2006) and the mechanism by which they exert toxicity remains unclear (Fischer et al. 1998). Inhibition of intercellular communication has been suggested as one potential mechanism of the promoting activities which have been demonstrated by PCBs (Knerr and Schrenk 2006). Hansen (1998) asserts that it has now been well documented that Phenobarbital-like induction increases CYP2A1, CYP2B1, CYP2B2, and CYP3A. It is also of note that most toxicity evaluation of PCBs has been based on toxicity of DL-PCBs, however NDL-PCBs represent the majority fraction of PCBs which are found in the tissue of both wildlife and humans (Fischer et al. 1998).

Further complicating matters, PCBs are typically found in the environmental as a complex mixture of multiple congeners. This makes assessment of toxicity of individual isomers a challenge and often little known about the contribution of the individual isomers to the toxicity of mixtures. Furthermore, Strathmann et al. (2006) assert that recent reports have demonstrated that PCB mixtures produce a variation of interaction responses including additive, synergistic or antagonistic in regard to tumor promoting activities. In some instances, studies using the same model chemicals have reported conflicting results. For example, the authors point out that studies such as Bager et al. (1995) have reported that PCB 153 and 126 have additive or synergistic effects, however conflicting information demonstrating antagonistic effects was disclosed in studies conducted by Dean et al. (2002) and Haag-Gronlund et al. (1998). More recently, a study conducted by Jeong et al. (2008) found PCB 126 and PCB 153 produced a synergistic effect on toxicity.

PCB congeners are spread to the environment through a variety of means, including transport by air and water, followed by deposition in soils and sediments, concluding with possible dietary intake (ASTDR 2000; Kimbrough and Krouskas 2003; Ross 2004; Yang et al. 2008). Although production of PCBs was halted in the late 1970's, PCBs remain a contaminant of concern because of their ubiquitous nature in the environment. For example, traces of PCBs have been found around in the world atmospheric testing even though PCBs are no longer produced (Chen et al. 2009; Du et al. 2009).

Another route of exposure for PCBs is in soil and water from fluids leakages, spills and improper disposals (ASTDR 2000; Foster and Cui 2008). Many studies have focused on investigating PCB exposure related contaminated sediments. Selected concentrations of PCBs in sediment that have been reported in literature are reported in Table 2.

A review of studies of sediments from New Bedford harbor in Massachusetts observed PCB concentrations as high as 100,000 mg/kg (Ford et al. 2005). USEPA (2000c) researchers reported PCB concentrations up to 220 mg/kg in sediment from the Sheboygan River in Wisconsin. The Hudson River in New York represents one of the largest volumes of PCB contaminated sediment in the United States. The US EPA (2002) has measured total PCB concentrations over 50 mg/kg in the Upper Hudson. Stalling and Mayer (1972) noted that environmental PCBs can be concentrated in fish to 40,000 times the original exposure and adverse reproductive effects could occur at levels as low as 5 µg/L. Table 3 notes selected concentrations of total PCBs in fish species. Selected levels of PCBs that have been reported in fish range from 13 ppb to as high as 28 ppm.

Table 2. Selected concentrations of total PCBs in sediment reported in literature are displayed.

Reference	Location	PCB Concentration
Manyin and Rowe 2006	Baltimore Harbor	152 ppb
Fish and Principe 1994	Upper Hudson River, NY	25 ppm
Bzdusek et al. 2006	Sheboygan River, WI	161,000 ppm
Ingersoll et al. 2002	Grand Calument River/Indiana Harbor Canal	56 ppm
Imamaglu et al. 2004	Fox River, WI	0.2 – 18 ppm

Table 3. Selected concentrations of total PCBs in fish reported in literature are displayed**.**

Reference	Location	Species	Total PCB Concentration
Perrson et al. 2007	Baltic Sea	Atlantic salmon	13 – 171 ppb
Stalling and Mayer, Jr at al. 1972	Lake Michigan	Lake trout	28 ppm
Stalling and Mayer, Jr at al. 1972	Lake Michigan	Coho salmon	13 ppm

Toxicity in aquatic receptors

Attempts to link sediment and water column contamination to abnormalities in aquatic receptors began during the 1970s. McCain et al. (1977) reported on the presence of liver neoplasms in the form of hepatomas in English sole, which were collected from the Duwamish River estuary (Seattle, Washington). It was noted that 32% of the fish that were collected were found to have liver hepatomas and in rare instances, those fish with hepatomas were found to have either fin erosion or greatly distended gall bladder as well. The authors noted that the cause of the hepatomas was unknown, however they suspect that PCBs, which are present in large amounts in the Duwamish may have been the cause.

In a more detailed accounting of the same study, Pierce et al. (1978) revealed additional liver abnormalities which were found to be present in the Duwamish English sole. Gross abnormalities included liver enlargement and abnormal coloring of the liver. In addition, the authors observed increased fatty vacuolation of the hepatocytes microscopically, as well as necrosis and loss of liver structure in some instances. The authors also noted that although the liver damage in the Duwamish River fish has not been causally linked to PCBs, the results did generally resemble previously described damage in fish that has been attributed to PCB and other chlorinated hydrocarbons.

McCain et al. (1982) conducted field and laboratory studies investigating the pathology of two species of flatfish in urban estuaries located in Puget Sound. While the field studies evaluated wild-caught fish, the laboratory studies included both acute studies, which involved injection of fish with sediment extract fractions, as well as chronic studies, which were conducted by maintaining fish in field-collected sediments in the laboratory for up to three months. The two species of fish that were used in the study include English sole and starry flounder. In addition, studies investigating the uptake of BaP from sediment by sole were also conducted.

Results of the study found neoplastic, preneoplastic and non-neoplastic lesions in the liver. Other types of lesions that were found included lesions present in the gills, kidney and skin. Liver lesions were the most prevalent in urban estuaries compared to fish from cleaner reference locations, while lesions in other organs were not significantly

different between locations. The most frequently occurring lesions of the liver were fatty change, hepatocellular necrosis, hemosiderosis and blood cysts. The authors felt that most of lesions observed in the study were likely the result of prolonged exposure, as fish with lesions were more likely to be older fish (4 to 6 years), although in isolated incidences fish as young a 1 year were found to have lesions. In regard to concentrations of contaminants found in fish tissue, PCB concentrations were generally reflective of the amount of PCBs found in sediment, while aromatic hydrocarbons were largely non-detects, likely due to rapid metabolism, with the exception of naphthalene. The authors notes that this finding is consistent with other laboratory findings, which have noted that flatfish are unable to metabolize naphthalene as rapidly as other high molecular weight aromatic hydrocarbons. Laboratory studies failed to induce the pathological conditions that were observed in field-collected fish and metabolism studies clearly demonstrated that BaP was readily taken up by English sole from sediment.

Malins et al. (1982) published a study on chemical concentrations and abnormalities in fish and invertebrates in Puget Sound. Results of the study revealed that ecological receptors such as worms, shrimp, clams and crab from urban sampling areas contained levels of aromatic hydrocarbons that were as much as 11 times higher (17 ppm to 1.5 ppm) than those sampled in nonurban locations. PCB concentration were also higher in urban than nonurban locations among sampled biota, as concentrations of PCBs in English sole liver were as much as 15 times higher than reference locations (24 ppm to 1.6 ppm). Receptors such as bottom fish, crabs and shrimp exhibited a variety of pathological abnormalities. Liver neoplasms in English sole were found to be correlated with relative sediment contaminant concentrations of both aromatic hydrocarbons and metals, while levels of preneoplastic lesions were associated only with levels aromatic hydrocarbons present in the sediment. The authors also noted that the organs that were most often found with histopathological lesions in shrimp and crab were the gills, hepatopancreas, midgut and antennal gland.

Later, Malins et al. (1985a; 1985b) conducted two additional studies further investigating the relationship between hepatic lesions in English sole following exposure to Puget Sound sediments. The first study was conducted by capturing fish from the

Puget Sound at Mukilteo, Washington and a cleaner Puget Sound control site. The contamination present in Mukilteo sediments was predominately aromatic hydrocarbons and, to a lesser extent, PCBs. Results revealed that English sole captured at Mukilteo had higher concentrations of contaminants in stomach contents, higher concentrations of metabolites in bile, and higher prevalence of hepatic lesions than English sole captured from the reference lacation. The second study examined the same relationship using fish captured from Eagle Harbor in Puget Sound and a cleaner Puget Sound reference location. The authors note that Eagle Harbor sediments are heavily contaminated with creosote. Results indicated that higher concentrations of aromatic hydrocarbons were found in food organisms in the stomachs of Eagle Harbor English sole versus controls, higher concentrations of aromatic hydrocarbon metabolites were found in bile of Eagle Harbor English sole versus controls, and that Eagle Harbor English sole were found to have higher prevalences of hepatic lesions than English sole captured from the reference location. The study findings supported previous data suggesting an association between accumulation of aromatic hydrocarbons and formation hepatic lesions in English sole.

Varanasi et al. (1986) examined the susceptibility of two fish species and one rat species to BaP-induced hepatocarcinogenesis. Following exposure to BaP, livers from English sole, starry flounder and Sprague-Dawley rats were dissected and DNA binding was investigated. The findings revealed that both fish species demonstrated substantially higher levels of DNA binding than rats. The authors then suggest that both fish species may then be more susceptible to BaP-induced carcinogenesis than rats.

Immunosuppression is also an effect that has been studied in aquatic receptors. Arkoosh et al. (1991) collected Chinook salmon from the Duwamish Waterway, an urban estuary, and a non-urban reference location. Results indicated that immunological memory was suppressed in Chinook salmon from the Duwamish Waterway. Salmon liver collected from the Duwamish Waterway fish were contaminated with three times greater PCBs and 24 times greater aromatic hydrocarbons than Chinook salmon livers from the reference location. The authors also assert that the results are not with precedent, as a previous study by Jones et al. (1979)

indicated that upon injection of a commercial PCB mixture into channel catfish (*Ictalurus punctatus*), a reduction in disease resistance was observed.

In addition to immunosuppression, another potential effect of exposure to contaminants is altered function of the reproductive system in fish. Johnson et al. (2002) state that in addition to other adverse effects such as liver disease, English sole in the Puget Sound region have been demonstrated to suffer from several forms of reproductive impairment. Included among those impairments mentioned are female English sole being less likely to enter vitellogenesis, lower concentrations of reproductive hormones, inhibited spawning ability and reduced egg viability. It is also of note that juvenile Chinook salmon, which were fed PAHs during a laboratory feeding study, were found to have depressed blood chemistry parameters, as well as reduction of biomass and lipid stores (Meador et al. 2006). The authors noted that the effects were similar to effects that would be experienced in fish that had been undergoing starvation. In this study, the starvation-like effects were noted even though the fish were fed throughout the study period and ingested all food presented to them. If the reduction in lipid stores that was observed in these juvenile Chinook salmon also occurs in the natural environment, it could have a substantial effect on the likelihood of survival as Meador et al. (2006) assert that reduced lipid reserves in juvenile fish exiting the estuary will likely be more susceptible to predation and less successful at capturing prey.

Assessment of sediment toxicity

If considered alone, chemical analysis data on sediments and mixtures does not provide enough information to properly determine the risk of adverse effects to organisms (Long et al. 1995). For this purpose, more interpretive tools are needed make the appropriate decisions for protection of ecosystems and human health, as well as management decisions regarding topics such as dredging, source control and remediation (Adams et al. 1992).

Quantitative structure activity relationships (QSAR)

QSAR are a tool used by many toxicologists when little is known about the toxicity of a chemical. Landis and Yu reviewed the concept in their book (2003). This process uses

the physical and structural makeup of a contaminant to estimate the toxicity. Each distinct substructure in the molecule contributes to the toxicity in a specific way, thus each part of the molecule can be assigned a numerical value to represent the contribution to toxicity. If little toxicity data exists for the contaminant of concern, data from similar chemicals with similar modes of action can be used to help form the quantitative toxicity estimate for each substructure of the contaminant.

Mutagenicity and genotoxicity assays

While toxicity tests using whole sediment exposures come closest to simulating *in situ* conditions, in many instances due to cost and time considerations, organic extracts have been used as a screening tool to assess the ecotoxicological impact of contaminated sediments (Kosmehl et al. 2004). A variety of bacterial and cultured cell-based genotoxicity assays have been used to test the genotoxicity of sediment extracts, however, the most commonly performed procedure is the modified Ames *Salmonella* assay (Jarvis et al. 1996). The modified protocol was published in 1983 (Ames and Maron 1983). The tester strains used in the assay are mutated in order to ensure that they are not able to produce histidine (Jarvis et al. 1996). When the tester strains are exposed to mutagenic contaminants, a reverse mutation occurs, which then allows the cells to produce histidine again (Houk 1992; Jarvis et al. 1996). The mean number of revertants can then be quantified and compared with controls so the samples can be classified into categories. For example, Papoulias and Buckler (1996) used three categories to classify Great Lakes sediment extracts including mutagenic if revertants from extract samples were greater than or equal to double the number in controls, toxic if revertants were less than half the number on solvent control plates, and no-effect. In general, the most commonly used tester strains for the Ames *Salmonella* assay include TA98, TA100, TA97, and TA102, however for analysis of environmental samples, TA98 and TA 100 are the most frequently used strains due to their ability to detect frameshift and base-pair mutagenic activity (Chen and White 2004). It is important to note that this prokaryotic system lacks many metabolic enzymes that are found in mammals, so in order to mimic mammalian metabolism *in vitro* rat liver homogenate (S9) is often added during the assay (Houk 1992).

Used now for several decades, the Ames *Salmonella* assay has become a benchmark assay for detection of genotoxins (Johnson 1991). Advantages include that Papoulias et al. (1996) adapted the assay in order to optimize it for use with aquatic sediment extracts. The changes implemented included the use of gel permeation chromatography prior to performance of the assay in order to remove macromolecules. In addition, the authors found that results were optimal when a 30% S9 mix. In addition to sensitivity to genotoxic contaminants, the use of this assay as a valuable screening tool for sediments is based on the low cost (relative to other more time consuming alternatives) and rapid data generation. Ho and Quinn (1993) estimate the time needed for data generation using the Ames *Salmonella* assay at 76 hours. Limitations include that the Ames *Salmonella* assay is labor intensive, in that is requires technical expertise in the form of microbiological training and aseptic technique to prevent contamination (Jarvis et al. 1996). Cost associated with continuous culture of bacteria is also of concern (Johnson and Long 1998). An additional concern with using the Ames *Salmonella* assay is that some genotoxic substances will not ultimately lead to the formation of mutations, thus a portion of genotoxic contaminants will go undetected (Kosmehl et al. 2004).

Another commonly used bacterial assay is the Microtox® assay which was developed by Beckman Inc. (Bulich 1984). The Microtox® assay has been summarized by Giesy and Hoke (1990). This method is a bacterial luminescence assay which is often used as a screening tool because it can be conducted faster than standard aquatic toxicity tests. The test was completed through measurement of bioluminescence of the marine bacterium *Photobacterium phosphoreum,* which is reduced upon exposure to contaminants (Giesy and Hoke 1990). More recently, the marine bacterium *Photobacterium phosphoreum*, which is used in the Microtox® assay has been referred to as *Vibrio fisheri (Doherty 2001)*. Studies such as the one conducted by Day et al. (1995) have been successful at correlating Microtox® results with results from standard whole sediment toxicity test, such as decreased survival in *Chironomus riparius* and reduced growth in *Hyalella azteca, among others*. However, it should be noted that techniques such as the Microtox® assay have not been universally accepted as accurate indicators of toxicity and the relevance of bioluminescence as and endpoint has been the subject of considerable debate.

Closely related to the Microtox® assay is the Mutatox® assay, which uses a dark mutant strain (M169) of the same *Vibrio fisheri* bacteria that is used in the Microtox® assay (Ho and Quinn 1993). The method for the Mutatox® assay was first published by Ulitzur et al. (1980) who found that in some instances, the Mutatox® assay was capable of detecting mutagenic activity of contaminants at concentrations 100 times lower than the Ames *Salmonella* assay. Kwan et al. (1990) report that the Mutatox® assay is sensitive to contaminants which are DNA damaging agents, DNA intercalating agents, DNA synthesis inhibitors, and direct mutagens.

Johnson (1991) points out that the Microtox® assay and the Mutatox® assay should not be confused, as the Microtox® assay is conducted by exposing glowing luminescent bacteria to contaminants which decrease light activity as an indicator of toxicity, while the Mutatox® assay is conducted by exposing nonglowing luminescent bacteria to contaminants which increase light activity as an indicator of toxicity. A typical classification of the results, such as the one used by Johnson and Long (1998) would classify a positive response as a light value of 100 or more and at least three times more than the control. Samples would be considered genotoxic if they tested positive at two or more dose levels, while one positive test would result in a suspect classification, and no positive test for a sample would be classified as negative in regard to genotoxicity.

The Microtox® assay and Mutatox® assay share many advantages and disadvantages as they are similar assays. Johnson and Long (1998) noted that the sister assays are ideally suited for tandem testing and hold several advantages over other assays because they both generate data in less than 24 hours and are sensitive to a wide variety of organic contaminants. In addition, both assays have been applied in the assessment of sediment toxicity (Ho and Quinn 1993; Johnson and Long 1998). Other advantages for the Mutatox® assay include small sample size requirements relative to the Ames *Salmonella* assay and that aseptic technique is not required (Chen and White 2004). In addition, because the bacteria come freeze-dried and use many pre-packaged elements, the assay is technician friendly requires little technical training (Johnson 1991). One limitation is that in some instances, blanks have tested positive for mutagenicity in the Mutatox® assay even though no known mutagens were present

at detectable levels in GC/MS analysis (Ho and Quinn 1993). In addition, the Mutatox® assay has shown variable sensitivity when compared to results of the Ames *Salmonella* assay, so if definitive mutagenic assessment is needed, it has been recommended that at least a sub set of samples be run on the Ames *Salmonella* assay as well for confirmation (Jarvis et al. 1996).

An additional method of toxicity assessment for sediments is found in examination of DNA strand breaks. The most commonly used method of assessment of DNA strand breaks is the alkaline single cell gel electrophoresis assay (Ohe et al. 2004), which is often referred to as the comet assay. Although previously methods had been published to examine double DNA strand breaks (Ostling and Johanson 1984), Singh et al. (1988) were the first to publish a method for examination of single DNA strand breaks from single cells.

The comet assay method was summarized by Ohe et al (2004) in a review on mutagens in surface water. The method typically involves nuclear DNA being unwound and then electrophoresed under alkaline conditions. This causes DNA to fragments to migrate away from the nucleus towards an anode. The distance traveled by the DNA fragments can then be quantified and used as an indicator of DNA damage, as DNA strand breaks could potentially be pre-mutagenic lesions.

Advantages of use of the comet assay as a screening tool include that the assay is sensitive and versatile, in that many types of cells can be used, including cells from aquatic species which were exposed in field studies (Mitchelmore and Chipman 1998). Limitations of the comet assay include non-specificity of the assay, as many factors other than xenobiotic exposure may affect the results of this assay, thus it is recommended that additional assays be performed to determine the specific causes of strand breakage if needed (Mitchelmore and Chipman 1998).

Another option among the many mutagenicity assays currently available is the micronucleus (MN) assay, which can provide and *in vivo* or *in vitro* assessment of chromosome damage (Ohe et al. 2004). The MN assay was originally developed using mammalian species, including an *in vivo* mammalian bone marrow assay which was used to characterize the genotoxicity of a wide array of contaminants (Heddle et al.

1983), however more recently peripherereal erythrocytes from fish blood samples have been frequently selected for use in this assay (Al-Sabti and Metcalfe 1995). Minissi et al. (1996) feel that fish are an appropriate test species as they provide information related to the complex mixtures that are found in aquatic environments.

Condensation of chromosomal fragments or whole chromosomes which were not included in the main nucleus following cell division results in formation of micronuclei (Al-Sabti and Metcalfe 1995; Heddle et al. 1983). A typical method used to employ the MN assay, such as the one used by Minissi et al. (1996), would first involve blood smears being put on microscope slides. Next, the slides would be stained and then examined under a microscope and the number of micronuclei per 10,000 erythrocytes would be reported. In many other studies, micronuclei were reported per a smaller number of erythrocytes, such as 1000 erythrocytes (Matson et al. 2005) or 4000 erythrocytes (Moraes de Andrade et al. 2004), however in this case Minissi et al. (1996) examined a larger number to increase the chances of observing weak mutagenic responses. Various studies have indicated that erythrocytes in fish have a higher incidence of micronuclei following exposure to contaminants in both field studies and laboratory experiments (Al-Sabti and Metcalfe 1995).

Advantages of the MN assay include that it has been successfully applied in studies as it is a simple, reliable and sensitive assay (Minissi et al. 1996). In addition, many standard methods, such as the one by Al-Sabita and Metcalfe (1995), are available for application of the MN assay, both in test species that were exposed in the field and for spiked exposures in the laboratory. Potential drawbacks of using this approach include that the selectivity and sensitivity are not ideal as current research is aimed at improvement in these areas (Al-Sabti and Metcalfe 1995)

Physical and chemical manipulation

Toxicity identification evaluation (TIE) is another method toxicity assessment that may be applied to sediments which are composed of complex mixtures. The TIE process is typically divided into three phases consisting of phase I characterization, phase II identification, and phase III confirmation (Ho et al. 2002), and multiple guidance documents are available from the USEPA (Burgess et al. 1996; Durhan et al. 1992;

Mount and Anderson-Carnahan 1988, 1989; Mount et al. 1993; Norberg-King et al. 1991a; Norberg-King et al. 1991b). Phase I TIE typically involves fractionation of the samples through a variety of physical and chemical manipulations in order to help identify which classes of contaminants are responsible for toxicity of the sediment (Kay et al. 2008). TIEs are useful for many purposes including providing information that will help make remediation decisions, identify contaminants for which discharges should be stopped, provide information for sediment guidelines and aid regulators in determining contaminants that should be banned (Ho et al. 2002).

Benchmarks

Benchmarks such as sediment quality criteria (SQC) and sediment quality guidelines (SQG) were created to help relate sediment chemistry data to potential for the occurrence of adverse effects in receptors (Chapman 1989; Jones et al. 1997; Long et al. 1995). Chapman (1989) discusses the requirement by congress that the USEPA produce water quality criteria (WQC), as part of PL92-500. In addition, the USEPA also has the authority to develop and implement SQC under the Clean Water Act, section 304(a) (Chapman 1989). SQC are an important addition to WQC, for the following reasons: (1) toxicants may be found in trace levels in the water column, but accumulated to elevated levels in sediment, (2) sediments can be a reservoir as well as a source of contaminants for the water column, (3) sediments represent exposures over time, while the water column is more indicative only of recent exposure, (4) sediments, as well as the water column, can adversely affect benthic and sediment-associated organisms, and (5) sediments are vital to the aquatic environment, through provision of habitat, feeding and rearing areas for aquatic organisms (Chapman 1989).

In addition, contamination at hazardous waste sites often contains complex mixtures composed of hundreds of chemicals. Thus, it is necessary to screen hazardous waste sites for potential contaminants of concern (COC) during ecological risk assessment (ERA) (Jones et al. 1997). Screening is often accomplished by using toxicological benchmarks such as SQGs, which allow the comparison of collected field data with benchmark levels, in order to prioritize sites for further investigation and to aid in management decisions (Long and MacDonald 1998). It is also recommended that

multiple benchmarks be employed simultaneously to strengthen the evidence on the likelihood of adverse effects (Jones et al. 1997).

Sediment quality guidelines

An important focus in management of aquatic ecosystems is assessment of sediment quality (Smith et al. 1996). Professionals responsible for management of aquatic ecosystems require the use of effective practical and scientific tools for evaluation of the potential adverse effects of sediment-associated contaminants (Smith et al. 1996). Although multiple methods of deriving sediment quality measures have been introduced, there is currently no consensus on which method provides the most appropriate estimate of toxicity. Although none of the currently available methods have been implemented as nationwide legal standards in the United States, various SQGs have been used by remedial project managers in order to prioritize or rank chemicals of concern at specific sites or regions (Long and MacDonald 1998). This prioritization can be accomplished in several ways. First chemical concentrations in field samples can be compared to SQGs. In addition, total number of individual contaminant SQGs exceeded in field samples and mean SQG quotients can be compared with the probability of toxicity as calculated in aquatic toxicity tests (ER Long et al. 1998). These rankings can then be used to identify which areas require further investigation or corrective action (Long and MacDonald 1998).

Many of the currently available methods use effects-based data in order to derive SQGs. Often, these SQGs will be derived by making comparisons between chemical concentrations in sediments and occurrence of adverse biological effects in organisms following exposure to contaminated sediments (Long and MacDonald 1998). Three sources of data often used in derivation of effects-based SQGs include equilibrium-partitioning (EqP) modeling, laboratory bioassays, and field studies (Long et al. 1995).

Background sediment chemistry approach

Perhaps the simplest method of evaluating sediment quality is the background sediment chemistry approach. This method involves comparison of chemical concentrations found in contaminated samples to chemical concentration found in reference sites from

more pristine areas (Chapman 1989). Advantages include simplicity, low cost and minimal data requirements, however disadvantages include lack of accounting for differences in sediment characteristics, such as grain size and total organic carbon (TOC), lack of distinguishing the bioavailable contaminants from those that are not, and difficulty of legal defense (Chapman 1989).

Equilibrium partitioning approach

EqP theory has been used to predict the bioavailability of PAHs and other nonpolar organics (Ingersoll et al. 1997). The EqP approach attempts to model the tendencies of chemicals to transition between various environmental compartments (Shea 1988). The EqP methodology for evaluation of contaminated sediments is based on relating the bioavailable fraction of contamination, or the contamination which is dissolved in porewater, to the potential of that sediment to cause toxicity (New York State Department of Environmental Conservation 1999). EqP modeling states that nonionic chemicals in contaminated sediment "partition between sediment organic carbon, sediment interstitial (pore) water, and benthic organisms" (USEPA 2003).

The partitioning coefficient (K_p) is calculated by dividing the concentration of contaminants that are sorbed onto the sediment by the concentration of contaminants that are dissolved in water (New York State Department of Environmental Conservation 1999). However, there is strong evidence suggesting that the most important component of sediment in regard to the binding of nonionic, hydrophobic organic compounds is organic carbon (Shea 1988). Thus, the organic carbon partition coefficient (K_{oc}) is used to relate the chemical concentrations present in porewater and bulk sediment samples (Adams et al. 1992). The K_{oc} represents the ratio of the contaminant concentration present in porewater to the contaminant concentration sorbed to organic carbon in sediment (USEPA 2003). As a result, at equilibrium, the mass of chemical in any of the three phases can be used to estimate the mass of contaminant present in the other phases (Adams et al. 1992).

Each approach to deriving values that may be used for SQGs has advantages and disadvantages. One key advantage of using the EqP approach is that it takes various site-specific characteristics into account and in some instances, these site-specific

characteristics may be associated with fraction of the contamination which is bioavailable (MacDonald 1994). One key disadvantage of the EqP approach include that like many other approaches, it fails to take mixture interactions between contaminants of concern into account (USEPA 2003).

Acid volatile sulfides (AVS)/simultaneously extracted metals (SEM)

In sediments which are contaminated with metals, the ratio of AVS to SEM has also been used as a SQG in some studies. The method was first published by Di Toro et al. (1990). The study demonstrated that dose-response curves for toxicity among sediments contaminated with Cadmium varied as the amount of AVS present in the sample changed. Hinkey and Zaidi summarized the method in their recent study (2007). The authors point out that AVS in contaminated sediments react with SEM and insoluble metal sulfides. This is of note because these compounds are then not available for uptake into ecological receptors and are therefore less likely to cause toxicity. Thus, sediments in which the ratio of AVS to SEM is greater than 1 are less likely to exhibit metal-related toxicity in ecological receptors. In addition, sediments in which the ratio is less than 1 are like to have more free metal available for uptake and ecological receptors are more likely to experience metal-related adverse effects. While this ratio undoubtedly provides a useful tool for evaluation of sediment toxicity, as with other SQGs, accuracy of the method is somewhat questionable. As Fang et al. (2005) point out, sediments in which the AVS/SEM ratio is less than 1 do not always cause metal-related toxicity. The authors note that studies have proven that additional binding phases may be present in sediments, thus resulting in an overestimate of metal bioavailability in some instances.

Sediment toxicity bioassays

Characterization of the composition of chemical components in any contaminated matrix is a first step in establishing a risk estimate. Although chemical characterization provides important information, it does not provide data regarding the potential of a chemical mixture to produce adverse effects in exposed receptors or the bioavailability of organic contaminants present in benthic compartments. Aquatic toxicity bioassays of whole sediments or sediment extracts (e.g., porewater, elutriates) are often used to

estimate the impact that contaminants have on a variety of ecologically relevant species, or bioaccumulation of target compounds in benthic invertebrate sentinels (Hose et al. 2006; Ingersoll et al. 1997). These toxicity tests may involve exposing various aquatic test species to contaminated sediment samples in the laboratory for specified periods of time.

The two main approaches to aquatic toxicity bioassays including the bulk sediment toxicity testing (BSTT) approach and the spiked-sediment toxicity test (SSTT) approach were summarized by Lamberson and Swartz (1992) and Thomas et al. (1992) respectively. The BSTT approach typically involves collection of sediments in the field, followed by a return to the laboratory where aquatic organisms are exposed to the collected sediments and monitored for toxicological responses. The SSTT approach typically involves spiking clean sediments with a known contaminant or mixture of contaminants, followed by the addition of aquatic organisms. The aquatic organisms would then be monitored for toxicological responses, just as they are in the BSTT approach.

Endpoints for these toxicity tests typically include measuring the test species' survival, growth, reproduction or bioaccumulation over the course of the experiment (Chapman and Anderson 2005; Reynoldson et al. 2002; USEPA 2000b). Sediments from a clean reference sites are typically tested as well for comparison (Adams et al. 1992). For example, a 20-25% difference in survival, growth, and/or reproduction between the organisms exposed to reference sediment and the organisms exposed to the contaminated sediment is often used to assess ambient sediment toxicity biologically relevant (Chapman and Anderson 2005; Ingersoll et al. 1997). When sediments are identified as toxic but the chemical constituents are unknown, porewater, elutriate and whole toxicity identification evaluation techniques have been developed to identify causative toxicants (Ho et al. 2002; USEPA 2000b).

Advantages of these aquatic toxicity testing approaches include that they are able to directly answer the question of whether or not sediments from the sampled location are toxic to the test species (McCauley et al. 2000) and that they are particularly effective as a screening tool for identification of problem sediments (Chapman 1989). Another advantage to performing these assays is they can be conducted successfully regardless

of which contaminants are present on site or in the case of the SSTT, the spiking can be conducted with any contaminant of interest (Adams et al. 1992). In addition, these assays can be conducted with a wide variety of aquatic species in various life stages, and in the case of BSTT, often test species which occur naturally at the site of interest can be used (Adams et al. 1992). Aquatic sediment bioassays also take also take all exposure routes into account (Chapman 1989). SSTT also can produce valuable dose-response data (MacDonald 1994). Finally, there are many standardized methods available which have been the subject interlaboratory comparisons (McCauley et al. 2000) to address quality assurance and quality control concerns.

Disadvantages of these sediment evaluation methods include that the exposures may not express chronic values (Chapman 1989) and that it can be labor and cost intensive to implement the assays using a variety of test organisms. While BSTT assays fail to provide information regarding which one of the contaminants is responsible for causing the toxicity, SSTT can be used to help provide information regarding bioavailability and causation (McCauley et al. 2000). An additional concern is that the assay is conducted under the assumption that the testing conducted in the laboratory is providing a true measure of what effects would be experienced *in situ* (Chapman 1989), however in reality this often may not be the case.

Two of the most commonly used aquatic species for assessment of ambient freshwater sediment toxicity include *Chironomus tentans* and *Hyalella azteca* (USEPA 2000b). You et al. (2004) selected these two test organisms in a recent study because of their wide distribution in North America, sensitivity to contaminants, ease of culture and the ability to compare results with existing data from other researchers. Among test organisms, Chironomids are considered to be among the most useful aquatic bioassays due to their sensitivity to contaminants and tolerance of various sediment types (Petanen et al. 2003; Traunspurger and Drews 1996). *Chironomus tentans* is an ideal test species for accessing the toxicity of sediments because it is in contact with the contaminants not only through physical contact, but also through ingestion of sediment particles, which allows for specific considerations of dietary exposure routes (Ingersoll et al. 1997; Oberholster et al. 2005).

Wang et al. (2004) believe that sediment toxicity testing using *Hyalella azteca* provides an ideal testing method for measurement of toxicity and bioaccumulation of contaminants among freshwater sediments. *Hyalella azteca* is also a useful bioassay organism because the benthic community is its natural habitat and its epibenthic life history habits can lead to appreciably interactions with sediment contaminants at the sediment-water interface (Borgmann et al. 2005). Conducting toxicity test on contaminated sediment using *Hyalella azteca* provides an important tool for risk assessment and investigating the adverse effects that contaminated sediment could potentially cause in the environment (Borgmann et al. 2005). Thus, a combination of *Hyalella azteca* and *Chironomus tentans* is often preferred in risk assessments of contaminated sediments to evaluate multiple routes of contaminant exposures (e.g., dietary, uptake from porewater).

Direct measurement of interstitial water approach

Another potential measure of sediment quality involves comparing the concentration of contaminants in interstitial or porewater with EPA water quality criteria (WQC) (Jones et al. 1997). One such measure, that is derived using EqP theory and the porewater method, is the sediment quality advisory level (SQAL). Bolton et al. (1985) are the first credited with using this method, however it was also used in more recent publications (USEPA 1997; Zarba 1992). The toxicity values which were used in derivation of SQALs included both final chronic values (FCV), from USEPA ambient freshwater quality criteria, and secondary chronic values, which were produced as part of the USEPA's Great Lakes Water Quality Initiative (Zarba 1992). SQALs were intended to be used only as screening values, which could help identify if a site needed further investigation (USEPA 1997).

The advantages of deriving SQGs from measurement of porewater include that the WQC are set in order to protect 95% of aquatic life, so only 5% of species would be at risk of adverse affects, and that it uses a major toxicological dataset so collection of porewater from sediments is the only data that must be obtained from the contaminated site (Chapman 1989). Disadvantages include that in some cases, such as that of PAHs, WQC may not be available (Chapman 1989). Debate among scientist about the most appropriate method of porewater analysis (Jones et al. 1997) and lack of

application of the data to complex mixtures are among other disadvantages (Chapman 1989).

Another method of sediment toxicity evaluation using porewater is to employ the TIE approach, which was discussed earlier for use on sediment samples. This variation of the approach involves isolation of porewater, followed by the performance of toxicity tests on the porewater sample, and eventually separation of the porewater sample into various fractions composed of the major chemical classes (Ankley and Nelson 1992). Finally, TIE procedures can be performed on the porewater fractions in order to determine the contribution of each contaminant to toxicity (Ankley and Nelson 1992). Key advantages of this approach include that it can be used to perform both acute and chronic toxicity tests with many different species and that this method does not require normalization of bulk sediment contaminant concentrations (Adams et al. 1992). A key disadvantage is that large amounts of sediment must be collected in order to obtain the appropriate amount of porewater to conduct toxicity tests. Adams at al. (1992) point out that availability of porewater is often the limiting factor in regard to which and how many toxicity tests can be run. Other disadvantages include that aquatic test organisms may be over exposed as they are not normally exposed to 100% interstitial water in the environment and that other potentially important routes of exposure, such as sediment ingestion and direct contact with sediment particles, are not accounted for when this method of sediment toxicity evaluation is employed (Ho et al. 2002).

∑*PAH model*

The ∑PAH model was developed and summarized by Swartz et al. (1995) and serves as an example of a SQG that can be derived using data obtained from sediment aquatic toxicity bioassays. The basis of the ∑PAH model comes from combination of EqP, quantitative structure activity relationships (QSAR), toxic units (TU), additive contaminant interactions and dose-response models. This model uses three different kinds of data including percent mortality from aquatic sediment toxicity test, concentrations of contaminants in chemical analysis of bulk sediments and interstitial water, and organic carbon (OC) measurements. Chemical analysis of field-collected sediments and OC measurement is the first step of SQG calculation. These measurements then use the EqP theory to predict interstitial water concentrations.

Using the toxic unit model, porewater concentrations are then divided by 10 day lethal concentrations for 50% of the test population (LC50) that were derived from QSAR studies found during a literature search. Next the toxic units were summed using the additivity model for interaction of contaminants. SSTT were then conducted with a limited number of PAHs and these test provided dose-response data, which is then integrated into the model. The final output of the ∑PAH model is a prediction of the probability of acute toxicity to aquatic organisms and this is classified into three categories including toxic (>24% mortality), not toxic (<13% mortality), or uncertain (between 13% and 24% mortality). The SQGs that are generated by the ∑PAH model include a ∑PAH toxicity threshold and a ∑PAH mixture LC50 (Swartz 1999).

Advantages and disadvantages were also summarized by Swartz et al. (Swartz et al. 1995). The main advantage of the ∑PAH model is that the model has shown accuracy at prediction of acute toxicity when compared with BSTT. However it is of note that the accuracy of toxicity predictions decline when PAHs are not the major contaminant of concern (Swartz et al. 1995). Another advantage is that the model incorporates data based on a variety of approaches including TUs, which is simply the process of dividing the concentration of contaminant present in the sample by some toxicity reference value (Kay et al. 2008). Disadvantages include that the model does not account for differences among species of aquatic organisms used in toxicity testing and the model fails to address the chronic effects of PAHs, which may result in an underprotective estimate of toxicity. In addition, because the ∑PAH model incorporates many other models, such as EqP, QSAR, toxic units, dose-response and additivity, the ∑PAH will exhibit many of the advantages and disadvantages that are associated with these models as well.

Tissue residue approach

The tissue residue approach (TRA) of SQG derivation involves calculation of sediment contaminant concentrations that would result in acceptable levels of contaminants being present in exposed aquatic organisms (Cook et al. 1992). This approach typically includes two steps. The first step is linking toxic effects to organism contaminant residues, while the second step is linking organism contaminant residues in specific organs to levels of contaminants present in sediment (Adams et al. 1992). Another

definition for this approach is that TRA is simply using the concentrations of contaminants in tissues as the dose metric for characterization of toxicity (Meador et al. 2008). Although there is a multitude of research involving tissue levels in aquatic organisms, it is rare for this information to then be used for derivation of SQGs. However, use of levels of contaminants in tissues as a dose metric for toxicity bioassays has been gaining acceptance over the last 20 years (Meador et al. 2008). This approach has the potential for success with chemicals such as nonpolar organics, because contaminants that bioaccumulate in aquatic organisms will also typically be found in sediment (Cook et al. 1992). One potential application of TRA, would be to derive a maximum permissible tissue concentration (MPTC) for each contaminant. This can be accomplished by multiplying WQC by a bioconcentration factor (BCF) that is specific to the aquatic organism which tissues are being collected from (Cook et al. 1992).

The main advantage to the TRA is that levels of contaminants in tissues give a better estimate of the biologically effective dose (BED) than estimates of dose which were derived from sampling of environmental media such as sediment and water (Meador et al. 2008). This serves to reduce uncertainty, especially when *in situ* studies are conducted (Cook et al. 1992). Additional strengths of this approach include that it is well suited for evaluation mixture toxicity and reduction of spatial and temporal variability, which is accomplished because the approach integrates water column and sediment exposure over time and space (Meador et al. 2008). Disadvantages for this approach include that for some contaminants, such as PAHs, rapid metabolism may cause the parent compounds to be biotransformed or eliminated quickly, causing levels of parent compounds to misrepresent the exposure level (Meador et al. 1995). Other potential complications for these chemicals would include variation in uptake of contaminants related to lipid content in the aquatic organism as well as various environmental factors such as temperature and salinity (Meador et al. 1995; Meador et al. 2008).

Apparent effects threshold approach

The diversity of opinions on the appropriate methods of sediment quality evaluation is emphasized by the fact that the EPA prefers an EqP based model, while the state of

Washington issued sediment quality standards that were derived using the apparent effects threshold approach (AETA) (Adams et al. 1992). Tetra Tech Inc. (1986) is credited with developing the AETA during its study of sediment contamination in the Puget Sound area of Washington State. The AETA is based on relationships between measurements of contaminant concentrations in sediment and biological effects which are observed in aquatic organisms (MacDonald 1994). In short, the apparent effects threshold (AET) would identify the contaminant concentrations at which adverse biological effects would always be expected to occur (Malek 1992).

Calculation of AETs is a three step process. First, matched sediment chemistry and biological effects data must be collected or obtained (Adams et al. 1992). The second step involves classification of the sites which the matched data was collected from as either impacted or nonimpacted (Malek 1992). Impacted sites would exhibit statistically significant adverse biological effects when compared with reference sites (Adams et al. 1992). The third step is conducted by looking at the nonimpacted sampling stations and identifying the highest concentration for each individual contaminant at which no statistically significant adverse biological effects occurred (Malek 1992). Typically results are reported specific to the adverse biological effect that is being investigated and are expressed on a dry weight basis, rather than normalized to organic carbon (Chapman 1989).

Continuing the use of the AETA in the Puget sound, the Washington Department of Ecology (WDOE) used the approach to establish legally binding sediment management standards (SMS) in Washington State (1990). These legally-enforceable sediment quality standards were implemented with the intention of aiding in effective long-term management of sediments, management decisions regarding discharge of contaminants in coastal waters, identification of contaminated sites, and establishment of appropriate cleanup levels (MacDonald 1994). The AETA was reviewed by the USEPA Science Advisory Board (1989) and was found to be appropriate for site-specific SQGs in the Puget Sound, however the board did not recommend the use of the AETA for establishment of SQGs on a nation-wide basis (MacDonald 1994; Malek 1992). The most recent update of the SMS in Washington State were completed in 1995 (Washington Department of Ecology).

Although this method does not identify the causative factors, it does demonstrate which contaminants are most likely associated with specific adverse biological effects (Chapman 1989). Another advantage of AETA is versatility in regards to the types of data that can be used to derive AETs, as a diverse array of adverse biological effects from both field studies and spiked-sediment laboratory experiments can be employed (MacDonald 1994). The requirement of a large database can be both an advantage and a disadvantage for the AETA. The advantage of use of a large database is that it is assumed that the effects of mixture interactions are likely to be accounted for, while the disadvantage is that a large amount of data must be collected or acquired to perform the analysis (Chapman 1989).

An additional application of the AETA was summarized by Ingersoll et al. (1996). Among the SQGs that were derived in this study was the no effect concentration (NEC). The authors derived the SQG from a process similar to the AETA, however Ingersoll et al. used both contaminant concentrations in porewater and whole sediment, while the AETA typically uses just whole sediment results. Other modifications which were made included the use of a smaller number of samples (<25) and calculation of effects relative to a control, instead of to a reference sediment as in the AETA.

Screening level concentration approach

Development of the screening level concentration approach (SLCA) is credited to Neff et al. (1986), who used the method in preparation of a report being prepared for the USEPA. This method of SQG derivation is centered on protection of benthic organisms (MacDonald et al. 2000b). The SLCA matches sediment chemistry data from field samples with biological effects data that was collected simultaneously in order to calculate screening level concentrations (SLCs) (MacDonald 1994). The SLC is typically an estimate of the maximum concentration that a pre-determined percentage (95% for example) of benthic species can tolerate (MacDonald 1994). The protection level of 95% is often chosen, due to the water quality criteria (WQC) goal of 95% protection of aquatic biota (Chapman 1989).

The first step in calculation of the screening level concentrations (SLCs) is to build or obtain a database containing matched data on concentrations of contaminants in

sediments and surveys of benthic organism presence (MacDonald et al. 2000b). The benthic organism surveys must indicate both occurrences of species on site as well as species abundance. It also important to note that a minimum of 10 stations must be sampled for the data to be eligible for inclusion in the database (MacDonald 1994). The second step involved is calculation of individual species screening level concentrations (SSLCs) (MacDonald et al. 2000b). The SSLCs can be derived by plotting a cumulative frequency distribution for each species detailing the sampling stations where the species was present versus the organic carbon normalized concentration of the chosen contaminant of concern at that station (Swartz 1999). This plot is then used to obtain the SSLC through selection of a contaminant concentration level at which the species was present at 90% of the stations surveyed (Neff et al. 1987).

One major advantage of using the SLCA is versatility, in that the approach can be used with any contaminant, and the fact that it can be conducted using previously existing data (MacDonald 1994). Among the disadvantages for the SLCA are that a precise level of taxonomic identification is required to execute the method, the SLCA does not establish a method to identify the contributions of individual contaminants toward toxicity and SLCs may be affected by contaminants that were not measured during the SLCA process (Chapman 1989). In addition, there is concern that this method may not exhibit an appropriate level of protection (Jones et al. 1997) as the endpoint being used to derive the SLCs would not detect sub-lethal adverse biological effects.

A variation of the SLC approach was developed by Environment Canada and implemented in 1992 (St. Lawrence Centre and Ministère de l'Environnement du Québec) at a location contaminated with heavy metals. A short summary of the SQGs developed was published by the St. Lawrence Centre and Ministère de l'Environnement du Québec (2009). SQGs included in that summary consisted of three measures of sediment quality including the no effect threshold (NET), the minimal effects threshold (MET) and the toxic effect threshold (TET). While the NET was derived from a variety of methods, the MET and the TET were derived based on the SLC approach. The MET represents the level of contaminant concentration that is expected to be tolerated by 85% of benthic species, while the TET represents the contaminant concentration that is expected to be tolerated by only 10% of benthic species. Sediments which exhibited

levels of contaminant concentrations between the MET and the TET warrant further study to ascertain the safety of the site, while sediments found to have contaminant concentrations which exceed the TET, will need some form of remediation or containment (St. Lawrence Centre and Ministère de l'Environnement du Québec 2009). However, it is of note that due to problems implementing these interim criteria, these SQGs were recently replaced by Environment Canada and the Ministère de l'Environnement du Québec (2007). The new SQGs are based on a SQG derivation approach developed by Long and Morgan (1990) and will be discussed later in this document.

An additional variation of the SLC approach can be found in SQGs published by Persaud et al. (1993) for sediment management in Ontario. SQGs included in the publication were the no effect level (NEL), the low effect level (LEL) and the severe effect level (SEL). The NEL is the level at which contaminant concentration in sediments is not of concern. These sediments are considered clean. The LEL is the level adverse biological effects become apparent in ecological receptors. The SEL is representative of a level at which most of the benthic organisms present in the sediment may not survive. While the NEL is derived using EqP theory, the LEL and the SEL are derived using the SLC approach. The LEL for a given contaminant is simply selecting the 5^{th} percentile on the SLC, while the SEL is simply selecting the 95^{th} percentile of the SLC. The concentrations of contaminants that are found in a given sediment sample are normalized for OC and then compared the NEL, LEL and SEL in order to make management decisions.

Sediment quality triad approach

The sediment quality triad approach (SQTA) uses multiple styles of investigation to evaluate sediment quality. The theory behind the SQTA is based on the relationships between three different measures of sediment quality including sediment chemistry, sediment aquatic toxicity bioassays and *in situ* studies, which measure biological effects (MacDonald 1994). Sediment chemistry measures the amounts of contaminants present in the sediment, while sediment aquatic toxicity test measure effects of contaminant exposure under standardized conditions in the lab, and *in situ* studies

measure field conditions such as resident community alteration or biomarkers of exposure and effect (Chapman 1996).

Advantages of this approach include that it integrates data from three different types of measurements and provides data for a weight of evidence approach to sediment guideline development (MacDonald 1994). The weight of evidence approach could simply be defined as reaching conclusions after taking all available information into account (Chapman 1996). Disadvantages include that is fails to establish causation, thus the adverse biological effects may be caused by contaminants which were not even measured (MacDonald 1994). In addition, statistical methods used to quantify the SQTA results require further development (Adams et al. 1992). While simple in nature, the SQTA is holistic and appears to be quite useful (Chapman 1996).

Weight of evidence approach

Long and Morgan (1990) are credited with developing the weight of evidence (WOE) approach during their work for the National Oceanic and Atmospheric Administration (NOAA) on the National Status and Trends Program (NSTP). The WOE approach, also called the effects range approach by some, was originally created as informal guidelines using data from many different effects-based methods of SQG derivation, however later the database which was used to derive the sediment quality guidelines was updated and the approach those guidelines were derived from was further refined (MacDonald 1994). This approach is based on identification of ranges of contamination which are associated with adverse biological effects through evaluation of the WOE provided by data from multiple studies (Long and MacDonald 1992). The effects ranges that are identified represent ranges in which effects rarely occur, occasionally occur, or frequently occur (MacDonald et al. 1996). These ranges are also sometimes called the no-effects range, possible-effects range, and the probable-effects range (Long and MacDonald 1992).

The three basic steps of the WOE approach are summarized by Long et al. (1995). The first step involved collection and evaluation of all available studies which matched sediment contaminant concentration data and biological effects data. Data from laboratory spiked sediment bioassays, EqP studies, as well as field studies was

included. This data was then assembled into a database named the biological effects database for sediments (BEDS). The second step is determining the ranges of contaminant concentrations that were, rarely, occasionally, and frequently associated with adverse biological effects, and the final step was determining the incidence of adverse biological effects with in each of the ranges for each contaminate in order to evaluate the accuracy of the SQGs.

MacDonald (1994) described the first two SQGs derived from the WOE approach consisting of the effects range-low (ERL), effects range-median (ERM). The first step needed to calculate the ERL and ERM is to sort the individual contaminant concentrations (where adverse biological effects occurred) for each contaminant in ascending order. Following this data organization, the 10^{th} and 50^{th} percentile is determined, and they are termed the ERL and ERM, respectively.

These two SQGs delineate three different concentration ranges for each contaminant that were summarized by Long et al. (Long et al. 1995). The first contaminant concentration range is termed the minimal effects range. Concentrations in this range are below the ERL and adverse effects would be expected to occur rarely. The second contaminant concentration range is the possible effects range. Concentrations in this range would be greater than or equal to the ERL, but less than the ERM, and effects would be expected to occasionally occur. The last contaminant concentration range would be the probable-effects range and this would consist of concentrations that are equal to or greater than the ERM. If concentrations were found to be in this range, effects would be expected to occur frequently.

Strengths of the WOE approach were summarized by Smith et al. (1996). These strengths include that the WOE approach compiles large amounts of data from a variety of study types and locations. These sources include many field studies, where mixtures and a variety of different sediment types are likely to be found. This means that even though factors affecting bioavailability, such as TOC, grain size and acid volatile sulfide (AVS), are not directly measured, mixture interactions and bioavailability are considered implicitly. The diversity of geographic locations in which data was compiled from also leads to broader applicability for the WOE approach. Another reason that the WOE approach is broadly applicable is that data are compiled from a variety of species and

endpoints. MacDonald (1994) also discusses strengths of the approach, including the methods use of existing data without new data collection being required, and that the database is expandable, so that new information or additional geographical areas could be included.

Many of the strengths of this approach also can be sources of weakness. For example, the diverse background from which data is compiled may introduce bias related to differences in data quality and compatibility (MacDonald 1994). In addition, the method does not directly address mixture interactions (MacDonald 1994). This lack of availability of information related to bioavailability necessitates that values derived from the WOE approach be reported on a dry weight basis rather than normalized to organic carbon (Smith et al. 1996). Also, like many other methods of SQB derivation, the WOE approach also fails to support a quantitative evaluation of the cause and effect relationship between individual contaminant concentrations and adverse biological responses (MacDonald et al. 1996). Lack of availability of chronic exposure data in regards to marine and estuarine organisms is another weakness of the WOE approach (MacDonald et al. 1996).

Later, MacDonald et al. (1994) used a modified WOE approach to create two additional SQGs called the threshold effects level (TEL) and the probable effects level (PEL) in their study of Florida's marine coastal waters. Smith et al. (1996) then applied the method in derivation of TELs and PELs in freshwater ecosystems. MacDonald et al. (1996) also described these modifications and the procedure for interpretation of the new SQGs. In this new modified approach, data was sorted into two different data sets, one for data in which adverse biological effects were observed, and one for data in which adverse biological effects were not observed. This is unique from the ERL and ERM approach, because with those SQGs, no-effects data was not used in the derivation of the values. The criteria for selection into the effects data set included that the contaminant concentration must have been a 2 fold or more increase above the reference samples. Following a data organization similar to that of the original WOE approach for each database, both the TEL and PEL were calculated. The TEL was derived by calculating the geometric mean of the 15^{th} percentile from the effects data set and the 50^{th} percentile of the no-effects data set. Similarly, the PEL is derived by

calculating the geometric mean of the 50th percentile of the effects data set and the 85th percentile of the no-effects data set. As with ERL and ERM, the TEL and PEL are intended to define three contaminant concentration ranges including those associated with effects occurring rarely (below the TEL), occasionally (equal to or above the TEL but less than the PEL), or frequently (equal to or greater than the PEL) (MacDonald et al. 1996).

Yet another application for the TEL and PEL method was executed by Ingersoll et al. (1996; USEPA 1996a). In this variation of the method, TELs and PELs were calculated from individual toxicity test, which were conducted using the amphipod *Hyalella azteca*. The test length used in derivation of the SQC was 28 days. The SQGs derived from the tests were later termed the TEL-HA28 and the PEL-HA28, respectively by MacDonald et al. (2000b) in a study comparing consensus-based SQGs. The TEL-HA28 represents the contaminant concentration below which adverse biological effects such as significantly reduced growth or survival would occur rarely in a 28 day *Hyalella azteca* sediment toxicity test. The PEL-HA28 represents the contaminant concentration above which adverse affects such as significantly reduced growth or survival would occur frequently in a 28 day *Hyalella azteca* sediment toxicity test. It is important to note that the TEL-HA28 and the PEL-HA28 were calculated using geometric means to help account for the lack of even distribution in the two data bases used in the process.

Another method used in the prediction of sediment toxicity is to simply compare the number of SQGs that were exceeded among different samples. In an analysis of predictive ability, based on comparison of sediment chemistry data with actual BSTT performed on the same samples, Long et al (1998) found that in general, the incidence of toxicity rises with increased number of contaminants exceeding SQGs in samples. While number of SQGs exceeded certainly has some predictive ability, sediment quality guideline quotients (SQGQs) provide yet another application of the WOE model. SQGQs were first used by two different groups in 1996 publications (Carr et al. 1996; Ingersoll et al. 1996). The process of calculation SQGQs simply involves dividing the concentration of individual contaminants by publishes SQGs, such as ERMs, for each contaminant. The resulting quotient for each contaminant can then be summed with

other contaminants in the same chemical class, or all the given SQGQs from a sample can be summed to form a cumulative SQG index (Carr et al. 1996).

Another expansion of the WOE approach is evaluation of sediment toxicity involves averaging of SQGQs in order to determine mean sediment quality guideline quotients (mSQGQs) for each sample. Long et al. (1994), Sapudar et al. (1994), Ingersoll et al. (1996) and Carr et al. (1996) are some of the first to be credited with using this approach. Long and MacDonald (1998) feel that this approach helps to address the consequences of mixture interactions, which is important as toxicity may vary between samples based on the unique levels of contaminants present in sediments. Calculation of mSQGQs is accomplished by first dividing the contaminant concentrations by their individual SQGs to get the SQGQs. The SQGQs can then be summed and divided by the number of contaminants to get the mSQGQ. It is important to note that only contaminant concentrations for which a SQG is available can be used in this process.

Many studies which seek to evaluate the success of SQGQs at predicting toxicity have used the same criteria set by Long et al. (1995) for evaluation SQGs. This criteria is that the guideline is considered predictive if the toxicity in BSTT was <25% in samples in which all contaminant concentrations fall below the ERL or TEL and >75 when one or more contaminant concentrations exceed the ERM or PEL. These criteria seek to keep the target level for both false negatives and false positives at 25% or less (Long et al. 1995). Studies have found that mSQGQs <.1 typically exhibit toxicity in roughly 10-12% in BSTT, while samples in which mSQGQs were >1.0 or >1.6 exhibited toxicity in 60-80% of samples (ER Long et al. 1998; Long and MacDonald 1998). These results confirm that calculation of mSQGQs is an important step in the toxicity evaluation process, as Long et al. (1998) demonstrated that probability of observing toxicity was related not only to the number of SQGs exceeded, but also to the extent to which those SQGs were exceeded.

Consensus-based sediment quality guidelines

Swartz (1999) is credited with development of the first consensus-based SQGs. Swartz (1999) was concerned about the mixture paradox as it applies to PAHs, as these compound are almost always found in mixtures rather than individual compounds. The mixture paradox that Swartz described is that on one hand, any SSTT of an individual contaminant is going to underestimate the toxicity in nature because it won't account for the effects of the mixture. On the other hand, any BSTT performed on field samples will over estimate the toxicity of individual contaminants because it was actually the toxicity of the mixture rather than that of the individual contaminants.

In light of this mixture paradox, Swartz summarized a method to use a consensus-based SQG for total PAHs rather than looking at the individual PAHs (1999). The first step was to assemble the currently available SQGs for each PAH and then sum the individual PAH SQGs for 13 PAHs in order to yield total PAH SQGs. Next these SQGs were split into three groups based on their intended uses. The first group, threshold effect concentration (TEC) values, included TELs, EqP SQGs, ∑PAH toxicity thresholds, ERLs and SLCs. The second group, median effects concentration (MEC) values, included PELs, low AETs, ∑PAH mixture LC50s, and ERMs. The third group, extreme effects concentration (EEC) values, included only high AETS. Following this data organization, an arithmetic mean was found for each category to yield a total PAH consensus TEC, a total PAH consensus MEC and a total PAH consensus EEC.

MacDonald et al. (2000a) applied the concept of consensus-based SQGs to several classes of chemicals using a similar technique with a few modifications. The first modification was that the SQG data which was assembled was only categorized into two groups. The first group, TEC values, consisted of LEL, TEL, ERL, TEL-HA28, MET, and SQAL values. The second group, probable effect concentration (PEC) values, consisted of SEL, PEL, ERM, PEL-HA28, and TET. In this instance, the TEC represents a threshold below which adverse biological effects are not expected to occur and PEC represents a level above which adverse biological effects are expected more often than not.

In addition to expanding the consensus approach to other chemical classes, the other main differences implemented by MacDonald et al included use of a geometric mean instead of an arithmetic mean and reporting the consensus-based TECs and PECs for both individual contaminants and for chemical classes.

MacDonald et al. (2000b) and others have applied the mSQGQ approach to consensus based SQGs such as PECs. In their study regarding the predictive ability of this approach, MacDonald et al. (2000b) calculated that mean probable effect concentrations (mPECQs) of less than 0.5 were not predicted to be toxic, while mPECQs of >0.5 were expected to exhibit toxicity. These predictions were found to be accurate as the study found that only 30 of 174 samples with mPECQs <0.5 were found to be toxic (83% predictive) when compared to BSTT and 147 of 173 samples with mPECQs >0.5 were found to be toxic (85% predictive) when compared with BSTT. The authors go on to state that it is apparent that a mPECQ value of 0.5 can be used to accurately predict the toxicity of sediment samples (MacDonald et al. 2000b).

SQGs summary

Numerical SQGs serve as important screening tools for regulatory agencies and remedial project managers who must make decisions regarding the assessment and management of contaminated sediments (Smith et al. 1996). Several of the above discussed SQGs are displayed below in Table 4.

Table 4. Selected Sediment Quality Guidelines (SQGs) are displayed. Sediment chemical analysis data can be compared to SQGs in an effort to assess risk of adverse effects of ecological receptors. With the exception of the WDOE Impact Zone/Cleanup sediment quality standards, the SQGs listed are all derived for use in marine and estuarine systems.

	Washington Department of Ecology 1995 ppm OC		Long and Morgan 1990 ppb dry wt			Long et al. 1995 ppb dry wt		McDonald 1994 ppb dry wt	
	Marine	Impact Zones/Cleanup	ERL	ERM	AET	ERL	ERM	TEL	PEL
LMW PAHs	370	780	--	--	--	552	3160	312	1442
HMW PAHs	960	5300	--	--	--	1700	9600	655	6676
Total PAHs	--	--	4000	35000	22000	4022	44792	1684	16770
Total PCBs	12	65	50	400	370	22.7	180	21.6	189

SQGs provide a basis for interpretation of sediment chemistry data through identification of concentrations of contaminants which have the potential to cause toxicity in the form of adverse biological effects (MacDonald et al. 2000b). However, remedial project managers must also consider the reliability and predictive power of individual SGQs when making judgments regarding potential toxicity of contaminated sediment (Long and MacDonald 1998). For example, for samples in which PAHs exceed SQGs, the probability of experiencing toxicity would be relatively high, however for other contaminants this statement may not always be valid (Long and MacDonald 1998).

As toxicity is both a function of total concentration of contaminants and bioavailability of contaminants among other various factors such as mixture interactions, prediction of toxicity in sediments will never be made with 100% certainty, regardless of how many SQGs are exceeded (Long and MacDonald 1998). Measured contaminant concentrations exceeding SQGs will simply indicate that there is potential for adverse biological effects to occur (Smith et al. 1996). It is also important to note that in some cases, contaminants for which there are no SQGs may be toxic to sediments (Long and MacDonald 1998).

SQGs such as ERMs and PELs have been used to identify areas in which elevated contaminant concentrations require further investigation (ER Long et al. 1998). SQGs are similar to many other methods of sediment evaluation in that they fail to establish causation. However SQGs can help identify the chemicals that are most likely associated with toxicity and these causation relationships can be further investigated through the use of spiked-sediment aquatic toxicity tests (Long and MacDonald 1998). Further, it is recommended that SQGs are most appropriate when used in conjunction with other tools in a weight of evidence approach (Long and MacDonald 1998) such as *in situ* biological analysis or other toxicity tests (ER Long et al. 1998). In summary, while SQGs have many strengths and limitations, SQGs provide a reasonably accurate assessment of contaminant concentrations that would exhibit either toxic or nontoxic effects in laboratory based sediment toxicity bioassays (ER Long et al. 1998).

Biomarkers of exposure and toxicity in ecological receptors

Accumulation of contaminants in tissues has often been used as a biomarker of exposure. Varanasi et al. (1989c) demonstrated that carcinogenic PAHs are extensively metabolized on most species of fish and characterized the distribution of those metabolites. In general, the highest levels of metabolites were found in bile, followed by liver, skin and muscle. This trend remained the sample regardless of exposure route or type of dosage (acute or chronic). Often, due to extensive metabolism of higher molecular weight PAHs, only the lower molecular weight PAHs, such as naphthalene will be detected in fish muscle tissue (Varanasi and Stein 1991).

Although measuring contaminants such as PAHs in tissue may reflect recent exposures, it is also possible that due to rapid metabolism, ecological receptors such as fish could exhibit effects due to exposure and contain little to no contamination in tissues (Varanasi et al. 1989c). This may occur because certain contaminants, such as PAHs, are rapidly taken up from water by fish and are then metabolized and excreted in bile (Varanasi et al. 1989c). Due to this rapid metabolism of PAHs, field studies often use biomarkers to provide an index of the total amount of chemical that is biologically available to the exposed organism, or in other words, the ”real-world” exposure (Altenburger et al. 2003). Biomarkers that are frequently used in aquatic systems to measure exposure to contaminants, such as PAHs and PCBs, include assays that measure Cytochrome P450 (CYP450) induction, DNA adduct formation, and levels of PAH metabolites that are present in fish bile (Altenburger et al. 2003).

The CYP4501A subfamily is of interests as a biomarker due to the role that it plays in biotransformation of various contaminants of concern including dioxins, furans, PAHs and PCBs (Sarkar et al. 2006). Following exposure to contaminants such as those described above, CYP1A is induced via a pathway involving the cytosolic aryl hydrocarbon receptor (AhR), and subsequently, induction of CYP1A has been used as a biomarker for pollution monitoring in several species of fish when organic contaminants are of concern (Sarkar et al. 2006). It is, however, important to note that while CYP1A induction may indicate exposure, it likely will not always present a linear dose-response relationship, as complex mixtures present in sediments are likely to

have both CYP1A inducers and inhibitors present, and the two may act simultaneously (Sarkar et al. 2006).

One such assay typically used to measure CYP450 induction is ethoxyresorufin-O-deethylase (EROD). This analysis can be performed using fish liver and has been shown to be a very sensitive biomarker, making it useful in ecological risk assessment (Altenburger et al. 2003). The EROD assay typically involves combining the microsome that was prepared as described above with appropriate cofactors and measuring the CYP1A mediated deethylation of a phenoxazone ether substrate, 7-ethoxyresorufin (7-ER), to the metabolite resorufin (Whyte et al. 2000). This assay has traditionally involved measurements using two separate protocols, one to measure the formation of resorufin, and another to measure protein content in the microsomes for standardization. Burke and Meyer (1974) are often cited as a source for the measurement of resorufin protocol, although this protocol allows for only small numbers of samples to be tested at one time, while Bradford (1976) is often cited for the method to measure protein. More recently, Kennedy and Jones (1994) published a protocol in which simultaneous measurement of CYP4501A activity and total protein is possible with larger numbers of samples using a fluorescence plate reader.

Other useful tools in determining PAH accumulation include measuring metabolic products of PAHs such as the levels of fluorescent aromatic compounds in bile or PAH-DNA adduct levels (Meador et al. 1995). PAH-DNA adducts are formed when PAHs covalently bind with DNA. The ^{32}P-Postlabeling DNA adduct method was developed by Reedy and Randerath (1986). This method provided advantages over other methods of DNA adduct measurement because it required a small amount of DNA, as little as 10 µg, and was highly sensitive in detection of DNA adducts as detection limits are as low as 1 adduct per 10^9 nucleotides. In addition, Varanasi et al. (1989a) used a combination of *in vitro* and *in vivo* studies to demonstrate that measurement of DNA adduct levels may provide information on relative exposure of fish to hydrophobic genotoxic compounds. This measurement provides a tool for comparison of contaminated sites. Other findings included that major DNA adducts persisted in English sole liver for 60 days.

Bile fluorescence is a reliable and easily measured marker of recent PAH exposure, while DNA adducts offer a marker of cumulative exposure (Meador et al. 1995). Collier and Varanasi (1991) measured levels of fluorescent aromatic compounds (FACs) in both laboratory induction studies and in wild-caught fish. Among captivity studies, feeding was found to be a major factor in levels of FACs measured, as fish that were not feeding saw a 7-fold increase in levels of FACs measured at the BaP wavelength over a 28 period, while animals which were given feed saw a 70% decline of FACs in bile over the 28 day exposure period. In laboratory induction studies in which fish were injected with model compounds and sediment extract from the Duwamish Waterway. Both the BaP injected exposure group and the Duwamish Waterway sediment extract injected exposure group showed increased levels of FACs in bile with FACs reaching maximum levels after 4 days for each treatment group.

Research goals and objectives

Based on the review presented above, it is clear that an abundance of potential tools are available for evaluation of sediment toxicity. However, because most contaminated sediments contain complex mixtures making toxicity assessment particularly challenging, the accuracy of many of these tools remains in question. The goal of this research is to provide information which can improve the ability of risk assessors to accurately characterize toxicity associated with complex mixtures which are often associated with sediment contamination. With improved risk characterization, site managers could make more informed decisions regarding the need for clean up actions at hazardous sites. A series of studies has been conducted each July from 2003 through 2007 at contaminated stations in the Lower Duwamish Waterway, a estuarine Superfund site in the Pacific Northwest. The studies focused on the use of *in situ* caged juvenile Chinook salmon and Pacific staghorn sculpin for biomonitoring. In addition, a laboratory calibration study has been conducted using model PAHs, PCBs, a binary PAH mixture and a coal-tar mixture. The calibration study involved collaboration between five university-based Superfund Research Programs (SRPs). Each university received the model chemicals and performed an assay that has been developed at their university using funding from the Superfund Research Program. The calibration study is a first step, with the intent to eventually collect sediment samples from a Superfund

site and distribute an aliquot to each participating laboratory for analysis. Finally, in 2008, a study was conducted to evaluate the effectiveness of a passive sampling technique in characterization of levels of contaminants present in sediment and the water column at the LDW Superfund site.

Specific aims of this research include:

1. Investigate the utility of using SRP-developed assays to characterize the toxicity of complex mixtures in sediment through analysis of model PAHs, PCB, a binary PAH mixture and a coal-tar mixture. The eventual goal of the project is to improve characterization of ecological risk assessment associated with complex mixtures in contaminated sediments.

   Hypothesis 1: SRP-developed assays will be able to detect adverse effects related to chemical contaminants and mixtures of chemical contaminants at lower concentrations than traditional standard aquatic toxicity bioassays which are typically used to assess toxicity of contaminated sediments. In addition, SRP-developed assays will employ more sensitive endpoints than traditional sediment toxicity test.

2. Examine the relationship between levels of contaminants present in environmental samples (sediment and surface water) and biomarker responses measured in juvenile Chinook salmon and Pacific staghorn sculpin which were caged *in situ* at contaminated sites.

   Hypothesis 1: Levels of DNA adducts in *in situ* exposed juvenile Chinook salmon and Pacific staghorn sculpin will provide and effective indicator of exposure and will correlate with levels of contaminants present in environmental samples.

Hypothesis 2: Western Blot analysis of CYP1A1 expression performed using livers of *in situ* exposed juvenile Chinook salmon and levels of contaminants present in tissue will provide an effective indicator of exposure compared to reference fish.

Hypothesis 3: Levels of contaminants present in tissue of *in situ* exposed juvenile Chinook salmon will provide an effective indicator of exposure compared to reference fish.

3. Investigate the utility of using *in situ* deployed solid phase microextraction (SPME) fiber samplers to characterize levels of contaminants present in sediment and the water column of the LDW, an urban estuary in the Pacific Northwest.

   Hypothesis 1: Levels of contaminants detected on *in situ* deployed SPME fiber samplers will correlate with levels of contaminants present in environmental samples.

   Hypothesis 2: Levels of contaminants detected on *in situ* deployed SPME fiber samplers will correlate with response of standard aquatic toxicity bioassays conducted on sediment samples which were collected from the deployment sites.

CHAPTER II

A CALIBRATION STUDY TESTING MODEL CHEMICALS, A BINARY MIXTURE, AND A COMPLEX MIXTURE IN A BATTERY OF BIOASSAYS

Introduction

As far back as 1998, the US EPA estimated that as much as 10 percent of the sediment which lies below surface waters in the United States is contaminated with toxic pollutants to an extent to which consumption of fish by wildlife and humans would pose risks of adverse effects (USEPA 1998). Contaminated sediments may have wide-ranging impacts on human and ecological health. These impacts may include both direct impacts on population health, as well as indirect economic and environmental impacts (e.g., limited productivity of contaminated sediments). Toxic or hazardous materials present in contaminated sediments may include both organic and inorganic substances that adhere to sediment particles with different strengths or affinities. Due to the complexity of contaminated sediments, risk assessors have traditionally used acute sediment toxicity tests and chemical analysis in site characterizations. While it is certainly helpful to know what chemical concentration causes mortality in an aquatic test species, this information alone, does not necessarily indicate the amount of clean-up action that would be required on the site (National Research Council 1997).

Exposure of aquatic organisms to toxic contaminants could potentially cause not only lethal effects resulting from high dose acute exposure, but could also cause adverse chronic effects, including many which will not cause mortality in the receptor. Chronic toxicity, typically characterized by low dose exposures over long periods of time, can lead to adverse health effects (e.g., abnormal growth and development) which could ultimately translate into impacts at the population or ecosystem level. Measurements of sublethal toxicity in ecological receptors could serve as warning sign for adverse effects that would normally not have been detected using traditional methods. Sublethal toxicity measures also could aid in the assessment of potential health risks to human populations that are either directly exposed to contaminated sediments or indirectly, such as the movement of contaminants from the sediments through the food chain.

Analytical chemistry methods employ a variety of different instruments and detectors to quantify different classes of compounds. In much the same way a battery of bioassays, capable of detecting a range of different endpoints, could be used to quantify the toxicity of different classes of complex mixtures. For more than 20 years, the NIEHS Superfund Research Program (SRP) has supported the development of bioassays to elucidate the cellular, genomic and biomolecular effects of environmental exposures. Many of these bioassays are capable of detecting biological effects at lower concentrations than those detected by traditional standard aquatic bioassay endpoints such as growth, reproduction and mortality. In addition, if integrated into risk assessment by decision makers, these more sensitive bioassays would provide a supplemental line of evidence in exposure assessment and could potentially improve methods for contaminated sediment site characterization.

Recently, a collaboration between several university-based Superfund Research Programs has formed as investigators at Texas A&M University, University of California at Davis, University of California at San Diego, Duke University, Michigan State University, University of Washington, and Baylor University have combined their efforts to yield a battery of highly sensitive screening tools for a variety of Superfund priority contaminants including polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). The hypothesis of this research is that chemical-class specific bioassays or biomarkers can identify degraded sediment quality at lower sediment concentrations than standard aquatic toxicity bioassays. The first phase of the project is reported in this manuscript and includes a calibration step testing model polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and a coal-tar sediment extract in the assays, while future plans will include collection of freshwater sediments, which will then be homogenized and aliquoted for testing in each of the developed bioassays, as well as standard aquatic bioassays. This “proof of concept” study was conducted to calibrate the bioassays with a series of model compounds and mixtures. The sensitivity and selectivity of each bioassay was evaluated using two model polycyclic aromatic hydrocarbons (PAHs), two model polychlorinated biphenyls (PCBs), a binary PAH mixture, and a complex PAH mixture. These bioassays, which were developed by investigators at university-based Superfund Research Programs, may provide advantages to environmental managers by improving information for risk

and site characterization, while decreasing the cost and time that are required to assess sediment contamination. Many of the bioassays may be used as screening tools which could help focus chemical analysis and bioassay efforts on certain areas of the site. Hence the development and use of protective, accurate, and sensitive tools can greatly enhance these efforts.

To be effective tools, these chemical-class specific bioassays or biomarkers should be able to identify degraded sediment quality either at lower sediment concentrations or using more sensitive endpoints than standard sediment toxicity bioassays, which typically measure mortality, reproduction or growth. These chemical specific bioassays may also be capable of detecting biologically significant endpoints, such as induction of receptors or inhibition of intracellular communication, which are not measured in standard sediment toxicity bioassays. While it is recognized that sediment toxicity bioassays are the foundation for ecological risk assessment, if appropriate endpoints are selected, chemical specific bioassays may provide complimentary data that can be cross-walked to estimate ecological risk. The comparative approach described in this study entails research translation that strategically co-joins basic science with the efforts of environmental managers, risk assessors and government regulators. The process includes gap analysis that sheds light on where additional work could be done to create an integrated suite of bioassays (i.e., a matrix of potentially complementary or synergistic biological tools that can help improve our understanding and approach to ecotoxicology and risk).

Materials and methods

Model chemicals and chemical mixtures

Four model chemicals, one binary and one complex mixture were used in this round robin testing. Benzo[a]pyrene (BaP), fluoranthene (Flu), a binary mixture of BaP and Flu, 3,3',4,4',5-pentachlorobiphenyl (PCB 126), and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) were purchased from Absolute Standards Inc. (Hamden, CT). The binary mixture of BaP and Flu was prepared by mixing equal volumes of both solutions to reach a final concentration that was equivalent to the concentration tested in the bioassay for the individual compounds. Dimethyl sulfoxide (DMSO) or isooctane was

used as the solvent to dissolve the chemical or mixture depending on investigator preference. The coal-tar mixture was purchased from the National Institute of Standards and Technology (Gaithersburg, MD) and is referred to as Standard Reference Material 1597a.

Bioassays

Embryo development and enzyme activity assays (Duke University)

This assay has been employed extensively in the Di Giulio laboratory using Atlantic killifish (*Fundulus heteroclitus*). Adult killifish were collected from a reference site on King's Creek on the lower Chesapeake Bay in Southeastern Virginia. Adults were maintained at 22-25°C in 25 ppt artificial seawater (ASW; Instant Ocean, Aquatic Ecosystems, Apopka, FL) and were fed a mixture of TetraMin® Tropical Flakes and a sinking Dense Culture Food (Aquatic Ecosystems). Eggs were manually collected from females and fertilized *in vitro* with milked sperm. Eggs were given at least 30 min for fertilization before a 30 sec 0.3% hydrogen peroxide wash. Eggs were then placed back into 25 ppt ASW and stored in an incubator at 28.5°C until dosing. Aquatic exposures of embryos to chemicals or sediment extracts were started at 24 hours post fertilization (hpf). Embryos were maintained under these conditions until analysis for *in ovo* CYP1A enzyme activity which was quantified as 7-ethoxyresorufin-*O*-deethylase (EROD) activity at 96 hpf. This assay has no observable effects on living embryos, and the same embryos are subsequently assessed for cardiovascular deformities at 144 hpf.

CYP1A enzyme activity was measured via an *in ovo* EROD assay which was originally modified from the methods of Nacci et al. (1998; 2004; 2005). A more detailed description of this method is provided in Wassenberg and Di Giulio (2004). Briefly, ethoxyresorufin is added at 24 hpf. CYP1A metabolizes ethoxyresorufin into resorufin, which is highly fluorescent. Resorufin accumulates in the urinary bladder and is quantified via fluorescent microscopy at 96 hpf. Cardiovascular deformities were quantified following the methods described in Matson et al. (2008). Embryos were scored as a 0, 1, or 2, based on the severity of heart elongation and function. The scale

represents relatively normal (0), mild/moderate deformities (1), and severe deformities (2). Severe deformities include a “tube heart” phenotype with no visible blood flow.

Gap Junctional Intercellular Communication (GJIC) assay (Michigan State University)

Gap junctional intercellular communication was assessed in WB-F344 rat liver epithelial cell line (Tsao et al. 1984). The WB-F344 cells are derived from the Fisher 344 rat, which is extensively used in *in vivo* toxicology assessments and tumor promotion assays. These WB-cells are diploid, non-tumorigenic, representative of epithelial cell types (Tsao et al. 1984), and their pluripotency was demonstrated by *in vivo* differentation into hepatocytes or cardiomyocytes (Coleman et al. 1993; Malouf et al. 2001). In addition, these cells have been extensively characterized for their expressed gap junction genes, as well as their ability to perform intercellular communication *via* all available techniques in the absence and presence of well-known tumor promoters (Trosko and Ruch 1998). They have also been tested for the ability of growth factors and oncogenes to modulate gap junction function (Trosko and Ruch 1998).

Tested chemicals or appropriate volume of solvent (vehicle control) were added directly to the confluent WB-F344 cells grown on 35 mm culture dishes and incubated for 30 min. GJIC was assessed using scrape loading/dye transfer (SL/DT) technique (Upham et al. 2008). Briefly, the method involved making 3 scrapes with a surgical steel blade through the monolayer of cells bathed in 1 mg/mL of Lucifer-Yellow in a solution of PBS supplemented with 0.46 mM calcium chloride and 0.49 mM magnesium chloride (CaMgPBS). After 3 min incubation at room temperature, the extracellular dye was discarded and the cells were rinsed 3 times with CaMgPBS and then fixed with approximately 0.5 ml of 4% phosphate buffered formalin. The migration of the dye through gap junctions was visualized with a Nikon Eclipse TE3000 phase contrast/fluorescent microscope and the images digitally captured with Nikon EZ Cool Snap CCD camera (Nikon Inc., Nikon, Japan). The distance of dye migration from the scrape line was determined using a data imaging system, Gel Expert (NucleoTech, San Mateo, CA). GJIC was reported as a fraction of the vehicle control.

All treatments were done at least in duplicate and all experiments were independently repeated 3 times. The results are reported as an average ± SD at the 95% confidence interval. ECx values were calculated from non-linear regression fitting curves obtained after percentage transformation of fraction of control data.

Estrogen responsive assay using breast cancer cell culture (Texas A&M University)

Standardized bioassays developed in the Safe laboratory (Abdelrahim et al. 2006; Li et al. 2006; Pearce et al. 2004) were used to screen the model compounds and mixtures for estrogenic activity. Activation of genomic pathways of estrogen action were determined in breast cancer cell lines transfected with $pERE_3$ or $pSpl_3$, which contain three Tandem estrogen responsive elements (EREs) and GC-rich (Sp binding) sites respectively. This assay also evaluated activation of ER_{2+} ERα (Sp) by the tested compounds or mixtures. In addition, the methods described by Li et al. (2006) were used to determine the effects of the tested compounds and mixtures on non-genomic pathways of estrogen action by measuring activation of mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3-K), protein kinase C (PKC), PKA, and calcium calmodulin-dependent kinase IV (CaMKIV).

P450 reporter gene system assay (University of California, San Diego)

The P450RGS assay was conducted using EPA Method 4425 (USEPA 2000a) by applying the model compounds and mixtures to the TV101 human cell line containing a stably transfected aryl hydrocarbon receptor-responsive reporter gene (firefly luciferase) containing the upstream regulatory elements from the human CYP1A1 gene (Postlind et al. 1993). The solvent volume was reduced to 1-mL and an aliquot of 2 to 20-µL of the sample was applied to each of three wells in a tissue culture plate. Each well contained cells in 2-mL of culture medium. The same solvent and volume were used for the three replicates of the solvent blank. The cells were then incubated for 16 hours at 37°C and 5% CO2. Exposures were conducted over two specific time periods (6 and 16 hours). In the presence of CYP1A1-inducing compounds, the enzyme luciferase was produced, and its reaction with luciferin was detected by measuring relative light units (RLUs) in a luminometer. The mean response, in RLUs, of the three sample replicates was divided by the mean response of three replicates of a solvent blank, yielding a "fold induction,"

which provided a measure of the increase of the sample response over the background response.

The P450RGS assay is used to *screen* samples for a range of organic compounds including: PCDDs, PCDFs, coplanar PCB congeners, and high molecular weight PAHs. The two exposure times used by this test allow the test to distinguish between PAHs and chlorinated compounds, since the PAHs reach maximum induction at 6 hours, while the peak in induction from chlorinated planar compounds (PCBs, PCDDs/PCDFs) is not until 16 hours. In addition, fold induction may be converted to toxic equivalents (TEQ) for PCBs and PCDDs/PCDFs, or BaP equivalents (BaPEq) for PAHs, based on the fold induction responses to standards containing a mixture of PCDDs/PCDFs, or BaP, respectively.

Chemical-activated luciferase expression (CALUX) assay (University of California, Davis)

The CALUX bioassay (the chemical detection component of USEPA Method 4435) is a recombinant mouse hepatoma (H1L6.1c3) cell-based bioassay containing a stably transfected firefly luciferase reporter gene under the trans-activational control of the aryl hydrocarbon receptor (AhR). This bioassay system is used to examine individual chemicals and complex contaminant mixtures in order to identify those capable of activating AhR-dependent gene expression (reviewed in Denison et al. (Denison et al. 2004)). The AhR binds and mediates the toxic and biological effects of toxic halogenated aromatic hydrocarbons, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related dioxin-like chemicals, and the biological effects of numerous other structurally-diverse chemicals (Denison et al. 1999; Denison and Nagy 2003). Stock chemicals and extracts for CALUX analysis were obtained in isooctane. A concentration dilution series of test compounds and the coal-tar extract was prepared in isooctane.

The isooctane dilution samples were mixed with 3.5 µL of DMSO, the isooctane removed by vacuum centrifugation and 350 µL of tissue culture media (α-minimal essential media and 10% fetal calf serum (Garrison et al. 1996)) added to each sample and thoroughly mixed.

An aliquot (100 μL) of the sample dilution or solvent control in media was added to individual wells of a white clear-bottom 96-well tissue culture plate containing 75,000 cells. Each 96-well plate also contained a TCDD standard solution as a positive control. The 96-well plates were incubated at 37ºC for 24 hours in a humidified (85%) incubator with a 5% CO_2 atmosphere. After incubation, the cells were examined microscopically for viability, the medium removed, cells washed twice with 100 μL of phosphate-buffered saline followed by the addition of 50 μL of Promega lysis buffer and shaking of the plates for 20 minutes to ensure complete lysis. Luciferase activity (light output) in each well was measured using a Berthold microplate luminometer with automatic injection of Promega stabilized luciferase reagent and activity expressed relative to that maximally induced by TCDD. At least triplicate determinations were carried out at each chemical/extract concentration. The Effective Concentration at 50% (EC_{50}) for those concentration-dependent curves reaching induction levels matching or exceeding the TCDD maximal induction levels were determined using the 4-parameter Hill plot (Brown et al. 2004) while EC_{50} values for curves that did not reach maximal induction were determined from direct comparisons to the TCDD standard curve.

Results and discussion

Embryo teratogenicity and in ovo EROD assay

Previous studies using the embryo teratogenicity assay indicate that the deformity scale correlates quite well with embryo hatch success. For example, embryos with severe cardiovascular deformities do not hatch, while embryos with moderate deformities have about a 50% hatch rate.

In the fish embryo teratogenicity assay, PCB 126 was the most toxic compound tested, while Flu and PCB 153 had no activity. PCB 126 was also the most toxic compound in the ***in ovo*** EROD assay. BaP was less toxic than PCB 126 in the ***in ovo*** EROD assay, while Flu and PCB 153 again exhibited no increase in activity (both are known inhibitors of CYP1A). The results from testing a binary mixture of BaP and Flu in the ***in ovo*** EROD assay are presented in Figure 2.

The response for BaP and the binary mixture were similar up to the 5 ppb (ng/mL) dose. However, the response observed for the BaP exhibits a plateau (dotted line above the 5 ppb dose), while binary mixture response declines as dose increases forming a bell shaped curve. These data indicate that the Flu is likely binding CYP1A protein, which is responsible for EROD activity. At a concentration of 400 ppb, Flu reduced EROD induction to less than the level observed for the control (DMSO).

Gap Junctional Intercellular Communication

The response observed in the GJIC was reported as a fraction of the vehicle control and the results are presented in Figure 3. Flu (EC_{10} = 1.13 ppm) and binary mixture Flu+BaP (EC_{10} = 1.38) inhibited GJIC with very similar dose-response curves where almost complete inhibition was achieved at dose levels 15 ppm and higher. GJIC was effectively inhibited also by PCB153 (EC_{10} = 1.049 ppm) and Coal Tar Extract (EC_{10} = 1.553 ppm), while BaP and PCB 126 exhibited minimal activity in the GJIC assay.

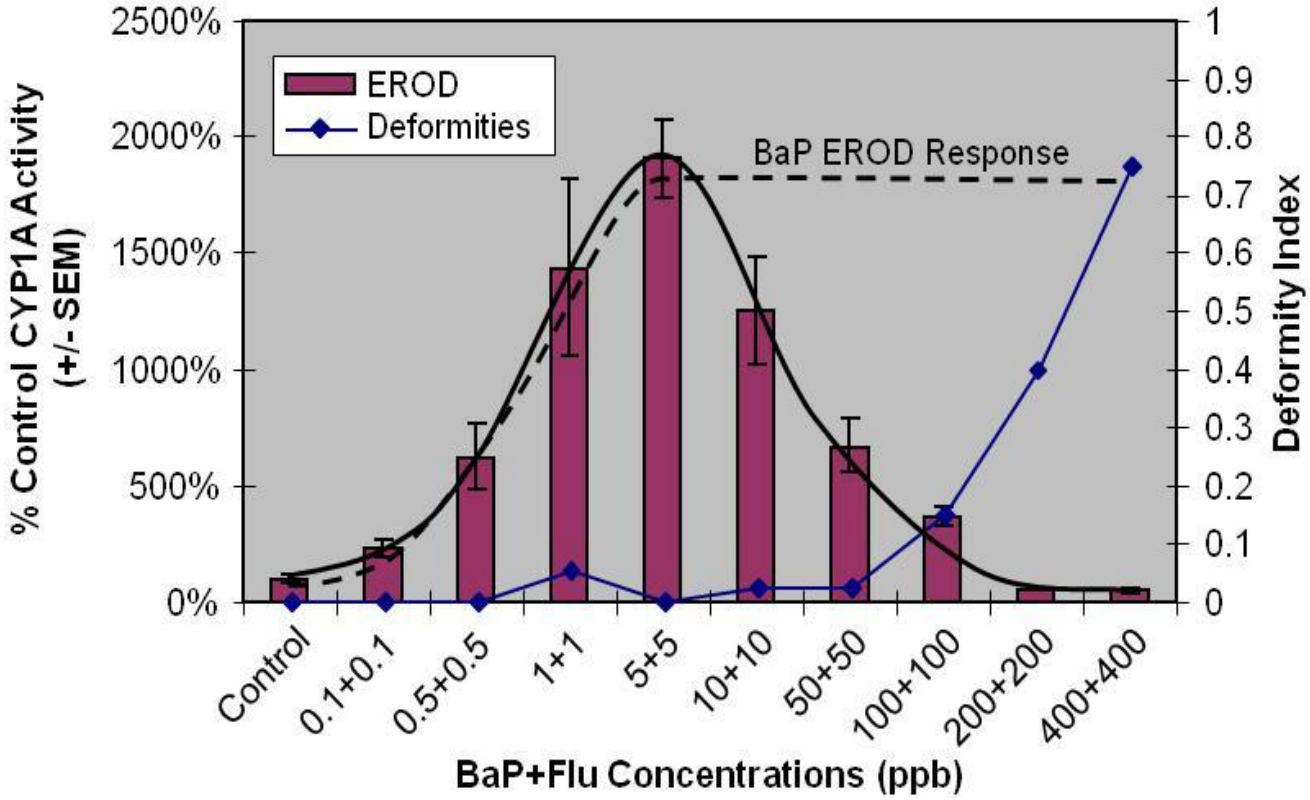

Figure 2. *In ovo* EROD activity in Atlantic killifish (*Fundulus heteroclitus*) (CYP1A) following exposure to BaP and a binary BaP/Flu mixture are displayed. In addition, a deformity index, calculated using the embryo teratogenicity assay, is displayed for the BaP/Flu binary mixture only.

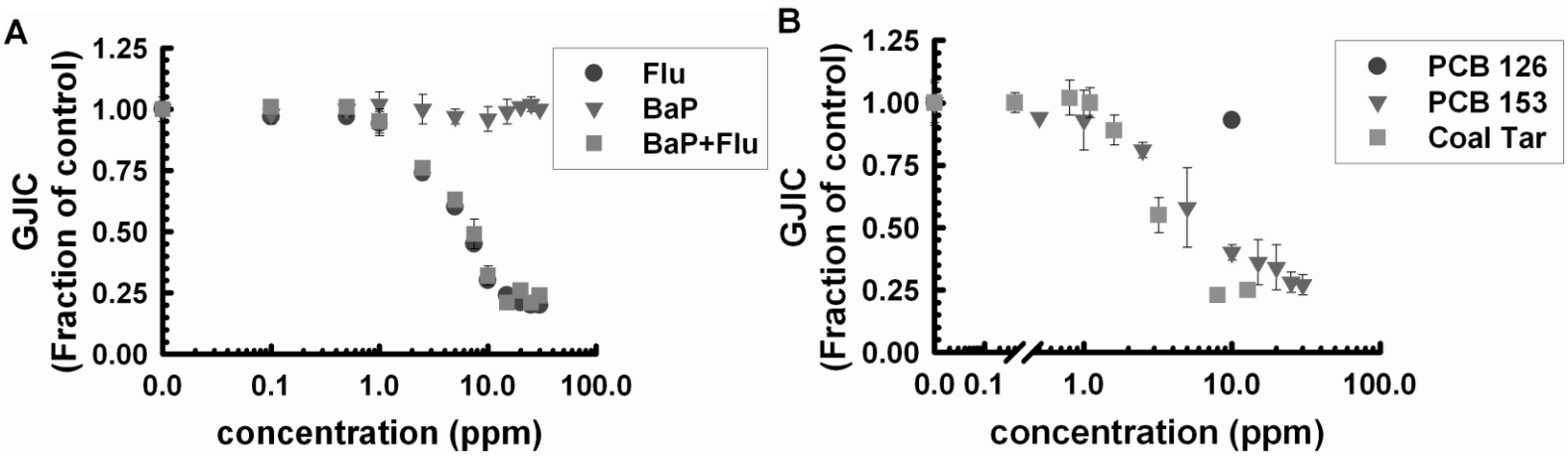

Figure 3. Gap junctional intercellular communication in a Fischer 344 rat liver epithelial cell line (WB-F344) is displayed following exposure to model PAHs, PCBs , a binary PAH mixture and a coal tar extract. The values are reported as a fraction of the vehicle control for: (A) effects of Flu, BaP, and Flu + BaP binary mixture, (B) effects of PCB 126, PCB 153 and coal tar extract.

P450 reporter gene system (P450 RGS) assay

PAHs, polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and PCBs induce the P4501A (CYP1A) gene subfamily via the AhR, leading to the production of CYP1A1 enzymes. CYP1A1 enzymes represent an important pathway for metabolism and removal of these compounds from cells in both ecological and human receptors (Jones JM et al. 2000; Postlind et al. 1993). The mechanism involves the binding of the ligand to an intracellular protein known as the AhR. The ligand-AhR complex is translocated to the nucleus and binds to specific enhancer sequences in the 5' flanking region of the CYP1A1 gene (known as the xenobiotic receptor elements or XREs). High affinity binding of the ligand to the cytosolic AhR is required for Aryl Hydrocarbon Hydroxylase activity, the enzyme activity associated with CYP1A1 (Postlind et al. 1993).

In Figure 4, average fold induction of the model chemicals and the binary mixture are reported. PCB 126 displayed the highest induction, while Flu and PCB 153 were found to have the lowest induction among those chemicals tested in the assay. In regard to the binary mixture of BaP and Flu, there appears to be antagonistic interaction between BaP and Flu in the P450 RGS assay at the highest dose level (1:100 dilution). However, further testing at additional dose levels is needed to get a more complete dose response curve. This would allow examination of whether or not this antagonistic trend continues at additional dose levels.

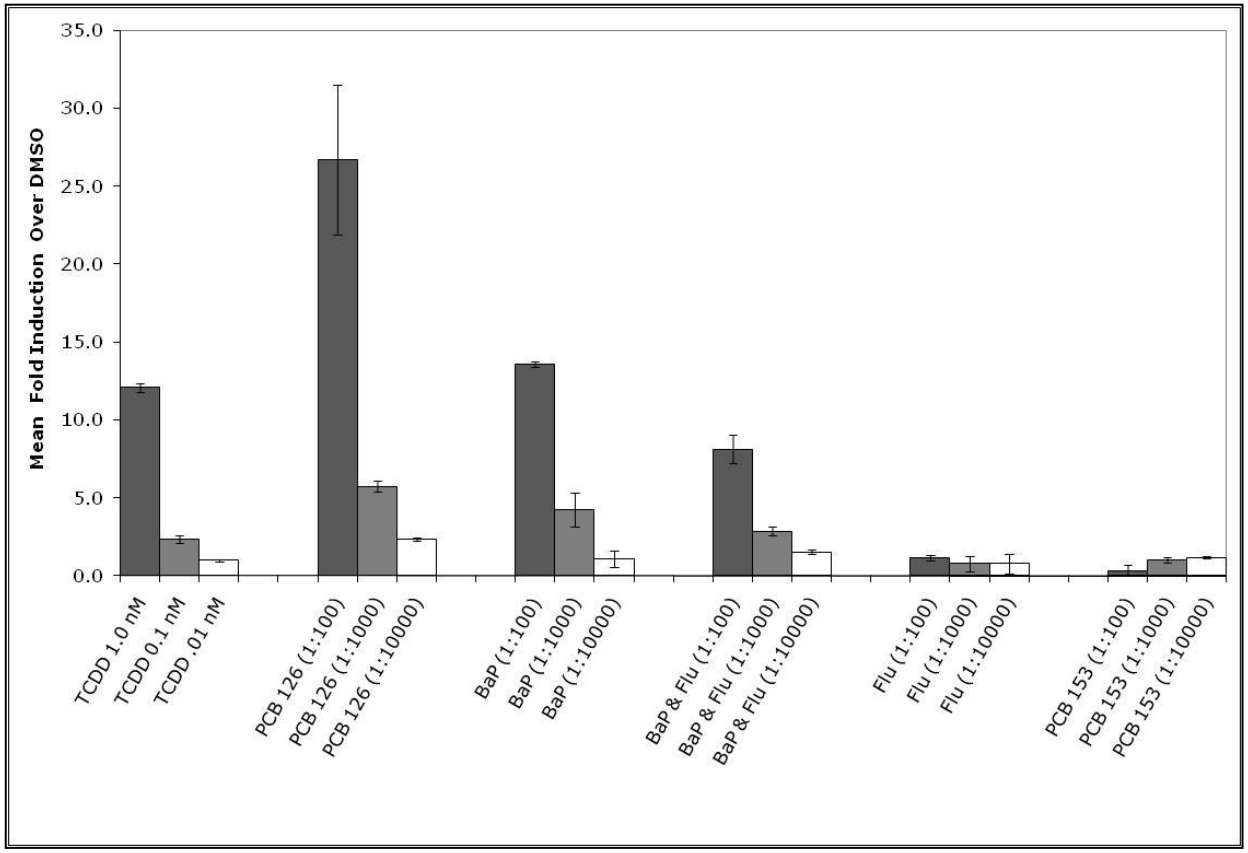

Figure 4. Average fold induction of luciferase activity, in a TV101 human cell line which has been stably transfected with an aryl hydrocarbon receptor-responsive reporter gene (firefly luciferase), following application of various doses of PCB 126, BaP, a BaP/Flu binary mixture, Flu and PCB 153. TCDD was included as a positive control.

CALUX assay

Concentration-dependent induction of luciferase activity by the positive control, TCDD, was determined to have an EC_{50} of 0.84 pg/assay (8 ppt) of TCDD per assay well. As previously observed (Garrison et al. 1996), the AhR agonist PCB 126 induced luciferase activity in the CALUX bioassay in a concentration-dependent manner to a maximal activity comparable to that of TCDD with the EC_{50} of 4.9 pg/assay (49 ppt), while PCB 153, which is not an AhR agonist, did not induce luciferase activity. The ability of the selected PAHs and a PAH binary mixture to induce luciferase activity in the CALUX bioassay is shown in Figure 5.

As expected, the PAHs were significantly less potent, inducing luciferase gene expression to between ~30-45% of that maximal induced by TCDD (higher concentrations were toxic to the cells (data not shown)); the isooctane solvent control was inactive. Flu was approximately 37- to 18-fold less potent as an inducer in the CALUX bioassay than BaP or the BaP and Flu binary mixture (compare EC_{10} values of 119 to 3.2 and 6.7 ppm, respectively.

While the relative potencies (EC_{40}s) of BaP and the BaP/Flu binary mixture are comparable, BaP was about 2-fold more potent than the BaP/Flu binary mixture when their EC_{10} values were compared. This suggests that at higher concentrations, there are some interactions occurring that result in an increase in the relative potency of Flu and/or BaP, otherwise it would be expected that the relative potency of the mixture would be intermediate between that of BaP and Flu alone.

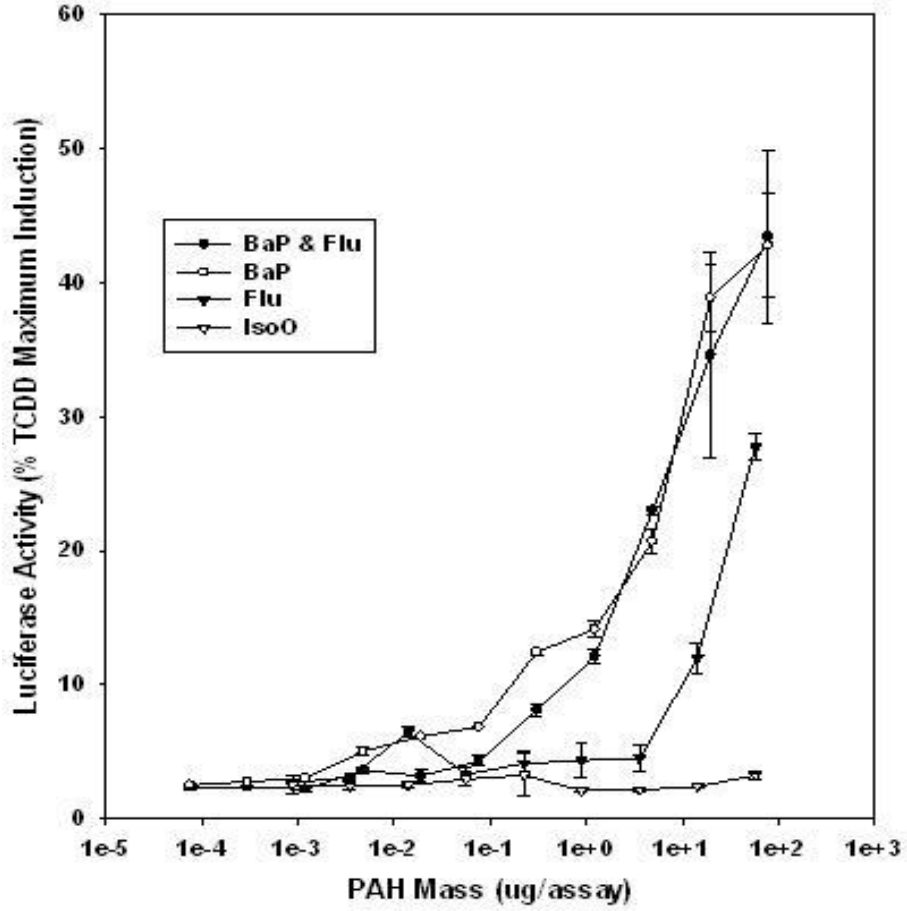

Figure 5. Concentration-dependent induction of luciferase activity in an AhR-responsive recombinant mouse hepatoma (H1L6.1c3) cell line containing a stably transfected firefly luciferase reporter gene following application of BaP, Flu and a BaP/Flu binary mixture. Isooctane was included as a control as the model PAHs were delivered in this solvent.

The complex coal-tar mixture was one of the most potent samples tested. As increasing concentrations of the extract were administered to the cell line, superinduction of luciferase activity was observed to a level that was more than 3-fold greater than that obtained with a maximal inducing concentration of TCDD. Superinduction of AhR-dependent gene expression (including the CALUX bioassay) has been previously observed and likely results from enhancement of AhR signal transduction through a mechanism that is independent of the chemicals ability to bind to the AhR ligand binding site (i.e., enhancing AhR transcriptional activation activity and/or the functionality of the transcriptional machinery at the luciferase gene promoter (Chen and Tukey 1996; WP Long et al. 1998; Long et al. 1999; Seidel et al. 2001)).

The observed interactions of the coal-tar extract on AhR-dependent gene expression is most likely due to the presence of chemicals that can not only interact with the AhR but also affect other cell signaling pathways. While the CALUX bioassay can be used to detect any AhR agonist, its use in USEPA method 4435 is specifically for the detection and relative quantitation of dioxin-like halogenated aromatic hydrocarbons (HAHs, like PCDDs, polybrominated dibenzo-p-dioxins (PBDDs), PCDFs and PCBs) and this requires that sample extracts be subjected to clean-up to eliminate nonHAH agonists of the AhR. Without sample cleanup, the AhR can be activated by a wide variety of structurally dissimilar chemicals (19, 20), many of which would be found in the coal tar extract.

Estrogen responsive assay in MCF-7 cell line

Binding with the estrogen receptor may have implications for reproductive health in both human and ecological receptors. There is a great deal of uncertainty in understanding the potential impact of chemical mixtures on the estrogen receptor. Many of the chemicals commonly detected in sediments have been found to activate endocrine signaling pathways.

The battery of test chemicals including PAHs and PCBs were repeatedly screened in the MCF-7 cell line for both Ah- and estrogen-responsive activity using reporter gene assays of CYP1A1 (Ah-responsiveness) (Figure 6). Major problems of reproducibility were observed in MCF-7 cells and this was attributed to passage-dependent changes in

expression of the AhR and ERα. Future studies will focus on adapting other breast cancer cell lines with more stable expression of both receptors, for example T47D cells, or cells with low endogenous expression of AhR and ERα and stably or transiently transfected as required.

Summary of results

A review of the literature was conducted to determine the response induced by the model compounds in standard aquatic toxicity bioassays. Table 5 presents LC_{50} values that were measured using *Hyalella azteca* or *Chironomus tentans*. Among sediment toxicity test that were conducted using water samples which were spiked with model PAHs, Flu was more toxic that BaP.

Results from toxicity tests using both *Hyalella azteca* and *Chironomous tentans* were consistent with this statement, as LC_{50} values for Flu were 7.3 ppb and 12.6 ppb, compared to an LC_{50} value of 32.4 for BaP (Buchman 1999; Hatch and Burton Jr 1999). Although a sediment LC_{50} was not located for BaP, sediment samples that had been spiked with Flu were much less toxic that water samples that were spiked with Flu. The LC_{50} values for Flu in sediment were 3248 ppb and 5200 ppb (Hatch and Burton Jr 1999; Verrhiest et al. 2001). LC_{50} values were not available for the two model PCBs that were selected for the round robin calibration, however an LC_{50} from a sediment toxicity test of a PCB mixture using *Hyalella azteca* was found to be 32.62 ppb (Buchman 1999), which would rank the toxicity of the PCB mixture almost equivalent to BaP.

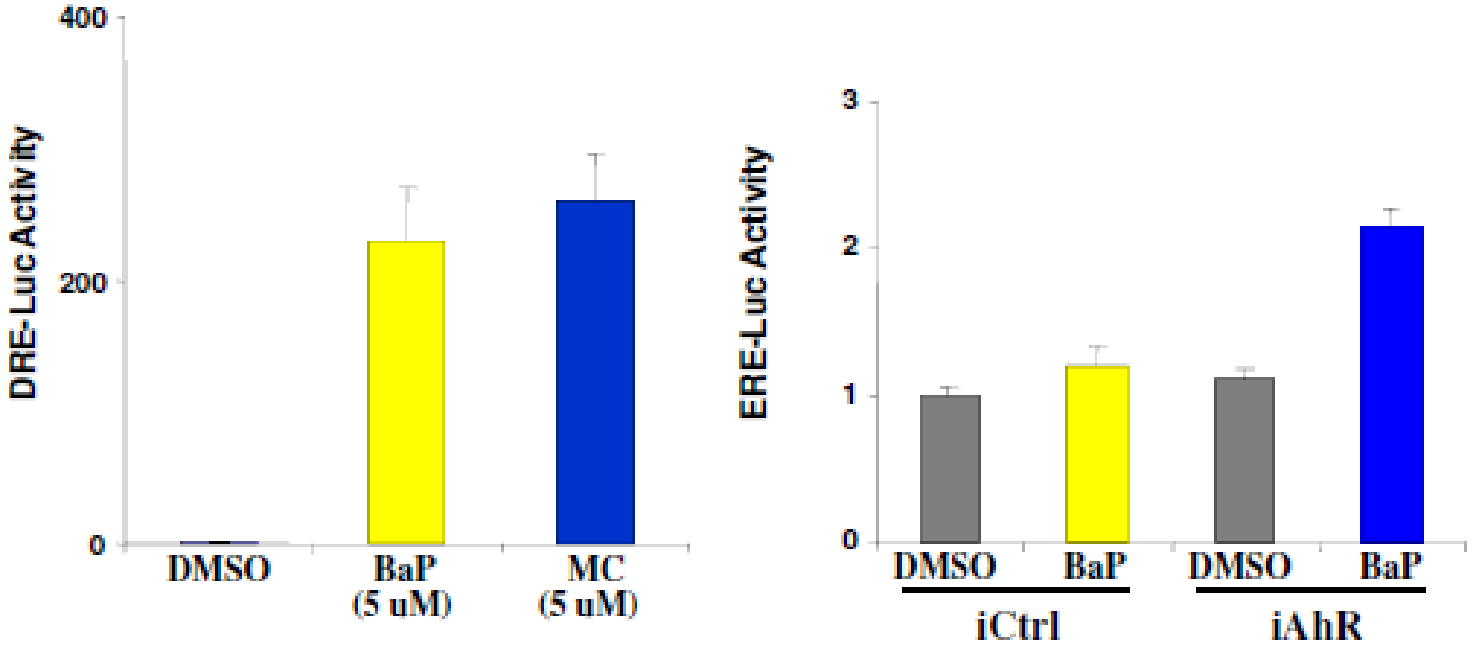

Figure 6. DRE-Luc (Ah responsive) and ERE-Luc (estrogen responsive) activity were measured in an MCF-7 cell line which was exposed to model compounds such as BaP. DMSO was included as a control because the model compounds were dissolved in DMSO. In addition, for DRE-luc activity, MC was included as a positive control.

Table 5. Historical data for sediment and aquatic toxicity tests which were conducted by exposing *Hyalella azteca* and *Chironomus tentans* to model PAHs and a PCB mixture are displayed.

Chemical	Test Organism	Media	LC_{50}	Reference
BaP	*Hyalella azteca*	Water	32.4 µg/L	(Buchman 1999)
Flu	*Hyalella azteca*	Water	7.3 µg/L	(Hatch and Burton Jr 1999)
Flu	*Hyalella azteca*	Sediment	3248 µg/kg	(Hatch and Burton Jr 1999)
Flu	*Hyalella azteca*	Sediment	5200 µg/kg	(Verrhiest et al. 2001)
Flu	*Chironomus tentans*	Water	12.6 µg/L	(Hatch and Burton Jr 1999)
PCB Mixture	*Hyalella azteca*	Water	32.62 µg/L	(Buchman 1999)

A summary of bioassay results compared to historical sediment bioassay data from literature is presented in Table 6. The GJIC assay data did not follow the pattern of results from the other vertebrate assays. The coal tar mixture proved to be the most toxic compound tested in regards to inhibition of GJIC, followed by Flu and PCB 153. BaP and PCB 126 did not appreciably inhibit GJIC at the doses tested in this project. The effects of binary mixture of Flu/BaP were similar to those of Flu only and also indicate an inactivity of BaP in GJIC assay. These results did correlate with the literature review of the standard invertebrate aquatic toxicity tests, as Flu and PCB 153 was more toxic than PCB 126 and BaP.

The CALUX bioassay does not provide a direct measure of toxicity of chemicals, but their ability to activate the AhR and AhR signal transduction pathway. While persistent activation of the AhR signaling pathway by metabolically stable ligands (i.e. dioxin-like HAHs such as TCDD and PCB 126) leads to AhR-dependent toxicity, metabolically labile AhR ligands (i.e. PAHs such as BaP) induce AhR-dependent signaling only transiently and do not produce direct AhR-mediated toxicity (although some PAHs can be metabolized by the induced enzymes into reactive carcinogenic chemicals). In the CALUX bioassay, PCB 126 was by far the most potent activator of the AhR pathway, followed by the coal-tar extract (~1000-fold less potent) and then the PAHs (another 1000-fold less potent). While the ability of the coal-tar extract to enhance AhR-dependent gene expression in the CALUX bioassay could suggest its potential to produce AhR-dependent toxicity, it remains to be determined if this extract would result in persistent AhR activation, a characteristic of AhR agonists that produce toxicity.

Table 6. Effected concentrations calculated from bioassays conducted using model compounds, a binary PAH mixture and a coal-tar mixture are presented for comparison with values from literature including lethal concentrations that were calculated by exposing *Hyalella azteca* to water spiked with model compounds. NA = No activity, -- = not tested.

	BIOASSAY				
Chemical	***Hyalella* (LD50) in water**	**In vivo EROD (EC50)**	**Fish embryo teratogenicity (EC10)**	**GJIC (EC50)**	**CALUX (EC50)**
BaP	32.4 ppb (Buchman 1999)	1 ppb	200 ppb	NA	405 ppm (EC40)
Flu	7.3 ppb (Hatch and Burton Jr 1999)	NA	NA	4.4 ppm	NA
BaP+Flu	--	1 ppb	100 ppb	4.8 ppm	422 ppm (EC40)
Coal-tar	--	.06 ppm	5 ppb	2.87 ppm	341 ppb
PCB 126	--	.03 ppb	0.1 ppb	NA	49 ppt
PCB 153	--	NA	NA	4.34 ppm	NA
PCB mix	32.6 ppb (Buchman 1999)	--	--	--	--

There were also a several trends present in the data from the EROD, fish embryo teratogenicity, and CALUX assays. In all three of these assays, Flu and PCB 153 exhibited no or very little toxicity. In addition, in each assay, PCB 126 was found to be the most active/toxic compound tested. In the EROD and fish embryo teratogenicity assays, BaP was less toxic than the coal-tar mixture, however in the CALUX assay, BaP was slightly more active than the coal-tar mixture.

Several of the assays measure induction of specific enzymes as an indicator of exposure and potential toxicity. The CALUX, P450RGS and *in ovo* EROD assays investigate activation or expression of Phase I enzymes that are involved in the metabolism and elimination of a broad range of toxic compounds. The assay using MCF7 cells measured activation of the ER and AhR and ER/AhR-dependent gene expression. The gap junction model system is a more general assay to assess compounds that disrupt homeostasis via the disregulation of intercellular communications within a tissue. Although disruption of intercellular communication is typically not a lethal effect in vertebrates, in small organisms it is feasible that xenobiotic concentrations could reach concentrations that disrupt a critical number of gap junctions resulting in a lethal metabolic reaction to the xenobiotic. This may explain the correlation observed between the gap junction assay and the standard aquatic toxicity bioassays.

The regulation of gap junctions is independent of AhR, thus the sensitivity of the GJIC bioassay system to various PAHs and PCBs would be expectedly different from the AhR-based bioassay systems. This bioassay system is a unique *in vitro* technique that takes a more systems biological approach to measuring adverse effects of multicellular organisms (Trosko and Upham 2005; Upham and Trosko 2009). Many environmental toxicants can disrupt homeostasis by causing an imbalance in the proliferation, differentiation and apoptotic events of cells in a tissue, which is dependent on a finite, but often, numerous signal transduction pathways (Trosko and Upham 2005; Upham and Trosko 2009). However, the homeostatic set point of cells in a tissue requires functional intercellular communication between cells. Although transient closure of channels in response to mitogenic regulators is normal, the chronic closure of gap junction channels will lead to adverse outcomes for the organism (Trosko and Upham

2005; Upham and Trosko 2009). Studies have correlated inhibition of GJIC with events such as enhanced proliferation, as well as inhibition of apoptosis and differentiation, prompting suggestions that inhibition of GJIC may play a role in carcinogenesis (Upham et al. 2008). The lack of activity of known AhR activators, PCB 126 and BaP, and strong effects of weak AhR ligands, Flu and PCB 153, in GJIC assay, indicates an importance of non-AhR dependent mechanisms of toxicity. Long ago, Wislocki et al. (1978) reported that bay region theory states that the presence of an epoxide which forms part of the "bay region", or a space between fused aromatic rings, on a saturated angular benzo ring of a PAH, results in an area of increased chemical activity and that diol-epoxides were prime candidates to be the ultimate carcinogenic PAH metabolites. More recently, it has been reported that three and four ringed PAHs which possess bay or bay-like regions (Bláha et al. 2002; Ghoshal et al. 1999; Upham et al. 1996; Upham et al. 1998; Weis et al. 1998) and non-coplanar PCBs (Machala et al. 2003) inhibit GJIC through a phosphatidyl choline specific phospholipase C mechanism, which has also been implicated various pathologies, such as cancer (Machala et al. 2003; Upham et al. 2008).

The embryo teratogenicity assay uses the same embryos as were evaluated to measure EROD induction, but is a more direct measure of toxicity to an ecological receptor. As would be expected, the response observed in the CALUX, P450RGS, *in ovo* EROD, and MCF7 assays are in general agreement. Each of these assays measures a similar endpoint and is responsive to similar classes of compounds. The similarity of the responses in the *in ovo* EROD and fish embryo toxicity assays indicates that BaP and PCB 126 induce their adverse response in the embryo following activation by a P450-dependent (CYP1A1) pathway.

This calibration study employed a variety of detectors to measure the toxicity of a small group of model compounds (two PAHs and two PCBs) and mixtures (a binary and a complex PAH mixture). This combination of bioassays expands the range of data typically obtained through the use of standard aquatic toxicity assays. This study's expanded suite of detectors added assay data that are mechanism-based and chemical-class specific. The benefits of this combination go beyond merely adding another set of tools to do the same thing. One of the advantages of using bioassays to

measure toxicity, as opposed to chemical analysis, is that the bioassays can measure interactions of mixture components. However, as mixture composition is likely to change due to spatial and temporal variations, the toxicity is also likely to change. These bioassays may also provide information that is relevant to the more complex organisms of an aquatic system. In addition, these assays may be useful for identifying risks to human populations that may be exposed to contaminated sediments either directly or indirectly.

When assessing the ecological or human health hazard associated with contaminated sediments, the sources of uncertainty are numerous and in some cases may be very large. A battery of biological tests, measuring a range of toxic endpoints may provide information which can be used to generate a more comprehensive characterization of contaminated sediments. The hypothesis of the National Bioassay Network is that collaboration among NIEHS Superfund Basic Research Programs, the EPA and other key stakeholders on a national level creates economies of scale and resources that can accomplish objectives unattainable by any separate entity. This study was conducted primarily to prove the concept that a battery of bioassays would provide more sensitive and selective information regarding complex mixture toxicity than chemical analysis alone.

The results have demonstrated a broad range of responses in the various bioassays. The data also suggest that bioassays provide valuable information that can be used to estimate the interactions of a complex mixture. The university-based researchers provided input to the current study with regards to toxic endpoints to measure, while the other key stakeholders provided guidance on cross-walking bioassay results to estimate an ecological risk. Previous studies suggest that this type of transdisciplinary collaboration can stimulate innovation, inspire new directions in the application of research results and enhance the positive role of science in society (Branscomb and Keller 1998; Juma and Yee-Cheong 2005; Whiteman 2007).

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CHAPTER III

BIOMONITORING USING AN *IN SITU* CAGED FISH EXPOSURE TO ASSESS TOXICITY RELATED TO SEDIMENT CONTAMINATION IN AN URBAN WATERWAY

Introduction

Contaminated sediments may have wide-ranging impacts on human and ecological health. These impacts may include direct impacts on population health and indirect impacts due to the limited productivity of contaminated sediments. Assessment of toxicity and risk related to contaminated sediments is particularly challenging as sediments are typically contaminated with complex mixtures rather than singular contaminants. Traditional assessment methods used by site managers have focused on the use of aquatic sediment toxicity bioassays. These bioassays are conducted in the laboratory and involve exposing aquatic organisms, such as the amphipod, *Hyalella azteca*, to field-collected sediments and measuring growth, reproduction and mortality over a specific period of time. More recently, various biomarkers have been demonstrated to be effective indicators of exposure (reviewed by Van Der Oost et al.) (2003). Although academics have frequently published manuscripts using these biomarkers, instances in which decision-makers use this information as a line of evidence in site management decisions seems to lag well behind their use by academics. Further study and method development is needed in order to incorporate these methods into the site management decision making process.

A series of *in situ* caged fish exposure studies were conducted in the Lower Duwamish Waterway, a estuarine Superfund site in the Pacific Northwest, each July from 2004-2007. The objectives of these studies include (1) characterization the extent of PAH and PCB contamination present in sediment and surface water samples from a contaminated industrial waterway, (2) measurement of levels of PAHs and PCBs present in fish tissue after a 8-10 day exposure period in a contaminated industrial waterway, (3) measurement of levels of DNA adducts present in fish hepatic and gill tissue after a 8-10 day exposure period in a contaminated urban waterway, and (4) measurement of enzyme induction in fish liver tissue following an 8-10 day exposure period in a contaminated urban waterway.

Materials and methods

Site description

The Lower Duwamish Waterway (LDW) refers to a 5.5 mile portion of the Lower Duwamish River (USEPA and WSDOE 2008). The LDW represents a transition zone or estuary, as it receives fresh water from the Green river, which is the primary source of water for the Duwamish River (Windward Environmental LLC 2007), and salt water from the Puget Sound, as the LDW flows into Elliott Bay (USEPA and WSDOE 2008). The LDW has been heavily modified from its original state to facilitate use as a navigational corridor (Windward Environmental LLC 2007). Today the LDW is characterized mostly by industrial activities such as shipping, boat manufacturing, marina operations, and airplane part manufacturing activities, among others (USEPA 2001), however, the residential areas of Georgetown and South Park are also located along or nearby the shores of the LDW. In addition to releases of pollution from industrial sources, combined sewer overflows (CSOs) and storm drains discharge into the LDW (USEPA 2001). Despite this pollution, the LDW is fished both commercially and recreationally and is part of historical fishing grounds for the Muckelshoot and Suqiamish Indian tribes (USEPA 2001). Currently, the LDW supports a commercial fishery operation by the Muckelshoot tribe which catches migrating salmon (Windward Environmental LLC 2007).

The City of Seattle began the first study to investigate water quality on the Duwamish estuary in 1949 (Gibbs and Isaac 1968). Adverse effects that have been documented in fish at the Lower Duwamish Waterway Superfund site include fin erosion (Wellings et al. 1976), accumulation of chemicals in fish liver and muscle tissue (Malins et al. 1984), and lesions in the liver, kidney and gills (Malins et al. 1984), among others. Rhodes et al. (1987) observed that presence in the LDW or Upper Duwamish Waterway (UDW) was a risk factor for several types of hepatic lesions. For example, odds ratios of 8.7 and 8.2 were observed for UDW and LDW English sole in regard to the presence of neoplasms. In addition, higher concentrations of xenobiotics, such as aromatic

hydrocarbons and metals were correlated with increased risk of development of hepatic diseases in fish located in the river Superfund site (Malins et al. 1984).

Additional research on the Duwamish was conducted by Varanasi et al. (1985). Various organisms including fish, shrimp, clams and amphipods were exposed in the lab to Duwamish River delta sediments. Results indicated that accumulation of aromatic hydrocarbons varied among species, as aromatic hydrocarbons were not detected in fish and shrimp, but aromatic hydrocarbons were successfully detected in clams and amphipods. In addition, prior to the exposure, radiolabeled BaP was added to the Duwamish River delta sediments and biliary metabolites of BaP were measured following the exposure period. Results revealed that all organisms tested were capable of uptake of BaP from the Duwamish River delta sediments. Particularly high levels of BaP metabolites were found in fish and shrimp, indicating extensive BaP metabolism, which likely accounts for the lack of detection of parent aromatic hydrocarbons in these organisms.

Krahn et al. (1986) studied English sole caught by trawling in the Duwamish Waterway and 10 additional sites around Puget Sound. Results of the study indicated a significant correlation between biliary metabolites of aromatic hydrocarbons in English sole and presence of hepatic lesions, however no correlation was found between levels of aromatic hydrocarbons in sediment and levels of aromatic hydrocarbon metabolites in bile. Eagle Harbor was found to have the highest mean concentration (2100 ng/g wet) of aromatic compounds measure at the BaP wavelength, while the Duwamish waterway was the second highest (1400 ng/g wet), however this role was reversed in respect to percent of neoplasms as the Duwamish Waterway had the highest (20.7%) and Eagle Harbor had the secone highest (18.2%).

Collier et al. (1986) examined xenobiotic metabolizing enzymes in spawning English sole following injection of sediments from the Duwamish Waterway, Eagle Harbor and a reference site in the Hood Canal. Interestingly, results indicated that even though Eagle Harbor sediments were found to have aromatic hydrocarbon levels 13-fold higher than the Duwamish Waterway sediment, spawning English sole which were intramuscular injected with sediment extracts from the Duwamish Waterway exhibited double the amount of aryl hydrocarbon hydroxylase (AHH) activity compared to their Eagle Harbor

counterparts. The investigators speculated that the result may be due to the presence of chlorinated hydrocarbons in the Duwamish Waterway sediments in contrast to the absence of chlorinated hydrocarbons in the Eagle Harbor and Hood Canal sediments. Other important study findings include that while AHH, which has been linked by various studies with activation of carcinogenic aromatic hydrocarbons, was rapidly induced, detoxifying enzymes such as epoxide hydrolase (EH) and Gluathione-S-transferase (GST) are induced at a much slower pace. The authors felt that this lag time between inductions of enzymes may represent a period of increased susceptibility to adverse effects in exposed fish.

Various studies have also characterized the extent of PAH and PCB contamination present in the Duwamish Waterway. During a review of a series of studies of Puget Sound sites conducted between 1979 and 1985, Malins et al. (1987) noted levels of aromatic hydrocarbons in the Duwamish Waterway at approximately 8 ppm and levels of PCBs at approximately 150 ppb. Another study found sediment from the Duwamish Waterway contained 8.7 ppm total PAHs and 460 ppb of total PCBs (Varanasi et al. 1989b).

Studies have also investigated DNA adduct levels in wild fish caught from the Duwamish Waterway. Varanasi et al. (1989b) examined levels of DNA adducts in English sole from the Duwamish Waterway and Eagle Harbor, in addition to white flounder (*Pseudopleuronectes americanus*) caught from Boston Harbor, in an effort to evaluate contaminant exposure and observed effects in marine organisms. The authors point out that previous studies have demonstrated that modification of DNA is likely a critical step in chemical carcinogenesis, thus it should provide a useful biomarker of exposure. Results of the study indicated that the exposed fish contained a suite of adducts indicative of exposure to genotoxic compounds. It is important to note that although the three locations studied contained various concentrations of PAHs in sediment, total adduct levels were comparable. Interestingly, although total adduct levels were similar, patterns of adduct levels varied by location and species of fish, indicating that DNA adduct results are both species and site specific. The authors suggested that factors such as contaminant structure, bioavailability and differences in metabolism and excretion among different species may affect DNA adduct levels. In

addition, the authors asserted that their finding that English sole samples immediately after capture from a site of very low contamination did not contain hepatic DNA adducts demonstrated the ability of the ^{32}P-postlabeling assay to distinguish between areas of high and low contamination. This study also examined fluorescent aromatic compounds (FACs) in bile from the exposed fish. Although there was some general agreement between levels of adducts in fish compared to levels of FACs present in bile, on an individual level, high concentrations of FACs did not always accompany high levels of adducts.

McCain et al. (1990) conducted a study investigating uptake of aromatic and chlorinated hydrocarbons by juvenile Chinook salmon in the Duwamish Waterway and a non-urban reference site. Stomach contents of the Duwamish Waterway salmon were found to contain a mean of 91 µg/gram dry weight of aromatic hydrocarbons and mean of 3.0 µg/gram dry weight of total PCBs. These levels were dramatically higher than those found among fish captured at the non-urban reference site (0.14 µg/gram dry weight and 0.82 µg/gram dry weight, respectively). The investigators also examined total PCB content in liver among the captured fish. Juvenile Chinook salmon from the Duwamish were found to have a mean total PCB content in liver of 2.6 ng/g dry weight, compared to means values of 0.68 and 0.90 ng/g dry at a hatchery and the non-urban control site. Another biomarker investigated was FACs in bile. Consistent with the results of stomach content analysis and the examination of PCB concentrations in the liver, samples from juvenile Chinook salmon captured in the Duwamish were found to have higher levels of biomarker response than fish captured at a non-urban reference location. Bile was measure at both the naphthalene and BaP wavelengths and mean levels in the Duwamish fish were found to be 1.3 and 446 µg/gram wet weight for BaP and Naphthalene respectively. In contrast, mean levels measured in reference fish were 0.05 and 59.0 µg/gram wet weight. Other important observations made by the authors include that the juvenile Chinook salmon captured during the study likely migrated through the Duwamish Waterway for an exposure period of between 1 and 6 weeks and that reports in the literature indicate that juvenile salmon exposed to aromatic hydrocarbons and PCBs in the water column can begin to take up substantial amounts of the contaminants in as little as a few days to a few weeks.

In a study of xenobiotic chemicals and metabolites in marine organisms, Varanasi and Stein (1991) reported levels of LMW PAHs and HMW PAHs detected in English sole tissue from various Puget Sound locations. Summed LMW PAHs from the Duwamish Waterway ranged from 8.8 to 26 ng/g of wet muscle tissue, while HMW PAHs detected ranged from 13 to 21 ng/g of wet muscle tissue.

In a study focusing on bioindicators of contaminant exposure, Stein et al. (1992) examined multiple biomarkers at up to five sites in the Puget Sound. The authors reported levels of sediment-associated contaminants as 3600 ng/g wet weight for total PAHs and 570 ng/g wet weight for total PCBs. In addition, among PAHs 4 to 5 ring PAHs were the predominant contaminants as they accounted for 2700 ng/g wet weight of the total PAHs present in the sediment sample. Another aspect of the study involved comparing biomarker responses in three species of flatfish collected from the Duwamish Waterway (a contaminated site) and a relatively clean reference site. Biomarker analysis performed included quantification of FACs in bile, PCBs in fish tissue, DNA adducts, AHH activity, EROD, and total hepatic GSH, while the three species of fish analyzed were English sole, rock sole and starry flounder. With the rare exception of bile FACs in English sole, each biomarker analysis for each fish species revealed elevated responses among Duwamish Waterway fish versus fish from the cleaner reference site. In fact, in most instances, the differences were statistically significant.

Varanasi et al. (1993) studied contaminant exposure and associated biological effects in juvenile Chinook salmon in various areas of Puget Sound. Endpoints measured by the group included PCBs in hepatic tissue, FACs in bile, and aromatic hydrocarbons in stomach contents. Results indicated that aromatic hydrocarbons and PCBs in stomach contents of juvenile Chinook salmon were significantly higher in the Duwamish Waterway and the Payallup estuary compared to a non-urban estuary and hatchery fish. In addition, levels of PCBs in hepatic tissue and FACs in bile were also significantly higher in Duwamish and Puyallup juvenile Chinook salmon compared to specimens collected from a non-urban estuary and a hatchery. The investigators also observed the same trend in relation to hepatic enzymes such as AHH, which is thought to play a critical role in activation of aromatic hydrocarbons to more toxic metabolites.

In another part of the study, contaminant effects on the immune system were measured by Varanasi at al. (1993) in both field-caught fish and in fish injected with contaminated sediment extract from the Duwamish Waterway. Results indicated suppression of the immune system in both assays. The final endpoint measured involved holding wild-caught fish in the lab for up to 80 days and results indicated that juvenile Chinook salmon from the Duwamish Waterway displayed significantly lower survival than specimens from non-urban comparison areas.

On December 1, 2000, it was proposed that the LDW be added to the National Priorities List (NPL), and on September 13, 2001, the LDW's listing as a NPL site became final (USEPA 2001). Concerns listed on the site narrative include sediments contaminated with semivolatile organic compounds, PCBs, inorganics and organotins (USEPA 2001). In 2002, a public health assessment was conducted and released for review and public comment by the Washington State Department of Health (2003). When the document was finalized in 2003, recommendations included that consumption of resident fish from the LDW should be limited to one 8 ounce meal per month due to concerns related to accumulation of PCBs in fish tissue. Salmon, which migrate through the LDW for short periods of time were not considered resident fish and thus were not included in this consumption advisory.

In August and September of 2004, additional marine sampling took place in the LDW for the purpose of updating the fish consumption advisory. In 2005, a report summarizing this data was released (Washington State Department of Health). The report found that PCB levels in fish were higher than historical levels and the recommendation was updated to suggest no consumption of resident LDW fish or shell fish. In 2007, a LDW fact sheet (Washington State Department of Health) was released which advised people to avoid eating any crab, shellfish or fish, with the exception of salmon, from the LDW. The fact sheet also provided recommendations on how to clean the salmon in preparation for cooking through fat removal, which could reduce the PCB exposure from salmon consumption by as much as 50%. Cleanup efforts have been extensive on the LDW since the site was added to the NPL. Some estimates put the total spent on cleanup so far at more than $70 million, although continued study is needed as the source of much of the pollution is still in question (McClure 2007).

Despite the considerable above summarized research and investment of resources, many questions related to the contamination in the Lower Duwamish Waterway remain unanswered. It is clear that contamination is present and that ecological receptors are likely at some level of increased risk of adverse health effects due to exposure to this contamination. However, especially in regard to complex mixtures, there is no universally accepted method of conducting toxicity assessments. This project was undertaken with the goal of evaluating selected tools for assessment of sediment toxicity and to potentially provide information to improve the accuracy of toxicity assessment of complex mixtures in sediment.

Collection of environmental samples

Sediment samples from each field site were collected from a boat using a petite ponar grab sampler (WILDCO, Buffalo, NY). Upon collection, the sediment samples were homogenized in stainless steel bowls and transferred to glass I-CHEM certified 1L sampling jars with Teflon lined lids (VWR, West Chester, PA). The samples were then shipped on ice overnight following chain of custody protocols to the laboratory at Texas A&M University, where they were stored at 4 ºC. Prior to extraction, each sediment sample was oven-dried overnight at 60 ºC, homogenized, ground in a mortar and pestle and then passed through an 850 µm sieve.

Water samples were collected at each site using a Beta bottle sampling device (Wildlife Supply Co., Buffalo, NY) placed just above the sediment surface within the exposure zone of the deployed cages on the day of cage deployment and immediately following cage retrieval. Samples were stored in 1 L I-CHEM certified amber bottles with Teflon lids (VWR, West Chester, PA) and were shipped on ice overnight following chain of custody protocols to the laboratory at Texas A&M University, where they were stored at 4 ºC until extraction.

Caged* in situ *exposure with juvenile Chinook salmon and Pacific staghorn sculpin

Each July from 2004-2007, juvenile pre-smolt Chinook salmon were obtained from the NOAA Mukilteo fish hatchery in Washington State for use in the *in situ* caged exposure study. In addition, in the July 2004 sampling event, adult Pacific staghorn sculpin (*Leptocottus armatus*) were used in the *in situ* caged exposure study in the Lower Duwamish Waterway. The Pacific staghorn sculpin were obtained with a scientific collection permit from Washington Department of Fish and Wildlife from a contractor (Bio-Marine Enterprises, Seattle, WA). The contractor collected the sculpin by trawling in the area of Holmes Harbor near Whidbey Island in Puget Sound (Seattle, WA). Following collection, the sculpin were held in chilled aerated coolers until cage deployment. Juvenile Chinook salmon were also held in chilled aerated tanks following pickup at the NOAA Mukilteo hatchery during transport to the exposure site.

The fish were transported to the exposure site in chilled, well-aerated coolers on a research boat. During transit, the fish were gradually acclimated to water conditions present in the LDW through addition of LDW to the water in the chilled aerated coolers. With the help of the US EPA Region 10 dive team, fish were then deployed to the river bottom in cages and exposed to contaminated sediments for seven to ten days depending on study year. The process had been performed in previous studies conducted by Donnelly lab personnel and fish survival has been 100% for this time period in most study years. The deployment process involved net transfer of fish into a 45.7 x 30.5 x 20.3 cm plastic-coated, wire mesh pinfish trap cage (Model 1264; FRABILL, Inc., Jackson, WI) that has been modified to prevent fish escape. Typically between 15 -25 fish were placed in each cage. Cages were typically deployed at four sampling in the historically polluted waterway (Figure 7) by slowly lowering the cage apparatus (with fish enclosed) to the bottom of the water column.

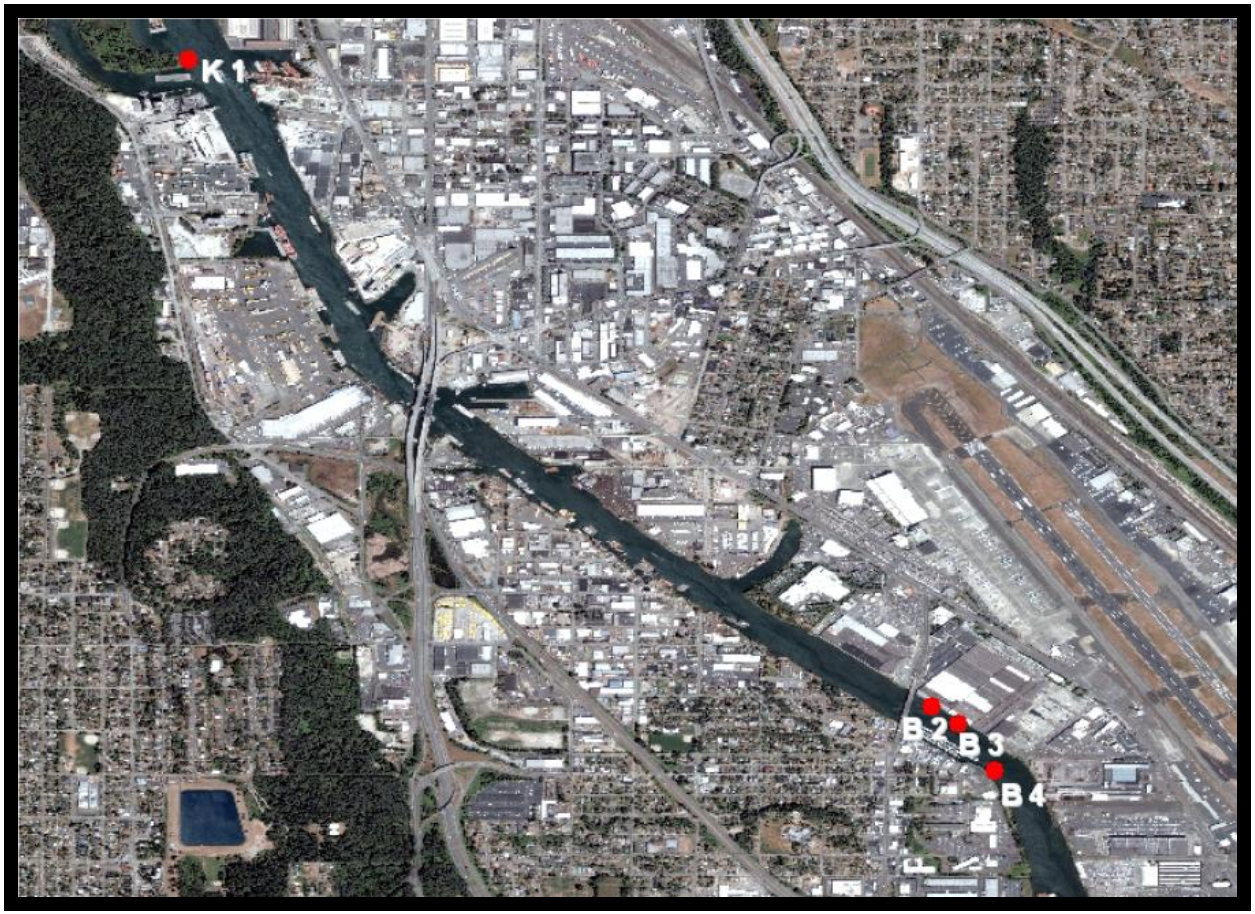

Figure 7. Displayed above are locations in the Lower Duwamish Waterway where samples of surface water and sediment were collected between 2004 and 2007. In addition, juvenile Chinook salmon (2004-2007) and Pacific staghorn sculpin (2004 only) were caged on the sampling locations for a period of 7-10 days.

Weights were attached to the bottom of the cages to order to keep the cage location stationary. During cage placement, surface water parameters directly pertinent to fish survival, such as dissolved oxygen, conductivity, pH, temperature, and total dissolved solids (TDS), were measured using a Hydrolab multiprobe (Hydrolab-Hach Company, Loveland, CO). Table 7 presents the typical physiochemical characteristics of each caging site. Between seven and ten days following placement of the cages, fish were retrieved from the sampling stations in the same order in which they were placed.

Finding an appropriate reference in close proximity to the exposed stations has represented a difficult challenge for the study. In the study's first year (2004), station NWW, near North Wind's Weir at river mile (RM) 5.2 was intended to serve as a reference site for comparison purposes. Unfortunately, only sculpin were able to survive at this location as salmon survival was 0% at this location. Inability of the Chinook salmon to survive at this location may have been related to the percent of salinity that was present at this location compared to other sampling stations. The salt-wedge must travel farther to reach the NWW station, thus the percent of salinity may have dropped substantially lower than that of other samping stations used in the study. In 2005, Station K1 was selected to be a reference station, based on consultation with the United States Environmental Protection Agency. Station K1 is located just offshore of Kellogg Island near (RM) 0.9 on the West side of the LDW. Kellogg Island currently represents the largest contiguous area of intertidal habitat in the LDW (Tanner 1991), thus the area was thought be a less contaminated location relative to other selected sampling locations. However, it was later determined that levels of contamination at station K1 were comparable and it would not be appropriate to consider station K1 as a reference station. Station K1 was sampled in the 2005 – 2007 sampling events and was considered to be an exposed station by study investigators.

Stations B2, B3 and B4 were sampled in each of the 4 study years and also serve as exposed stations. These sampling stations are located between RM 3.4 and 3.6 approximately, with stations B2 and B3 being closer to the East banks of the LDW and station B4 being closer to the West bank of the LDW. Boeing plant 2, South Park Marina and the neighborhood of South Park are all located in close vicinity to the sampling stations. The locations are contained within an area that was recommended

as early action area 4 (Windward Environmental LLC 2003b) out of 7 proposed early action areas on the LDW as the presence of PCBs, PAHs phthalates and metals has been previously demonstrated in the area (Windward Environmental LLC 2003a).

During the July 2007 sampling event, steps were taken to strengthen potential comparisons to controls, as cages of Chinook salmon were maintained at the hatchery in clean water. This was needed due to the lack of identification of an appropriate non-polluted reference site in the area. In order to facilitate examination of the effects of feeding on levels of contaminants in tissues and response in bioassays, control groups of fed and unfed fish were maintained. Upon completion of the eight-to-nine day caging period, all cages were retrieved by a US EPA dive team. Following retrieval, all fish, including field site exposed and caged controls were sacrificed by a lethal overdose of MS-222 followed by severing the spinal cord. Fish were then placed in an alphanumerically-coded zip-lock baggie, and quickly frozen on dry ice. Fish samples were then returned to the laboratory through overnight shipment on dry ice and were stored at -80°C until analysis. All animal care procedures were conducted in accordance with institutional animal care and use committees (IACUC) for Texas A&M University. Weights and lengths were recorded for all fish, followed by dissection of gills and liver. Following collection of tissue weights, dissected gills and liver were then placed in tissue bags and stored in a -80 °C freezer until later use in biomarker analysis.

Sample extraction

For sediment samples, approximately 10 grams of dried sample were extracted using methylene chloride in a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE). Liquid:liquid extractions were performed with methylene chloride according to US EPA standard extraction method 3510C (USEPA 1996b). Fish tissues were composited according to the sediment station where cages were placed. Compositing is necessary since analysis of individual fish is usually prevented by small fish masses and analytical detection limits. Therefore, average tissue PAH concentrations for each sampling station will be determined. Fish tissue samples were first composited in a stainless steel blender according to exposure station (treatment group). Next, blended fish tissue composites were weighed wet and then lyophilized overnight under vacuum using a Labconco Free Zone 12 L Freeze Dry System

(Labconco Corp., Kansas City, MO). Freeze-dried fish tissue composites were then re-weighed prior to extraction to determine the percent moisture of each composite. In addition, fish tissue composites were then ground into a powder-like consistency with a mortar and pestle to ensure optimal extraction.

Tissue samples were then extracted with 40 mL of methylene chloride using a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE). A surrogate spike solution with deuterated PAHs was added to each sample to correct for PAH recovery efficiencies. The ASE was operated at 1500 psi, and each sample was solvent flushed in two cycles at 50% cell volume and 60 sec purge time. The stainless steel extraction cells were heated to 100°C with 2 min pre-heat, 5 min heat, and 5 min static times for each cycle. Sample filters (Part No. 049458) will be grade D28 with a 1.983 cm diameter. A Zymark TurboVap LV evaporative solvent reduction apparatus (Zymark Corp., Hopkinton, MA) at 45°C for 13 min were used to concentrate the extracts to one mL. Extracts were further reduced to 0.5 mL under purified nitrogen.

Chemical analysis

Sediment extracts, water extracts and extracts from fish tissue composites were all analyzed for PAHs and other semivolatile organic compounds (SVOCs) using USEPA method 8270C (USEPA 1996c) and for total PCBs and PCB homologs following USEPA method 680 (Alford-Stevens et al. November 1985). Analysis was performed using an Agilent 5975 gas chromatograph with a mass selective detector in selected ion monitoring mode. A 60 m x 0.25 mm ID x 0.25 mm film thickness column (Agilent Technologies, Palo Alto, CA) was utilized. The injection port was maintained at 300 °C and the transfer line at 280 °C. The temperature program was as follows: 60 °C for 6 minutes increased at 12 °C/minute to 180 °C and then increased at 6 °C/minute to 310 °C and held for 11 min for a total run time of 47 minutes.

^{32}P-Postlabeling

DNA adducts were quantified in gill tissues, and liver tissue (only during the 2004 sampling event) following the ^{32}P-DNA postlabeling method of Reddy and Randerath (1986). Gills and livers were excised from individual fish and then composited

according to station with 3-8 fish per composite. The DNA was then isolated by solvent extraction combined with enzymatic digestion of the protein and RNA. The DNA is dissolved in 1.5 mM sodium chloride, 0.15 mM sodium citrate, and each concentration measured spectrophotometrically using a value of 20 A_{260} units/mg of DNA. The isolated DNA will be stored at -80°C until analysis.

DNA adducts were quantified using nuclease P1-enhanced bisphosphate ^{32}P-postlabeling. Approximately 10 µg of DNA from each tissue was digested by enzymatically hydrolyzing the DNA to normal and modified deoxyribonucleoside 3´-monophosphates using micrococcal endonuclease (Sigma) and spleen phosphodiesterase (WBC). The hydrolyzed DNA was treated with nuclease P1 (Calbiochem), which selectively hydrolyzes normal 3´-mononucleotides to nucleosides, thereby enriching the mixture in modified (adducted) 3´-monophosphates. The enriched modified nucleotides were converted to 5´-^{32}P-labeled 3´,5´-bisphosphate derivatives by incubation with carrier-free [γ-^{32}P]ATP (MP) and polynucleotide kinase (USB) mediated phosphorylation. The ^{32}P-labeled DNA adducts were purified and partially resolved by one-dimensional development using 2.3M NaH_2PO_4 (pH 5.75). An original area (2.8 x 1 cm) of the lower portion of cellulose map containing adducts were excised and the ^{32}P-labeled products were contact-transferred to fresh polyethyleneimine (PEI)-cellulose sheets. Labeled products were then resolved by two-dimensional thin-layer chromatography (TLC). The first dimension used 3.82M lithium formate + 6.75M urea (pH 3.35). The second dimension is obtained using 0.72M $NaH_2PO_4 \cdot H_2O$ + 0.4M TRIS + 7.65M urea (pH 8.2).

The ^{32}P-labeled products or radioactive spots on each chromatogram were quantitated by Instantimager (Perkin Elmer) (Zhou et al. 1999). Screen-enhanced autoradiography of the chromatograms was performed on Kodak XAR-5 X-ray (gray) film for 25 h at -80°C. The count rates of individual radioactive spots from fish tissue composites from each exposure group were used to calculate relative adduct labeling (RAL) values ± standard error of the mean (SEM) for each spot. RAL values were calculated according to the following:

RAL = Sample count rate [cpm] ÷ (DNA-P [pmol] * Spec. Act. $_{ATP}$ [cpm/pmol])

Where DNA-P represents the amount of DNA assayed (expressed as pmol DNA monomer units) and specific activity (Spec. Act.) of [γ-^{32}P]ATP used in the labeling reaction. Thus, for 10 µg DNA, and ATP with a specific activity of 4.5X10^{6} cpm/pmol, 145 cpm corresponds to an RAL value of 10^{9} (i.e., an estimated level of one modification in 10^{9} DNA nucleotides). Total RAL values were calculated by summing the values for individual spots from each DNA sample. Differences of mean DNA adduct levels between exposure groups or cage locations will be tested with ANOVA. The probability level used for determining significance will be $P < 0.05$.

Western blot assay

Western blot was used to determine the relative levels of CYP1A1 expression in liver samples of *in situ* caged juvenile Chinook salmon. In order to obtain the required amount of liver tissue for analysis, three livers from the same treatment group were composited for each sample.

Microsome preparation

The first step of the western blot method involves preparation of liver microsomes. To accomplish this, whole livers are placed in tubes containing 3mL of homogenize buffer (pH 7.4) which has been kept on ice. The homogenize buffer was made by mixing 1.21 grams of 10 mM Tris and 85.7 grams of 0.25 M sucrose. The pH is then adjusted to 7.4 and the total volume of brought to 1000 mL with distilled deionized water. Following addition of livers to the homogenize buffer, scissors are used to cut the livers into small pieces.

Following this procedure, the livers were homogenized and then centrifuged at 2500 rpm for 10 minutes. The supernatant was then transferred to fresh centrifuge tubes and spun again at 9000 rpm for 10 minutes. Next the supernatant was transferred to 50 mL centrifuge tubes. Following the transfer 5 mL of homogenize buffer and 125 µL of a 0.32 M CaCl and 50 mM Tris mixture was added. The samples are then spun at 14000 rpm for 20 minutes, following which the supernatant is stored at -80 °Celsius until use.

The next step involved adding 3 mL of wash buffer (pH 7.4) to the pellet and the gently mixing. The wash buffer was prepared by mixing 1.21 grams of 10 mM Tris and 11.18

grams of 150 mM KCl. The pH is the adjusted to 7.4 and the total volume of was brought to 1000 mL with distilled deionized water. Next the samples are again spun at 14000 rpm for 20 minutes and then 75-100 µL of suspension buffer (pH 7.8) was added to the samples, depending on the size of the pellet. The suspension buffer was made by mixing 3.03 grams of 50 mM Tris, 42.85 grams of 0.25 M sucrose and 100 mL of 20% glycerol. The total volume is then brought to 500 mL with distilled deionized water. Following addition of the suspension buffer, the samples are then homogenized and the microsome is stored at -80 °Celsius until use.

The next step in the process is to measure the concentration of protein in the liver microsomes. This process was begun by adding 2 µL of the sample to 400 µL of water. Samples were then added in duplicate to a 96-well plate, with the first two columns reserved for standards. Dye was then mixed by adding 40 mL of water and 10 mL of Biorad Protein Assay Dye Reagent Concentrate (catalog no. 500-0006). Following this step, 200 µL of dye was added to each well of the 96-well plate. Concentration of the samples is then read using a SpectraMax 340 (Molecular Devices, Sunnyvale, CA) at the 600 (nm) wavelength. The means of concentrations of protein in the samples were then determined.

Western blot analysis

The next step involved dilution of the microsomes such that each sample contained a minimum of 5 µg of protein for each µL of sample. This is followed by cleaning plates, setting up the gel apparatus and preparation of the separating gel momomer. The separating gel is a standard 10% SDS-polyacrylaminde gel electrophoresis (SDS-Page) preparation. This gel mixture for making two gels contains 23.8 mL of distilled water, 15.2 mL of 1.5 M Tris-HCL (pH = 8.8), 20 mL of acrylamide/bis-acrylamide (30%/0.8% weight to volume), 300 µL of 20% SDS, 600 µL of 10% weight to volume ammonium persulfate, and 24 µL TEMED. When TEMED is added, the gel was poured immediately in order to keep it from solidifying too early. Butomol is added on top of the gel in order to keep it even.

Next 6.25 µL of 5X buffer is added to a new vial. After this, 20 µL of sample was added and the mixture was spun down. The next step was preparation of the stacking gel.

The mixture for one stacking gel contains contains 6.8 mL of distilled water, 1.25 mL of 0.5 M Tris-HCL (pH = 6.8), 1.7 mL of acrylamide/bis-acrylamide (30%/0.8% weight to volume), 50 µL of 20% SDS, 100 µL of 10% weight to volume ammonium persulfate, and 10 µL TEMED.

Following pouring of the stacking gels, while the gel was setting, a syringe was used to poke holes in the tops of the sample vials. This was done in order to keep the tops from popping off during the next step, which involved heating the samples at 95 ºC for 3 minutes. Running buffer was mixed by combining 100 µL of10X buffer and 900 µL of distilled water. Next the running buffer was added to the tray. Next the samples were added to the wells and were run at 120 volts for 3 hours.

Transfer buffer was made by mixing 2.55 L of water, 450 mL of methel, 43.2 grams of glycine and 9.075 grams of Tris Base. The mixture was then stirred using magnet stir bars for 20 minutes. Next the membrane is cut, the stacker gel is discarded and the gel is placed in water. Markers are visible at this point and the membrane was placed in a Tupperware trey with methanol. For transfer, the black end piece goes on bottom, followed by the sponge, filter paper, the gel, filter paper, sponge, and end piece. The transfer is completed in the cold room because heat is produced during the transfer. The transfer process is completed on a shaker. Transfer buffer is poured into a trey, the cassette was loaded and the Biorad Powerpac HC was run at 25 volts overnight.

Blocking is the next step in the process. The first step of blocking involved mixing 3% milk with TBS. This was accomplished by mixing 24.2 grams of 200 mM Tris (pH 7.6), and 80 grams of 1.37 M NaCl. The membranes were then trimmed if needed. Next, 3% milk with TBS was added and the membranes were put on a shaker for 10 minutes. This step prevents binding at unoccupied membrane sites. The next step was addition of the primary antibody, CYP1A1 (H-70, rabbit polyclonal IgE, Santa Cruz Biotechnology catalog no. 20722). The membrane was then incubated with the 3% TBS/primary antibody mixture for 5 hours on a shaker at room temperature.

The next step was to wash the membrane three times in water for 2 minutes each time and then addition of the secondary antibody. The secondary antibody (goat, anti-rabbitt IgE – HRP, Santa Cruz Biotechnology catalog no. 2004) binds to the first antibody. After addition of the secondary antibody, the membrane was again place on a shaker for 2 hours at room temperature. This was followed by washing the membrane 3 times again with water for 2 minutes each wash.

The final step was addition of Western Lightning Oxidizing Reagent. This solution was poured over the membrane, ensuring that the membrane was completely covered. The membrane was then taken to a dark room and developed using Kodak film. Intensity of the bands was then quantified using scanned images of the developed film in the Image J program.

Results

July 2004 water quality parameters

The first task completed upon arrival at sampling stations was collection of water quality parameters in order to assess the ability of the fish to survive in cages at the selected locations for the exposure duration. These readings were collected just above the sediment surface, which is the same depth at which the fish were then exposed. Selected parameters such as temperature, pH, salinity and percent dissolved oxygen from the July 2004 sampling period are listed in Table 7, however other parameters such as turbidity and conductivity were also typically recorded.

Table 7. Water quality parameters from the July 2004 sampling period are displayed. These data were recorded prior to surface water and sediment sampling in order to ensure that conditions were acceptable for fish survival.

Site	Date Sampled	GPS Coordinates Lat. (°N)	Long. (°W)	Temp (°C)	pH	Salinity (pss)	DO (% saturation)
NWW	07/14/04	47.30.530	122.17.558	19.6	7.0	7.9	98.0
B2	07/14/04	47.31.759	122.18.783	20.9	6.8	3.9	97.0
B3	07/14/04	47.31.711	122.18.711	20.2	6.9	4.5	96.2
B4	07/14/04	47.31.595	122.18.614	20.4	7.1	5.8	95.2

While both Chinook salmon and Pacific staghorn sculpin were exposed at each of these stations, only the Chinook salmon experienced mortality during the exposure period. Chinook salmon at the North Wind's Weir were not able to survive and we speculate that the mortality may have been a result of the salt-wedge that is present in the LDW estuary. Although the reading taken at the time of cage deployment is well within the range that Chinook salmon have survived in during other study years, these readings only represent a snap shot in time and it is possible that the salinity conditions varied greatly during the exposure period.

Sediment analysis July 2004

One sediment sample was taken at each sampling location just prior to cage deployment. The levels of contaminants measured in those samples are listed in Table 8. All sediment values are reported on a dry weight basis.

The North Wind's Weir (NWW) site was intended to serve as a reference location and was found to have the lowest amount of total PAHs present (700 ng/g) and the lowest amount of total PCBs (non detect) as well. Site B2 was characterized by the highest total PAH exposure (1737 ng/g), while site B3 exhibited the highest total PCBs (2751 ng/g) present among the samples. Ratios of low molecular weight (LMW) PAHs to high molecular weight (HMW) PAHs varied among sampling stations, as the NWW and B3 sites had more LMW PAHs and the B2 and B4 had more HMW PAHs.

Table 8. Summary results for chemical analysis of sediment samples collected in the Lower Duwamish Waterway during July 2004 are displayed below. One sediment sample from each station was analyzed for PAHs and PCBs.

Date Sampled	Contaminants Measured (ng/g dry)	NWW	B2	B3	B4
7/14/04	Total PAHs	700	1737	1312	769
7/14/04	Carcinogenic PAHs	58	310	137	144
7/14/04	LMW PAHs	287	435	469	182
7/14/04	HMW PAHs	191	634	377	283
7/14/04	Total PCBs	nd	1586	2751	133

Surface water analysis

During the July 2004 sampling period, three surface water samples were taken at each site just prior to cage deployment and three additional surface water samples were taken just following cage retrieval. All water samples from each sampling site have been averaged together regardless and mean levels of contaminants measured in surface water are listed in Table 9. In regard to PAHs, the range between the sampling location with the least amount of PAHs detected and the sampling location with the highest amount of PAHs detected was only 76 ng/L (site B3) to 114 ng/L (site NWW), respectively. It should be noted that one sample from site B3 was found to have 11, 931 ng/L of total PAHs and was excluded from statistical analysis as an outlier. No other sample (out of 24) from the July 2004 sampling period was found to have levels of total PAHs more than 600 ng/L. The result was likely the result of more sediment particles being present in the sample, compared the rest of the samples taken, as surface water samples were not filtered prior to analysis. PCBs were not detected in water samples from the July 2004 sampling period.

Tissue composite analysis July 2004

Mean levels of PAHs and PCBs measured in fish tissue are presented in Table 10. Fish tissue samples were typically composites of 4-6 fish bodies, excluding the liver and gills which were dissected out for DNA adduct analysis. Mean weight and length for the juvenile Chinook salmon following sacrifice were 8.2 cm and 10.5 grams, while Pacific staghorn sculpin were 9.0 cm in mean length and 15.1 grams in mean weight.

Among sculpin, mean levels of total PAHs in tissue found in samples from exposure sites were higher than the mean level of total PAHs in control fish sacrificed prior to the exposure. In regard to Chinook salmon, however, this trend is not demonstrated as hatchery fish sacrificed prior to the exposure were found to have levels of total PAHs similar to those found in exposed fish.

Table 9. Results of chemical analysis of surface water samples collected in the Lower Duwamish Waterway during July 2004 are displayed below. At each sampling station three surface water samples were collected prior to cage deployment and three additional surface water samples were collected at cage retrieval. Results displayed are mean total PAHs, standard error of the mean and total PCBs of surface water samples collected from each station.

Date Sampled 7/14/2004 - 7/22/04	Mean Total PAHs (ng/L)	SEM	Total PCBs
NWW	114	20	nd
B2	107	8	nd
B3	76	11	nd
B4	102	10	nd

Table 10. In July 2004, Pacific staghorn sculpin and juvenile Chinook salmon were caged for 10 days at various locations in the Lower Duwamish Waterway. Results of chemical analysis of fish tissue composites from each sampling station are displayed below. Chemical analysis was perfomed for PAHs and PCBs.

Treatment Group	Species	Total PAHs (ng/g wet)	SEM	Total PCBs (ng/g wet)
Time = 0	Pacific staghorn sculpin	77	6	nd
NWW	Pacific staghorn sculpin	393	117	nd
B2	Pacific staghorn sculpin	167	85	nd
B3	Pacific staghorn sculpin	86	45	nd
B4	Pacific staghorn sculpin	288	--	nd
Time = 0	Chinook salmon	132	48	nd
NWW	Chinook salmon	--	--	--
B2	Chinook salmon	48	11	nd
B3	Chinook salmon	141	109	nd
B4	Chinook salmon	103	40	nd

It is also of note that one data point is missing as Chinook salmon experiences 100% mortality at the NWW sampling site. PCBs were not detected in either test species used in the exposure study.

DNA adduct analysis July 2004

Examples of the end product produced by ^{32}P-postlabeling analysis are presented in Figure 8. These autoreadiographs can be quantified using instant imager software (PerkinElmer) (Zhou et al. 1999). Statistical analysis of DNA adduct results was performed using SigmaPlot software version 11.0 (Systat Software, Inc., San Jose, CA). DNA adducts were quantified as relative adducts labeled (RAL) per 10^9 nucleotides. RAL and standard error of the mean (SEM) is presented in Table 11 for ^{32}P-postlabeled hepatic and gill tissue from both juvenile Chinook salmon and Pacific staghorn sculpin. Gill and hepatic samples represent composites of tissue from 4-6 fish per sample. Typically adduct analysis was performed on a minimum of four composites from each sampling station.

Levels of total RAL in composites from each station were compared for significant difference using one way analysis of variance (ANOVA). If an instance of statistical significant difference was observed among any of the treatment groups (fish caged at exposed stations and time = 0 hatchery fish) in the ANOVA analysis, a post-hoc test was selected for multiple comparisons versus the control (time = 0 hatchery fish). Typically the Holm-Sidak method was selected.

In both species of fish sampled, gill tissue exhibited higher levels of DNA adducts compared to hepatic tissue (Table 11).

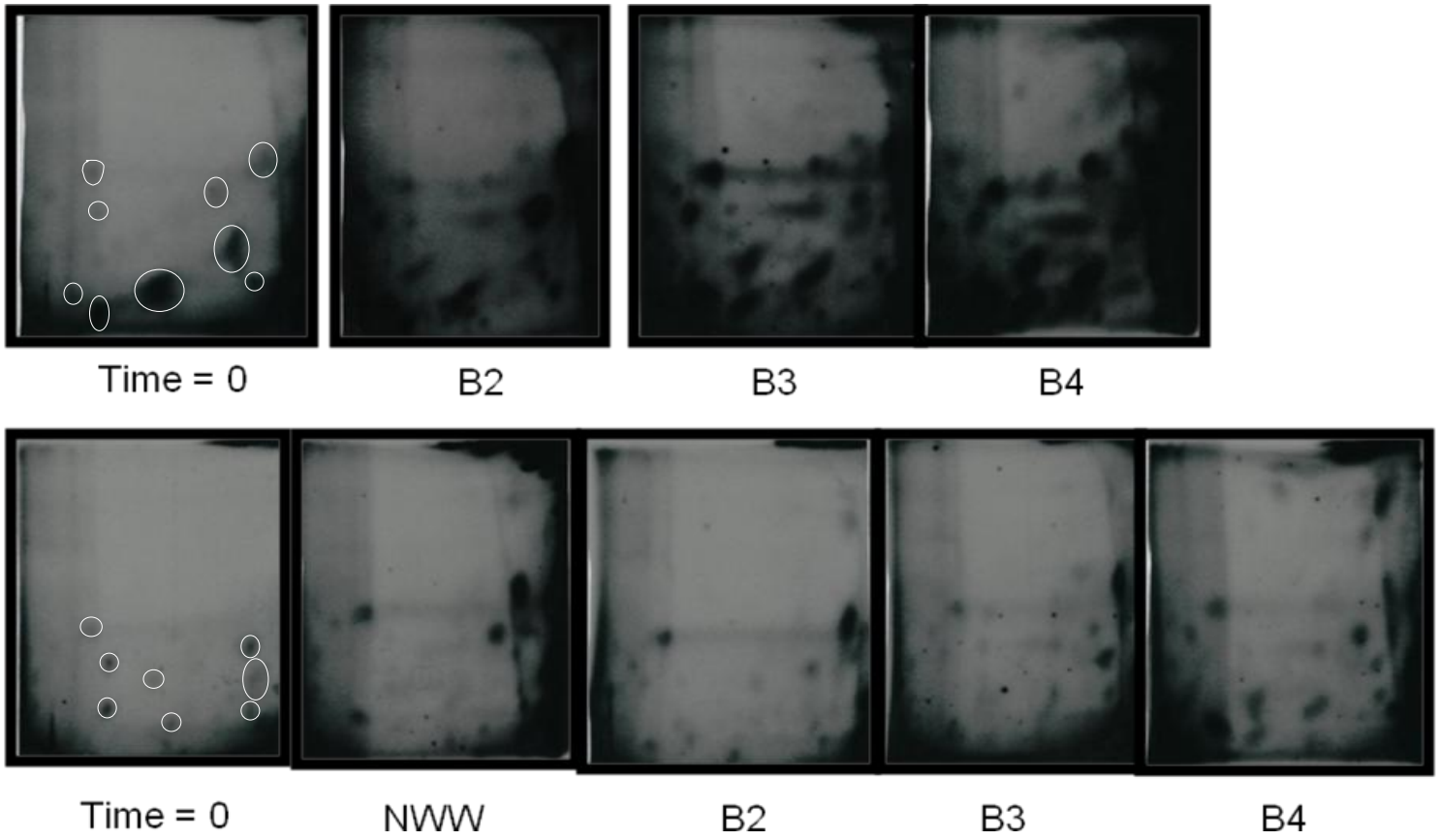

Figure 8. Pacific staghorn sculpin (bottom) and juvenile Chinook salmon (top) were caged in the Lower Duwamish Waterway for 10 days in July 2004. DNA adduct analysis was conducted using ^{32}P-postlabeling analysis. Representative autoradiographs of this analysis are presented above. During the quantification process, adducts (dark spots) are circled and the area and intensity are measured. Examples of adducts are circled on the time = 0 autoradiographs.

Table 11. Pacific staghorn sculpin and juvenile Chinook salmon were caged in the Lower Duwamish Waterway for 10 days in July 2004. DNA adduct analysis was conducted on hepatic and gill tissue composites using ^{32}P-postlabeling analysis. Typically tissues from 4-6 fish were used in each sample and a minimum of 4 samples were in each treatment group. Quantification of autoradiographs which were produced from this analysis are presented below. * Statistically significant difference from control ($p<0.05$)

Treatment Group	Species	Liver RAL x 10^9 Nucleotides	SEM	Gill RAL x 10^9 Nucleotides	SEM
Time = 0	Pacific staghorn sculpin	8	1	12	1
NWW	Pacific staghorn sculpin	21	4	21*	3
B2	Pacific staghorn sculpin	15	2	13	1
B3	Pacific staghorn sculpin	13	3	22*	5
B4	Pacific staghorn sculpin	12	3	22*	2
Time = 0	Chinook salmon	10	2	25	4
NWW	Chinook salmon	--	--	--	--
B2	Chinook salmon	12	3	86*	20
B3	Chinook salmon	11	1	42	10
B4	Chinook salmon	6	2	85*	9

In addition, a trend of higher levels of adducts in Chinook salmon compared to Pacific staghorn sculpin was observed. Among sculpin, livers dissected from fish caged in the Lower Duwamish Waterway were not found to have significantly higher levels of adducts than control Pacific staghorn sculpin which were sacrificed prior to exposure. In addition, gills dissected from sculpin caged at stations NWW, B3 and B4 in the LDW were found to have significantly higher levels of RALthan control Pacific staghorn sculpin, which were sacrificed prior to the exposure (p-value<.05).

Among Chinook salmon, gills dissected from fish which were exposed at sampling locations B2 and B4 were found to have significantly higher levels of DNA adducts (RAL) than hatchery control fish which were sacrificed prior to the exposure (p-value<.05). However, consistent with results from Pacific staghorn sculpin, no significant differences were observed in regard to levels of DNA adducts detected in Chinook salmon livers versus the control. Based on these results and sporadic availability of sculpin, future studies focused on DNA adduct analysis of juvenile Chinook salmon tissue and used gill tissue exclusively.

Water quality parameters 2004 - 2007

Water quality parameters that were collected during each sampling period from July 2004 – July 2007 are listed in Table 12. Stationary landmarks, such as building vents features and pilings were used in order to ensure that sampling locations were consistent between different sampling events, despite GPS coordinates that varied slightly from year to year.

Table 12. Water quality parameters from the July 2004 – July 2007 sampling periods are displayed. These data were recorded prior to surface water and sediment sampling in order to ensure that conditions were acceptable for fish survival. Depth of collection varied depending on tide conditions, but was typically just above the sediment surface where cages would later be deployed. -- Reading was not recorded in log book.

Site	Date Sampled	GPS Coordinates		Temp	pH	Salinity	DO
		Lat. (°N)	Long. (°W)	(°C)		(pss)	(% saturation)
NWW	07/14/04	47.30.530	122.17.558	19.6	7.0	7.9	98.0
B2	07/14/04	47.31.759	122.18.783	20.9	6.8	3.9	97.0
B3	07/14/04	47.31.711	122.18.711	20.2	6.9	4.5	96.2
B4	07/14/04	47.31.595	122.18.614	20.4	7.1	5.8	95.2
K1	07/12/05	47.33.399	122.20.665	12.9	7.0	27.2	81.0
B2	07/12/05	47.31.759	122.18.782	17.0	7.0	5.3	89.0
B3	07/12/05	47.31.719	122.18.711	18.0	7.0	5.0	89.0
B4	07/12/05	47.31.598	122.18.622	17.0	7.1	--	79.0
K1	07/26/06	47.33.401	122.20.657	13.1	7.1	27.9	82.9
B2	07/26/06	47.31.763	122.18.803	19.5	7.4	11.6	85.6
B3	07/26/06	47.31.727	122.18.747	19.7	7.4	11.2	84.1
B4	07/26/06	47.31.604	122.18.628	21.6	7.5	5.1	90.2
K1	07/31/07	47.33.407	122.20.668	13.4	7.7	28.5	90.0
B2	07/31/07	47.31.755	122.18.792	18.5	7.3	9.3	92.0
B3	07/31/07	47.31.709	122.18.716	19.1	7.4	3.4	89.0
B4	07/31/07	47.31.598	122.18.614	18.8	7.4	11.8	85.9

It should be noted that the sailinty levels temperature and salinity levels varied from year to year and even between stations depending on the time at which the station was sampled. Temperatures recorded ranged from 12.6 to 21.6° C, while sailinty levels ranged from 3.4 to 28.5 practical salinity scale (pss), with the highest levels being recorded at site K1.

Sediment analysis July 2004 – July 2007

Mean levels of contaminants measured in sediment samples from July 2004 - July 2007 are presented in Table 13. The NWW site was not included on this table as it was only sampled during the July 2004 sampling period. The K1 sampling station was sampled during the July 05 – 07 sampling events (n = 3), while B2, B3 and B4 were sampled during the July 04 – 07 sampling events (n = 4).

In regard to total PAHs, sampling stations B2 was found to have the highest levels as the mean of approximately 2.7 parts per million (ppm). Sampling station K1, originally intended to be a reference station, ranked second in regard to total PAHs as mean measured levels were found to be 1.78 ppm. An important distinction between these two sampling stations is that a PAH/PCB mixture is present at station B2 (1.2 ppm mean total PCBs), while station K1 contained only 16 parts per billion (ppb) of total PCBs. When the ratio of HMW PAHs to LMW PAHs across stations is examined, at each station HMW PAHs were more predominant in the sediment samples.

Table 13. Summary results for chemical analysis of sediment samples collected in the Lower Duwamish Waterway during July 2004 – July 2007 are displayed below. One sediment sample from each station during each sampling year and samples were analyzed for PAHs and PCBs. Means of all sampling years from each station and standard error of the mean are presented in order to summarize the data.

Date Sampled	Contaminants Measured (ng/g dry)	K1		B2		B3		B4	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
July 2004 - July 2007	Total PAHs	1782	741	2712	1373	1730	536	1395	448
July 2004 - July 2007	Carcinogenic PAHs	423	197	457	203	449	155	308	104
July 2004 - July 2007	LMW PAHs	267	125	541	248	253	107	231	111
July 2004 - July 2007	HMW PAHs	783	340	1159	644	770	273	604	201
July 2004 - July 2007	Total PCBs	16	8.2	1248	414	1752	895	383	264

Surface water analysis July 2004 – July 2007

Yearly mean levels of total PAHs measured in surface water samples collected during the July 2004 - July 2007 sampling periods and standard error of the mean are presented in Figure 9. The data is presented in this format to illustrate yearly variation.

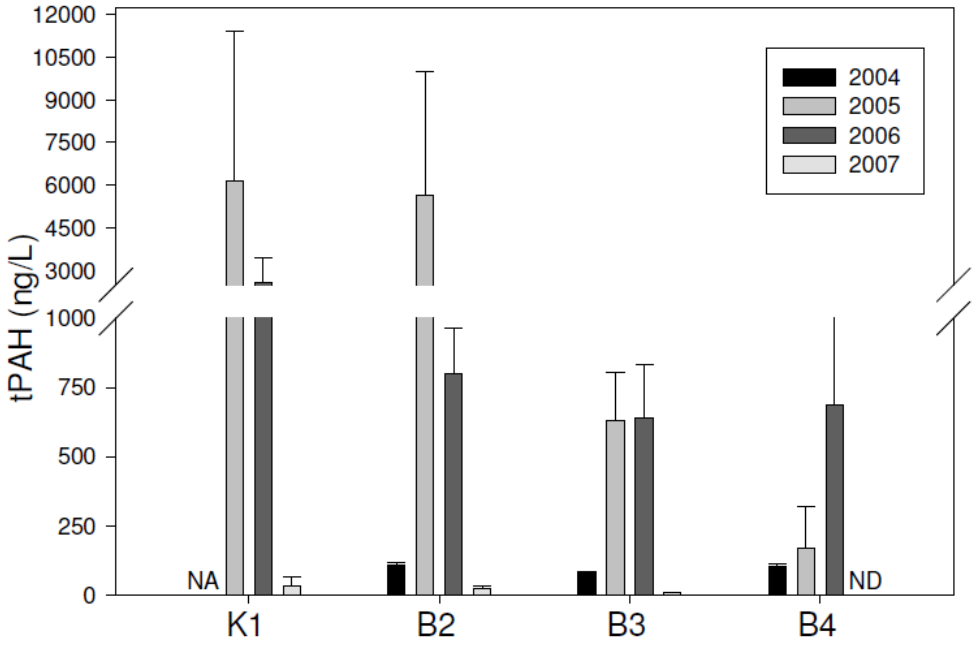

Figure 9. Surface water samples were collected each year from various sampling stations in the Lower Duwamish Waterway from 2004 – 2007. Results of chemical analysis of PAHs are presented above. Mean total PAHs and standard error of the mean are presented according to sampling year.

Exposure varied substantially from year to year. The highest levels measured during the study period occurred during the 2005 sampling period and likely may have resulted from a dredging event which had recently taken place on the LDW and stirred up the contaminated sediment. Water samples are not filtered prior to extraction, thus sediment particles are sometimes present in samples. Contaminants which are sorbed onto sediment particles in those water samples are thus extracted and likely account in part for higher levels of contamination reported in the LDW 2005 results. This could be prevented through filtration of water samples prior to extraction, however filtration of water samples would not provide a realistic picture of what contaminants may be available for uptake into receptors. For this reason, it is standard operating protocol on this project to extract the samples with out filtration. Further supporting this theory of altered results due to dredging activity is that during the 2005 study period, total PCBs were detected in most of the surface water samples, while every other study period produced only non-detects in this regard. The levels of total PCBs measured in 2005 LDW surface water are displayed in Figure 10.

The mean range of total PCBs detected is from 0.2 ppb to just over 6 ppb. The data does not appear to correlate with levels of PCBs detected in sediment as K1, which had minimal levels of PCBs in sediment, is one of two stations at which levels of 6 ppb of total PCBs were detected in 2005 surface water samples.

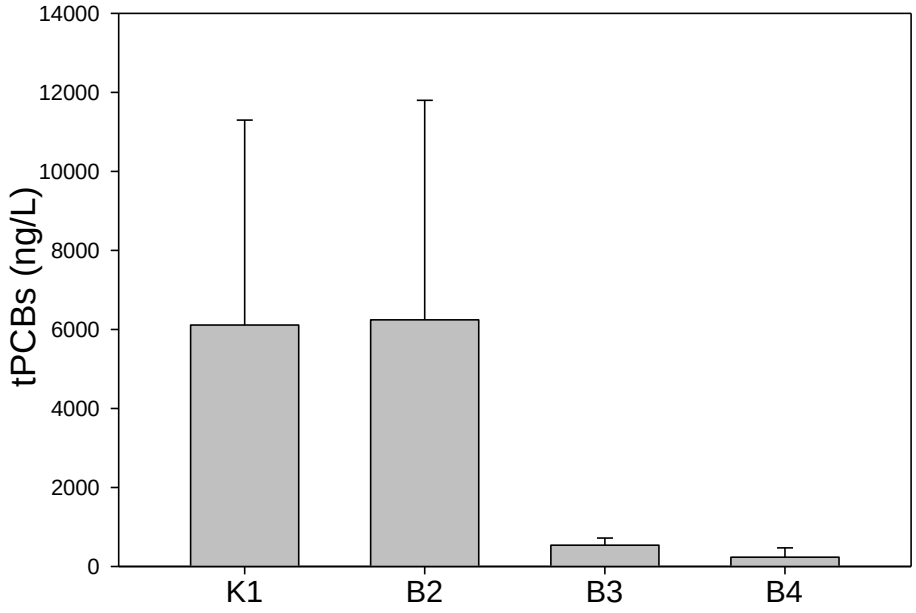

Figure 10. Surface water samples were collected each year from various sampling stations in the Lower Duwamish Waterway from 2004 – 2007. Results of chemical analysis of PCBs from the 2005 sampling event are presented above. Mean total PCBs and standard error of the mean are presented. Analyses from other sampling years (2004, 2006 and 2007) were not able to detect the presence of PCBs in any of the samples collected.

Sampling design varied from year to year in regard to surface water sampling due to a number of factors including equipment malfunction and input from groups such as the Lake Union Action Team. During the 2004 sampling period, 6 surface water samples were collected at each sampling location. Limited variation was observed among repeated samples so in subsequent years the sampling design was altered to include a smaller number of samples at each station. During the 2005 sampling event, one surface water sample was collected before cage deployment and a second sample was collected during cage retrieval at each sampling station. During the 2006 sampling

event, based on a request from the Lake Union Action Team, one surface water sample was collected from near the east bank, middle and west bank of the LDW to allow comparison of samples from the middle of the river to samples taken near the river banks at each station. These results are displayed in Figure 11.

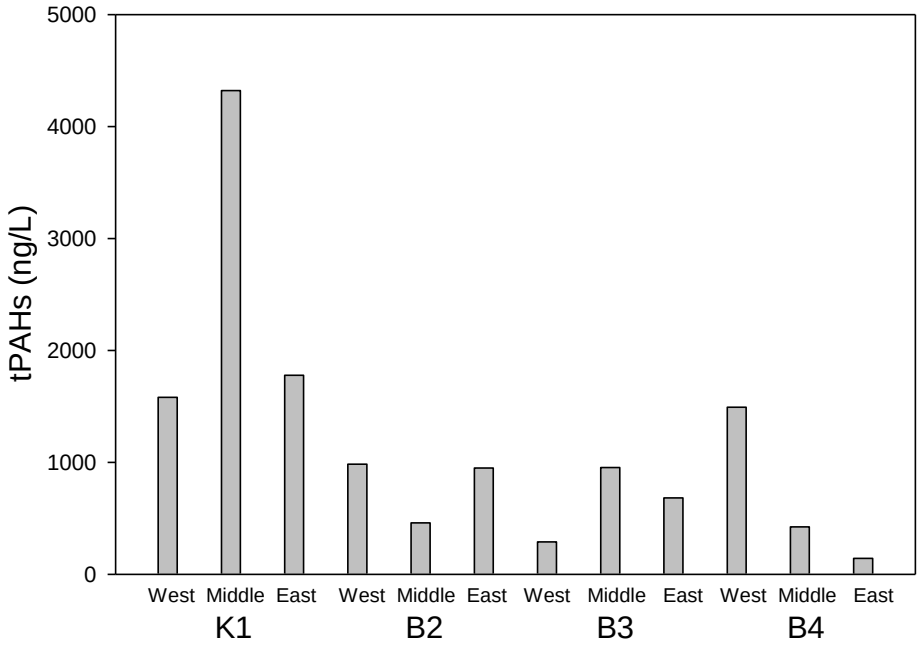

Figure 11. One surface water sample was collected on the west side, middle and east side of various locations in the Lower Duwamish Waterway during July 2006. Results of chemical analysis of total PAHs are presented above according to station.

During the 2007 sampling event, as in the 2005 sampling event, one surface water sample was collected before cage deployment and a second sample was collected during cage retrieval at each sampling station. The mean for each sampling location from each sampling year was calculated using all samples collected, regardless of sample design. The lowest levels of total PAHs in surface water were found in samples

collected during the July 2007 sampling event. This finding was consistent across stations and at sampling location B4, total PAHs were not detected in the 2007 samples.

Tissue composite analysis July 2004 – July 2007

Mean levels of total PAHs in Chinook salmon tissue samples from each sampling year are displayed in Figure 12. Among fish that were caged at the hatchery, one treatment was fed and another was not, in order to allow examination of the effects of feeding on levels of PAHs in tissue and biomarker responses. For samples from the B2, B3 and B4 treatment groups, means and standard error of the means from each sampling year from July 2004 – July 2007 are displayed, however analysis of the hatchery caged fed and hatchery caged not fed treatment groups was only completed during the July 2007 sampling event. In addition, results for the K1 treatment group are only displayed for the 2005 and 2006 sampling periods. This was necessitated because in 2004 station K1 was not sampled and in 2007, during cage retrieval, EPA Region 10 divers were unable to locate cage deployed at station K1.

For statistical comparisons, total PAHs detected in tissue composites collected from each sampling station from all study years were compared to fish tissue samples from Chinook salmon which were sacrificed prior to the exposure (time = 0) from all study years. A Kruskal-Wallis one way ANOVA was performed on ranks using SigmaPlot software (San Jose, CA) because the data failed to meet the assumption of a normal distribution. Results indicated that a significant difference between the treatment groups was present, thus a posthoc test using Dunn's method was conducted in order to make multiple comparisons versus the control.

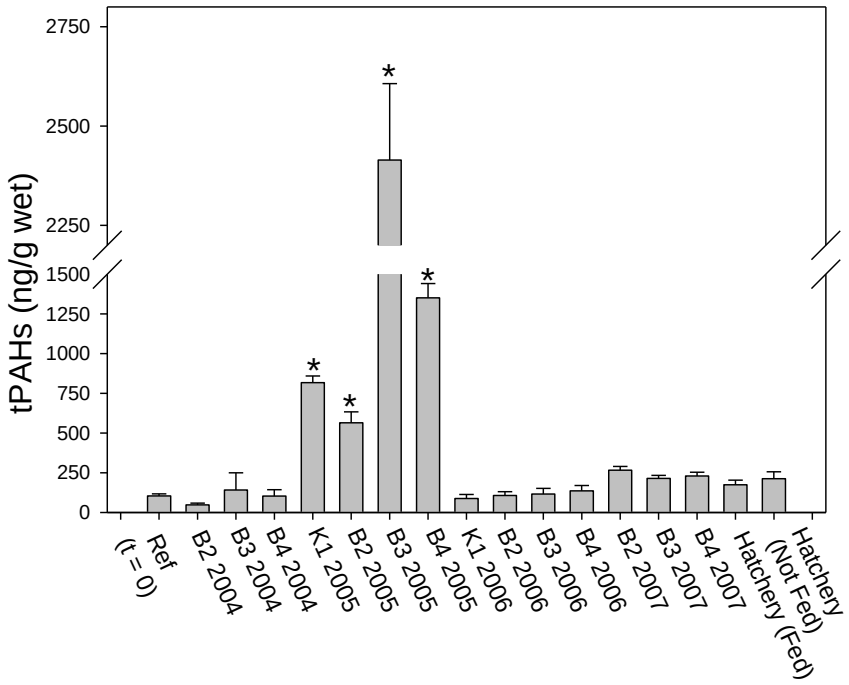

Figure 12. Juvenile Chinook salmon were caged at various locations in the Lower Duwamish Waterway for 8-10 days each July from 2004 - 2007. Results of chemical analysis of fish tissue composites (typically 3-8 fish per composite) from each sampling station are displayed below in the form of means and standard error of the mean. Chemical analysis was perfomed for PAHs and PCBs, however only results for PAHs are presented above as PCBs were not detected in any of the tissue composites. * Statistically significant difference from control ($p<.05$).

Results of the statistical analysis revealed that levels of total PAHs detected in tissue compositess from the juvenile Chinook salmon which were exposed at stations K1, B2, B3 and B4 during the 2005 sampling period were significantly elevated (p-value<.05) when compared to levels in tissue composites taken from salmon which were sacrificed prior to exposure (time = 0) . Although additional instances of elevated levels of total PAHs in salmon composites were noted, ANOVA statistical testing did not reveal any other occurances of significant difference among individual stations and control fish. Interestingly, among those fish caged at the hatchery, juvenile Chinook salmon which were not fed exhibited higher levels of PAHs than salmon which were allowed to feed.

This result may be due to effects of caloric restriction on metabolism of PAHs. Although levels of total PAHs in Chinook salmon tissues do not appear to be strongly correlated with environmental samples such as surface water and sediment, they do appear to be useful as an indicator of exposure.

DNA adduct analysis July 2004 – July 2007

DNA adducts were quantified as relative adducts labeled (RAL) per 10^9 nucleotides. RAL and standard error of the mean (SEM) is presented in Figure 13 for ^{32}P-postlabeled gill tissue from juvenile Chinook salmon. Gill samples typically represent composites of tissue from 4-6 fish per sample. In most instances, adduct analysis was performed on a minimum of four composites from each sampling station. Autoradiographs can be quantified using instant imager software. Statistical analysis of DNA adduct results was performed using SigmaPlot software version 11.0 (Systat Software, Inc., San Jose, CA). Levels of total RAL in composites from each station from each sampling year were compared for significant difference using one way ANOVA. The data failed to satisfy the assumption of a normal distribution, thus the one way ANOVA was conducted using ranked data. In addition, the Student-Newman-Keuls post-hoc test was selected for multiple comparisons versus the control (caged hatchery fish). DNA adduct analysis used Juvenile Chinook salmon, which were caged in clean water at the hatchery, as a reference for statistical comparisons. These reference fish were caged and exposed for 7 days at the hatchery and were not fed during this exposure time period, thus effects due to stress from caging or starvation during the experiment should be experienced in both experimental fish and reference fish. Levels of DNA adducts recorded from gill composites from fish exposed during the 2004 and 2006 study periods were all elevated compared to the reference fish, while levels of DNA adducts recorded from gill composites of fish exposed during the 2007 study period were all lower than levels of adducts in gill composites from reference fish. Adducts in gill composites from fish exposed in the LDW during the 2004 study period at stations B2, B3 and B4 were all significantly higher than adducts in the gill composites from reference fish.

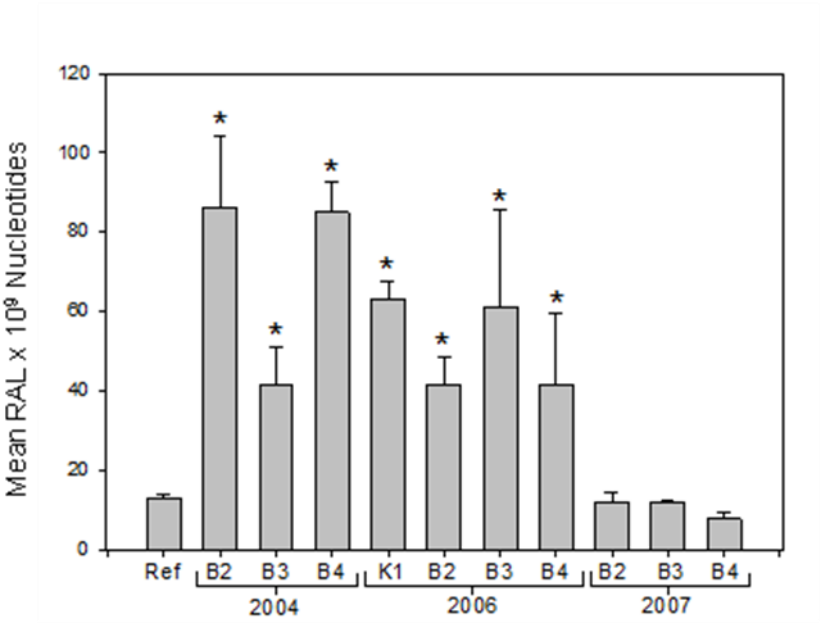

Figure 13. Juvenile Chinook salmon were caged at various locations in the Lower Duwamish Waterway for 8-10 days each July from 2004 - 2007. DNA adduct analysis was conducted on gill tissue composites (typically 3-8 fish per composite) using ^{32}P-postlabeling analysis. Quantification of autoradiographs which were produced from this analysis are presented below. * Statistically significant difference from control ($p<.05$).

In addition, significantly higher levels of adducts were found in fish exposed in the LDW at stations K1, B2, B3 and B4 from the 2006 study period. In contrast to the 2004 and 2006 LDW DNA adduct data, the 2007 DNA adduct data revealed no instances of elevated levels of DNA adducts compared to controls. In fact, DNA adducts detected in gill composits from fish exposed at station B4 were actually lower than levels of RAL in reference fish. Review of sediment and water data collected over the course of the study offers at least a partial explanation, as PAHs in both sediment and water were recorded at their lowest levels during the 2007 study period.

Western blot analysis

Western blot analysis was performed with livers from Chinook salmon which underwent the caged exposure procedure during the 2007 study period. The end product of the analysis is the scanned image of the Western blot band displayed below in Figure 14. Four composites from each sampling station were spotted.

The Image J program was used to quantify these results and the means and standard deviations of those quantifications are displayed in Table 14. Results indicate the highest levels of induction of CYP1A1 were present in livers of hatchery fish which were sacrificed prior to exposure on the first day of the study. These fish do not likely represent an appropriate reference as they did not experience caging, transport or starvation for the exposure period.

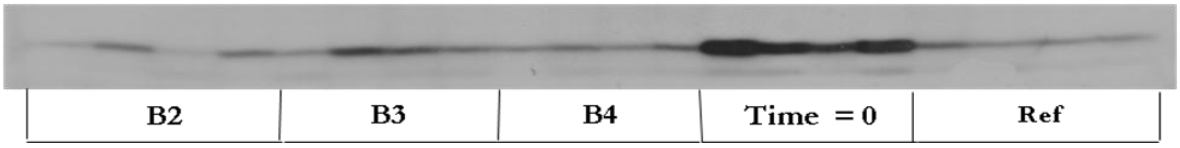

Figure 14. Juvenile Chinook salmon were caged at various locations in the Lower Duwamish Waterway for 8 days July 2007. Western blot analysis was performed using hepatic tissue composites (typically 2-4 livers per composite) to evaluate CYP1A1 expression. A scanned image of the western blot band is displayed above.

Table 14. Juvenile Chinook salmon were caged at various locations in the Lower Duwamish Waterway for 8 days during July 2007. Western blot analysis was performed using hepatic tissue composites (typically 2-4 livers per composite) to evaluate CYP1A1 expression. Results of quantification of the Western blot band are displayed below. These data represent the relative intensity of the band. Means and standard error of the mean are displayed as each treatment group included 4 composites.

Date Sampled	Assay	B2		B3		B4		t = 0		Ref	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
July 2007	Western Blot - CYP1A1	17	4	34	5	26	2	69	6	21	4

One potential explanation for the elevated response in these fish is that hatchery feed likely contains trace amounts of PCBs, which have the ability to induce CYP1A1. Reference fish which were caged at the hatchery on clean water for 7 days with out feeding provided a more appropriate reference and produced a response that was comparable to the response of samples from fish caged at experimental sampling stations.

The no treatment (time = 0) fish showed the highest CYP1A1 induction with a mean relative band intensity of 69, while sampling station B3 showed the highest mean CYP1A1 induction among the experimental stations. This was followed by station B4 with a mean of 34 and sampling station B2, which had the lowest CYP1A1 induction, with 17 being the mean.

Discussion

Comparison of sediment to SQGs

Numerous sediment quality quidelines (SQGs) are available to serve as screening tools to help estimate the likihood of toxicity among aquatic organisms and to identify areas of concern. Surface sediment samples collected from 2004-2007 are presented and compared to selected sediment quality guidelines in Table 15.

Table 15. Summary results for chemical analysis of sediment samples collected in the Lower Duwamish Waterway during July 2004 – July 2007 are displayed below. One sediment sample from each station during each sampling year and samples were analyzed for PAHs and PCBs. Means of all sampling years from each station are presented in order to summarize the data. Published sediment quality guidelines are also listed in order to facilitate comparison with results of the collected data. All units are in ppb. *Underlined = a minimum of 1 SQG exceeded.

	LDW 2004-2007 Sediment Means				Long, et al. 1995		McDonald 1994	
Site/SQG	K1	B2	B3	B4	ERL	ERM	TEL	PEL
LMW PAHs	267	541	253	231	552	3160	312	1442
HMW PAHs	783	1159	770	604	1700	9600	655	6676
Total PAHs	1782	2712	1730	1395	4022	44792	1684	16770
Total PCBs	16	1248	1752	383	22.7	180	21.6	189

Total PCB from sampling stations B2, B3 and B4 were substantially higher than either of the selected sediment quality guidelines. Note that in regard to total PAHs, each sampling station excceds a minimum of one recommended sediment qualifty guidelines with the exception of sampling station B4. Sampling station B2 reports higher levels for LMW PAHs, HMW PAHs, total PAHs and total PCBs when compated to threshold effect levels (TELs) that were calculated by MacDonald (1994).

Sediment PAH fingerprinting

PAH ratios were calculated to determine the possible source of the PAHs. Results indicate that the contamination in the Lower Duwamish Waterway likely comes from a variety of sources, as results did not consistently implicate one type of contamination. Neff et al. (2005) assert that a phenanthrene to anthracene (PH/AN) ratio of less than 5 is typical of pyrogenic assemblages, while a PH/AN ratio of greater than 5 is typical of petrogenic assembledges. In addition, pyrogenic assemblages typically have a fluoranthene to pyrene (FL/PY) ratio which approaches or exceeds 1, while FL/PY ratios which are substantially below 1 are typical of petrogenic assemblages (Neff et al. 2005). Results of PAH fingerprinting analysis are presented below (Table 16).

Table 16. Ratios of the PAH isomers phenanthrene to anthracene (PH/AN) and fluoranthene to pyrene (FL/PY) in sediments collected from various locations in the Lower Duwamish Waterway between July 2004 – July 2007 are presented below. PH/AN ratios of greater than 5 are typically petrogenic and those less than 5 are typically pyrogenic. FL/PY ratios which are substantially below 1 are typically petrogenic assemblages and ratios that approach or exceed 1 are typically pygrogenic assemblages.

	Site	PH/AN	FL/PY	Source
2004	B2	**9.02**	**1.29**	Petrogenic/pyrogenic
	B3	**11.96**	**1.70**	Petrogenic/pyrogenic
	B4	**9.82**	**1.55**	Petrogenic/pyrogenic
	NWW	**14.97**	**1.90**	Petrogenic/pyrogenic
2005	K1	**8.46**	**1.52**	Petrogenic/pyrogenic
	B2	**1.32**	**1.48**	Pyrogenic
	B3	**0.17**	**1.02**	Pyrogenic
	B4	**4.78**	**1.17**	Pyrogenic
2006	K1	**5.95**	**1.31**	Petrogenic/pyrogenic
	B2	**7.97**	**1.10**	Petrogenic/pyrogenic
	B3	**5.95**	**0.62**	Petrogenic
	B4	**12.34**	**1.24**	Petrogenic/pyrogenic
2007	K1	**2.26**	**1.37**	Pyrogenic
	B2	**17.18**	**1.84**	Petrogenic/pyrogenic
	B3	**2.62**	**1.29**	Pyrogenic
	B4	**4.50**	**1.33**	Pyrogenic

Chemical analysis of sediment samples collected during the 2004 sampling period indicates that contamination from both petrogenic and pyrogenic sources were present as the PH/AN ratios are all above 5 and the FL/PY ratios are all above 1, thus indicating a mixed exposure. Data from other study years supports the theory that the contamination is coming from a variety of sources as ratios varies between stations and even within the same station from year to year.

Regression analysis of environmental samples to relative adducts labeled in gill tissue composites

In order to examine the relationship between levels of PAHs present in environmental samples and levels of DNA adducts found in fish that were caged at the sampling locations, simple linear regression analysis was performed. Figure 15 depicts results of this analysis for the sampling periods for 2004, 2006 and 2007. In 2005, the exposure was conducted, as in other study years, however due to various issues including thawing and some deteriation of samples during shipment delays, DNA extracted from the gill samples was of questionable quality. Rather than attempt the postlabeling analysis with questionable DNA, study investigators decided to exclude the 2005 salmon from DNA adduct analysis. From each study year, one sediment sample was collected at each station and 4 composites of Chinook salmon gills were postlabeled.

The analysis reveals that there does not appear to be a linear relationship between levels of PAHs in environmental samples and levels of DNA adducts in caged fish which were exposed *in situ* to the sampling locations, as linear regression analysis was not able to reject the null hypothesis that the slope was equal to zero. In addition, correlation coefficients support this conclusion (R^2 = .122 for sediment and R^2 = .044 for surface water).

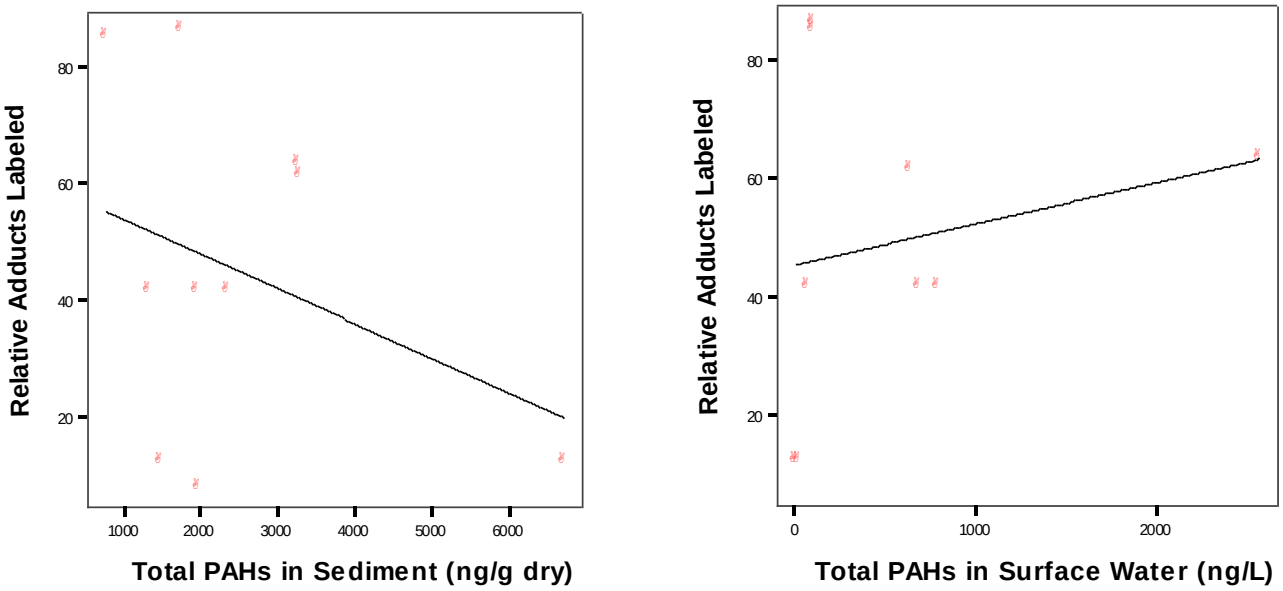

Figure 15. Plots of levels of total PAHs found in environmental samples (sediment and surface water) versus relative adducts labeled (per 10^9 nucleotides) in gills from Chinook salmon caged in the Lower Duwamish Waterway for 8-10 days are displayed above.

PAH/PCB interactions

In a previous study conducted by personnel in the Donnelly laboratory (Barbee et al. 2008), ^{32}P-postlebeling following a caged *in situ* fish exposure has been proven a sensitive marker of genotoxicity of PAHs in sediments. In that study, which employed the same caged fish exposure protocols with coho salmon, a correlation was observed between sediment PAHs and levels of DNA adducts in fish which were caged for 7 days at a contaminated site. There are several potential explanations for conflicting results between the current study and those obtained by Barbee et al. including species differences, and chemical interactions. The Barbee et al. study employed coho salmon instead of Chinook salmon and it is possible that species difference may account for some on the variation in results. However, the more likely explanation lies in chemical-chemical interactions. The Barbee et al. study was conducted at a freshwater location in which a point source contamination had resulted in an area highly contaminated with PAHs. In the LDW the sources of contamination appear to point and non-point, resulting in a more complex mixture of chemicals including PAHs, PCBs, PBDEs and metals among others. The focus our study was primarily PAHs and PCBs, however many additional contaminants are present which could potentially have affected results even though analysis was not performed on these chemicals.

The ability of PCBs to alter toxicity of other chemicals has been documented by previous studies. For examples, Shelton et al. (1983) showed that PCBs can either enhance or inhibit carcinogensis which is induced by aflatoxin exposure in rainbow trout (*Salmo gairdneri*). Stein et al. (1984) exposed English sole (*Parophrys vetulus*) to sediments spiked with radiolabeled PCBs and BaP for 10 days. Results indicated that simultaneous exposure increased the amount of BaP-derived radioactivity in bile. The authors suggest previous research has demonstrated that PCBs induce mixed function oxidases (MFO), which are responsible for metabolism of PAHs, thus potentially explaining why the results indicated increased metabolism of PAHs upon coexposure with PCBs. Collier et al. (1985) noted that exposure of coho salmon to PCBs substantially altered the biological fate of 2,6-dimethylnaphthalene (DMN). Overall effects noted by the study included that DMN metabolites in bile were increased and lower levels fof DMN were found in extrahepatic tissues.

In a another relevant study, Gillespie (2006) performed an animal experiment in which mice were exposed to a sediment extract which contained a complex mixture of PCBs and PAHs. PCBs were the predominant component of the sediment extract at 125, 409 ng/mg extract, while PAHs were present at 6284 ng/mg extract. Mice were exposed by dermal application at a shaved area on the mice's back and the sediment extract was dissolved in methylene chloride for application. At two different dose levels (10 nmol BaP and 100 nmol BaP) mice were exposed to BaP alone, as well as increasing doses (1.2 mg and 3.0 mg) of the predominately PCB sediment extract. Mice were then sacrificed, the skin where the sediment extract and BaP were applied was then dissected, and ^{32}P-postlabeling analysis was then performed to examine levels of DNA adduct formation. Results indicated that as the amount of PCB-containing sediment extract applied increased, DNA adduct levels were reduced. The trend held at both BaP dose levels. This finding is consistent with the theory that some sort of PAH/PCB interaction may be responsible for the lack of correlation between sediment PAHs and DNA adducts in fish observed in this study.

Additional collaborations related to this research

Juvenile Chinook salmon that were used in this study were provided by the National Oceanic and Atmospheric Administration (NOAA) and more specifically by Dr. James Meador. During the 2006 sampling year, Dr. Meador requested that a small portion of the *in situ* exposed juvenile Chinook salmon be returned to NOAA for analysis of fluorescent aromatic compounds (FACs) in bile. Following retrieval from the Lower Dwuamish Waterway by US EPA Region 10 dive team members, the fish designated for analysis by NOAA were loaded into aerated coolers and transported back to the NOAA lab for sacrifice. Levels of FACs detected by NOAA are presented in Table 17.

Table 17. FACs detected by NOAA in bile composites from juvenile Chinook salmon which were caged in the Lower Duwamish Waterway at various locations for 10 days.

Site	NPH Protein Corrected ng/mg protein	PHN Protein Corrected ng/mg protein	BaP Protein Corrected Ng/mg protein
K1	26,000	8,800	190
K1	45,000	15,000	290
K1	26,000	9,200	180
B3	30,000	11,000	190
B3	39,000	13,000	280
B3	40,000	15,000	270
B3	43,000	15,000	270
B3	40,000	14,000	270

Results of the FAC analysis revealed levels of FACs in the fish caged in the LDW for 10 days were high but with in the range of levels of FACs that have been previously detected in fish that were wild-caught by trawling near Kellogg Island in the LDW (personal correspondence, Dr. James Meador, NOAA National Marine Fisheries Science Center, Seattle, WA). This provides support for the theory that the exposure time used in the study is environmentally relevant compared to the exposure that salmon migrating through the LDW would experience.

In addition to analysis of FACs in bile, NOAA personell measured blood paramaters and intended to analyze the stomach contents of the juvenile Chinook salmon for levels of PAHs. In regard to analysis of stomach contents, upon dissection of fish for retrieval of the stomach content samples, NOAA investigators revealed no presence of aquatic organisms of the juvenile Chinook salmon, indicating that feeding ceased once the caged exposure period began (personal correspondence, Dr. James Meador, NOAA National Marine Fisheries Science Center, Seattle, WA). Previously, study investigators had assumed that small aquatic organisms would float through the cage and that there would be some dietary exposure. These results, combined with the observation that the juvenile Chinook salmon were not in direct contact with sediment during the caged exposure, indicated that the only exposures occurring were related to contaminants which were bioavailable in the water column.

Results revealed that blood parameters were depressed (data not shown), although it remains unclear if these effects are related to starvation during the exposure period or potentially caused by exposure to contaminants (personal correspondence, Dr. James Meador, NOAA National Marine Fisheries Science Center, Seattle, WA). Based on the depressed blood parameters, which may have potentially indicated starvation stress in the fish, the 2007 exposure period was reduced to 8 days instead of a 10 day exposure period which had been used in 2004 through 2006.

Further collaborations on the study occurred in 2007 when Dr. Evan Gallagher's laboratory at the University of Washington was given a small subset of fish from each sampling station. Personnel from Dr. Gallagher's laboratory dissected livers from *in situ* exposed juvenile Chinook salmon and flash froze the samples in liquid nitrogen in the field following cage retrieval. Later steady state hepatic mRNA expression of seven exposure biomarker genes was examined using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays. Results, presented in Browne at al. (2009), did not find significant difference in any of the seven genes examined between juvenile Chinook salmon which were exposed *in situ* to the LDW and reference juvenile Chinook salmon which were caged in clean water at the hatchery. There are several potential explanations for the lack of response in gene expression assays. The authors assert that levels of PAHs detected in sediment duing the LDW 2007 sampling period were with in the range of levels reported historically in the region, however they tended to be in the lower end of the range. In addition, mean levels of PAHs in surface water were detected at the lowest concentrations recorded out of the four years of sampling (<19 ppt at all stations sampled).

Conclusions

A series of caged *in situ* exposure studies have been conducted using juvenile Chinook salmon and Pacific staghorn sculpin in the Lower Duwamish Waterway. Examination of the data collected during the 2004 sampling period revealed information which determined the direction of the study in subsequent years. In regard to biomarker responses of *in situ* exposed juvenile Chinook salmon and Pacific staghorn sculpin, salmon exhibited a higher level of response than sculpin. In addition, DNA adduct analysis of livers and gills dissected from fish exposed in the LDW for 10 days revealed

higher responses in gills than in hepatic samples. Subsequently, sampling conducted from 2005 through 2007 used only juvenile Chinook salmon and DNA adduct analysis was only conducted using gill tissue composites. In 2007, Western blot analysis was used to examine CYP1A1 expression in hepatic tissue.

Results of Western blot analysis of CYP1A1 expression in hepatic tissue did not appear to be reflective of exposure. In fact, the highest response was demonstrated in hatchery fish which were sacrificed prior to exposure. Potential explanations for the elevated response in the hatchery fish which were sacrificed prior to exposure include the potential presence of trace amounts of contaminants such as PCBs, which have been shown to induce CYP1A1, in feed at the hatchery. Unlike time = 0 fish, field-exposed fish were subject to starvation during the exposure period. In addition, hepatic tissue composites from juvenile Chinook salmon which were exposed at sampling station B2 were found to have response that was lower than the response noted in hepatic tissue composites from fish caged in clean water at the hatchery.

In regard to levels of PAHs detected in tissue composites, levels of total PAHs in juvenile Chinook salmon which were caged *in situ* in the LDW from each of the four sampling stations were significantly elevated compared to levels of total PAHs detected in hatchery fish which were sacrifice prior to exposure during the 2005 exposure period. Results during other sampling years did not follow a similar trend and no other instances of significant difference were noted. In addition, surprisingly, examination of total PAHs in composites from salmon that were caged in clean water at the hatchery revealed that fed juvenile Chinooks salmon were found to have lower levels of total PAHs present than salmon which were exposed similarly without feed. This was unexpected as hatchery feed presents a potential source of PAH exposure to fish at the hatchery. Regression analysis revealed that as expected, potentially due to rapid metabolism of PAHs, no dose-response relationship was present between levels of PAHs in sediment or surface water samples and levels of PAHs present in fish tissue composites. Also of note, chemical analysis of fish tissue composites was not able to detect the presence of PCBs in fish tissue. This could potentially be explained by the short exposure times.

DNA adduct analysis results varied from year to year. Results from the 2004 samplng year revealed that gill composites from *in situ* exposed juvenile Chinook salmon and Pacific staghorn sculpin exhibited higher levels of RAL than hepatic tissue composites. In addition, juvenile Chinook salmon exhibited higher levels of RAL than Pacific staghorn sculpin. Statistical analysis of DNA adducts by sampling year revealed that at each station sampled in 2004 and 2006, levels of RALs were significantly higher than RAL detected in control salmon which were caged in clean water at the hatchery. However, in 2007, levels of DNA adducts detected in field-exposed fish were either comparable or less than hatchery-exposed reference fish. This result may be due to the lower exposure levels detected in environmental samples during 2007 sampling. In spite of this general agreement between levels of DNA adducts and environmental samples in 2007, a dose-response relationship was not present between adducts and levels of total PAHs in either sediment or surface water samples. This lack of correlation may be due to chemical-chemical interaction among the complex mixture of contaminants present in sediment in the Lower Duwamish Waterway. However, at sites at which environmental exposures are substantial, DNA adducts do appear to provide an effective indicator of exposure.

The utility of DNA adducts following a caged in situ fish exposure in future research should be determined on a site-specific basis. Due to potential chemical-chemical interactions, DNA adducts may be most appropriate for use at sites at which PAHs are the sole or predominant contaminant of concern. It would also be ideal if sampling locations provided a diverse exposure gradient in which many different levels of contaminant would be encountered. This would increase the likelihood of seeing a dose-response relationship.

Disclaimer: the views and opinions expressed in this manuscript do not necessarily reflect those of the US Environmental Protection Agency. In addition, although EPA provided research support for this work, their effort was not a part of the negotiated risk assessment for the Lower Duwamish Waterway Superfund site.

CHAPTER IV

USING SOLID PHASE MICROEXTRACTION FIBERS TO CHARACTERIZE SEDIMENT PAH CONTAMINATION AT A SUPERFUND SITE

Introduction

Characterization of sediments and waters containing complex mixtures of contaminants presents a unique challenge for scientist and site managers. Among the assessment tools available are chemical analysis, standard aquatic toxicity assays and biological monitoring. Standard aquatic toxicity bioassays typically measure endpoints such as survival, growth and reproduction in aquatic invertebrates. While certainly effective in detecting high dose acute toxicities, many of these assays may not detect more subtle adverse biological effects that are potentially associated with chronic low dose exposure. Biological monitoring may include analysis of biomarkers in wild-caught aquatic organisms or may include biomarker analysis of aquatic organisms which were subjected to caged *in situ* exposures. While all of these options have advantages, they can also be costly and labor-intensive. In addition, results in biological monitoring using fish may be subject to extensive variation depending on the species selected and even between individual organisms. In many circumstances, biotransformation of contaminants or effects of stress may lead to a lack of correlation between ambient exposures and levels of contaminant found in tissues (Petty et al. 2000).

An additional option available is the use of passive sampling techniques. This approach typically involves accumulation of contaminants through diffusive and partitioning processes (Harman et al. 2009) as a sampling device is deployed *in situ* for a specified period of time, followed by retrieval and then chemical analysis in the lab. Passive sampling provides an advantage over active sampling in that fewer numbers of samples are needed to characterize the site (Ouyang et al. 2007). Some of the most commonly used passive sampling devices include semi-permeable membrane devices (SPMD). SPMDs were first developed in 1990 (Huckins et al.) and provide major advantages over biological monitoring. Harman et al. (2009) point out that SPMDs are not subject to factors that affect organisms such as feeding, life stage and variation in condition between individual animals, while variation between individual SPMD samplers is also

inherently lower than aquatic organisms. Among exposure studies which have successfully implemented the use of SPMDs alongside biological monitoring, Verweij et al. (2004) showed a significant correlation between biliary PAH metabolites in caged carp and concentrations of aqueous PAHs which were estimated from SPMDs placed near the cage. Disadvantages associated with SPMD use include that SPMDs undergo extensive treatment following retrieval before results can be obtained (Zeng et al. 2004). These steps are needed to extract the contaminants from the lipid-containing memberane and to perform the cleanup that is necessary prior to sample analysis.

Solid phase microextraction (SPME) is another passive sampling technique that has gained attention recently. The technique was developed by Arthur and Pawliszyn (1990) and involves the use of a fused silica fiber which has been coated with a specific polymer phase (Zeng et al. 2004). Much of the original method development has been focused on SPME use in analysis of volatile organic compounds (VOCs) (Zeng et al. 2005a), more recently more effort has been put toward establishing SPME samplers as effective samplers of non-volatile organic compounds in aquatic environments (Zeng et al. 2004). Advantages of SPME use include that it is a versatile technique that has been used in analysis of a wide variety of contaminants and the analysis is performed with solvent-free extraction, thus it is environmentally friendly compared to traditional extraction methods (King et al. 2004). Other advantages include cost efficiency, as SPME samplers can be constructed for as little as $100 (Zeng et al. 2004), and SPME fibers can be used as many as 50 – 100 times before a replacement is needed (King et al. 2004). In some instances, SPMEs have been referred to as biometric devices, because they can be used as surrogates for aquatic organisms in biomonitoring studies (You et al. 2007). One disadvantage that has been associated with SPMEs thus far is that detection limits are higher than those achieved in SPMDs, mostly due to the smaller sorbent phase associated with SPMEs compared to SPMDs (Zeng et al. 2004).

The objectives of these studies include (1) characterization of the extent of PAH and PCB contamination present in sediment (collected at two depths including 0-15 cm and 15-30 cm), surface water and ground water samples from a the Lower Duwamish Waterway (LDW), a contaminated industrial waterway, (2) measurement of levels of PAHs and PCBs present on Solid Phase Microextraction (SPME) fibers which are

exposed *in situ* for a 30 day exposure period to LDW water and sediment in specially fabricated water column and porewater samplers, (3) measurement of survival and growth in *Hyalella azteca* exposed to sediments collected from the LDW, and (4) investigation of potential correlations between endpoints in aquatic bioassays and concentrations of contaminants measured using SPME.

Materials and methods

Site description

The Lower Duwamish Waterway (LDW) refers to a 5.5 mile portion of the Lower Duwamish River (USEPA and WSDOE 2008). The LDW represents a transition zone or estuary, as it receives fresh water from the Green river, which is the primary source of water for the Duwamish River (Windward Environmental LLC 2007), and salt water from the Puget Sound, as the LDW flows into Elliott Bay (USEPA and WSDOE 2008). The LDW has been heavily modified from its original state to facilitate use as a navigational corridor (Windward Environmental LLC 2007). Today the LDW is characterized mostly by industrial activities such as shipping, boat manufacturing, marina operations, and airplane part manufacturing activities, among others (USEPA 2001), however, the residential areas of Georgetown and South Park are also located along or nearby the shores of the LDW. In addition to releases of pollution from industrial sources, combined sewer overflows (CSOs) and storm drains discharge into the LDW (USEPA 2001). Despite this pollution, the LDW is fished both commercially and recreationally and is part of historical fishing grounds for the Muckelshoot and Suqiamish Indian tribes (USEPA 2001). Currently, the LDW supports a commercial fishery operation by the Muckelshoot tribe which catches migrating salmon (Windward Environmental LLC 2007).

The City of Seattle began the first study to investigate water quality on the Duwamish estuary in 1949 (Gibbs and Isaac 1968). Adverse effects that have been documented in fish at the Lower Duwamish Waterway Superfund site include fin erosion (Wellings et al. 1976), accumulation of chemicals in fish liver and muscle tissue (Malins et al. 1984), and lesions in the liver, kidney and gills (Malins et al. 1984). Rhodes et al. (1987) observed that presence in the LDW or Upper Duwamish Waterway (UDW) was a risk

factor for several types of hepatic lesions. For example, odds ratios of 8.7 and 8.2 were observed for UDW and LDW English sole in regard to the presence of neoplasms. In addition, higher concentrations of xenobiotics, such as aromatic hydrocarbons and metals were correlated with increased risk of development of hepatic diseases in fish located in the river Superfund site (Malins et al. 1984).

Additional research on the Duwamish was conducted by Varanasi et al. (1985). Various organisms including fish, shrimp, clams and amphipods were exposed in the lab to Duwamish River delta sediments. Results indicated that accumulation of aromatic hydrocarbons varied among species, as aromatic hydrocarbons were not detected in fish and shrimp, but aromatic hydrocarbons were successfully detected in clams and amphipods. In addition, prior to the exposure, radiolabeled BaP was added to the Duwamish River delta sediments and biliary metabolites of BaP were measured following the exposure period. Results revealed that all organisms tested were capable of uptake of BaP from the Duwamish River delta sediments. Particularly high levels of BaP metabolites were found in fish and shrimp, indicating extensive BaP metabolism, which likely accounts for the lack of detection of parent aromatic hydrocarbons in these organisms.

Krahn et al. (1986) studied English sole caught by trawling in the Duwamish Waterway and 10 additional sites around Puget Sound. Results of the study indicated a significant correlation between biliary metabolites of aromatic hydrocarbons in English sole and presence of hepatic lesions, however no correlation was found between levels of aromatic hydrocarbons in sediment and levels of aromatic hydrocarbon metabolites in bile. Eagle Harbor was found to have the highest mean concentration (2100 ng/g wet) of aromatic compounds measured at the BaP wavelength, while the Duwamish waterway was the second highest (1400 ng/g wet), however this role was reversed in respect to percent of neoplasms as the Duwamish Waterway had the highest (20.7%) and Eagle Harbor had the second highest (18.2%).

Collier et al. (1986) examined xenobiotic metabolizing enzymes in spawning English sole following injection of sediments from the Duwamish Waterway, Eagle Harbor and a reference site in the Hood Canal. Interestingly, results indicated that even though Eagle Harbor sediments were found to have aromatic hydrocarbon levels 13-fold higher than

the Duwamish Waterway sediment, spawning English sole injected with the sediment from the Duwamish Waterway exhibited double the amount of aryl hydrocarbon hydroxylase (AHH) activity compared to their Eagle Harbor counterparts. The investigators speculated that the result may be due to the presence of chlorinated hydrocarbons in the Duwamish Waterway sediments in contrast to the absence of chlorinated hydrocarbons in the Eagle Harbor and Hood Canal sediments. Other important study findings include that while AHH, which has been linked by various studies with activation of carcinogenic aromatic hydrocarbons, was rapidly induced, while detoxifying enzymes such as epoxide hydrolase (EH) and Gluathione-S-transferase (GST) are induced at a much slower pThe authors felt that this lag time between inductions of enzymes may represent a period of increased susceptibility to adverse effects in exposed fish.

Various studies have also characterized the extent of PAH and PCB contamination present in the Duwamish Waterway. During a review of a series of studies of Puget Sound sites conducted between 1979 and 1985, Mailins et al. (1987) noted levels of aromatic hydrocarbons in the Duwamish Waterway at approximately 8 ppm and levels PCBs at approximately 150 ppb. Another study found sediment from the Duwamish Waterway contained 8.7 ppm total PAHs and 460 ppb of total PCBs (Varanasi et al. 1989b).

Studies have also investigated DNA adduct levels in wild fish caught from the Duwamish Waterway. Varanasi et al. (1989b) examined levels of DNA adducts in English sole from the Duwamish Waterway and Eagle Harbor, in addition to white flounder (*Pseudopleuronectes americanus*) caught from Boston Harbor, in an effort to evaluate contaminant exposure and observed effects in marine organisms. The authors point out that previous studies have demonstrated that modification of DNA is likely a critical step in chemical carcinogenesis, thus it should provide a useful biomarker of exposure. Results of the study indicated that the exposed fish contained a suite of adducts indicative of exposure to genotoxic compounds. It is important to note that although the three locations studied contained various concentrations of PAHs in sediment, total adduct levels were comparable. Interestingly, although total adduct levels were similar, patterns of adduct levels varied by location and species of fish,

indicating that DNA adduct results are both species and site specific. The authors suggested that factors such as contaminant structure, bioavailability and differences in metabolism and excretion among different species may affect DNA adduct levels. In addition, the authors asserted that their finding that English sole samples immediately after capture from a site of very low contamination did not contain hepatic DNA adducts demonstrated the ability of the ^{32}P-postlabeling assay to distinguish between areas of high and low contamination. This study also examined fluorescent aromatic compounds (FACs) in bile from the exposed fish. Although there was some general agreement between levels of adducts in fish compared to levels of FACs present in bile, on an individual level, high concentrations of FACs did not always accompany high levels of adducts.

McCain et al. (1990) conducted a study investigating uptake of aromatic and chlorinated hydrocarbons by juvenile Chinook salmon in the Duwamish Waterway and a non-urban reference site. Contents of the Duwamish Waterway salmon were found to contain a mean of 91 µg/gram dry weight of aromatic hydrocarbons and mean of 3.0 µg/gram dry weight of total PCBs. These levels were dramatically higher than those found among fish captured at the non-urban reference site (0.14 µg/gram dry weight and 0.82 µg/gram dry weight, respectively). Cumaceans, amphipods and small fish were the most commonly found food organisms in the stomach contents of the Duwamish juvenile Chinook salmon. The investigators also examined total PCB content in liver among the captured fish. Juvenile Chinook salmon from the Duwamish were found to have a mean total PCB content in liver of 2.6 ng/g dry weight, compared to means values of 0.68 and 0.90 ng/g dry at a hatchery and the non-urban control site. Another biomarker investigated was FACs in bile. As in the previous analysis, samples from juvenile Chinook salmon captured in the Duwamish were found to have higher levels of biomarker response than fish captured at a non-urban reference location. Bile was measure at both the naphthalene and BaP wavelengths and mean levels in the Duwamish fish were found to be 1.3 and 446 µg/gram wet weight for BaP and Naphthalene respectively. In contrast, mean levels measured in reference fish were 0.05 and 59.0 µg/gram wet weight. Other important observations made by the authors include that the juvenile Chinook salmon captured during the study likely migrated through the Duwamish Waterway for an exposure period of between 1 and 6 weeks and

that reports in the literature indicate that juvenile salmon exposed to aromatic hydrocarbons and PCBs in the water column can begin to take up substantial amounts of the contaminants in as little as a few days to a few weeks.

In a study of xenobiotic chemicals and metabolites in marine organisms, Varanasi and Stein (1991) reported levels of LMW PAHs and HMW PAHs detected in English sole tissue from various Puget Sound locations. Summed LMW PAHs from the Duwamish Waterway ranged from 8.8 to 26 ng/g of wet muscle tissue, while HMW PAHs detected ranged from 13 to 21 ng/g of wet muscle tissue.

In a study focusing on bioindicators of contaminant exposure, Stein et al. (1992) examined multiple biomarkers at up to five sites in the Puget Sound. The authors reported levels of sediment-associated contaminants as 3600 ng/g wet weight for total PAHs and 570 ng/g wet weight for total PCBs. In addition, among PAHs 4 to 5 ring PAHs were the predominant contaminants as they accounted for 2700 ng/g wet weight of the total PAHs present in the sediment sample. Another aspect of the study involved comparing biomarker responses in three species of flatfish collected from the Duwamish Waterway (a contaminated site) and a relatively clean reference site. Biomarker analysis performed included quantification of FACs in bile, PCBs in fish tissue, DNA adducts, AHH activity, EROD, and total hepatic GSH, while the three species of fish analyzed were English sole, rock sole and starry flounder. With the rare exception of bile FACs in English sole, each biomarker analysis for each fish species revealed elevated responses among Duwamish Waterway fish versus fish from the cleaner reference site. In fact, in most instances, the differences were statistically significant.

Varanasi et al. (1993) studied contaminant exposure and associated biological effects in juvenile Chinook salmon in various areas of Puget Sound. Endpoints measured by the group included PCBs in hepatic tissue, FACs in bile, and aromatic hydrocarbons in stomach contents. Results indicated that aromatic hydrocarbons and PCBs in stomach contents of juvenile Chinook salmon were significantly higher in the Duwamish Waterway and the Payallup estuary compared to a non-urban estuary and hatchery fish. In addition, levels of PCBs in hepatic tissue and FACs in bile were also significantly higher in Duwamish and Puyallup juvenile Chinook salmon compared to

specimens collected from a non-urban estuary and a hatchery. The investigators also observed the same trend in relation to hepatic enzymes such as AHH, which is thought to play a critical role in activation of aromatic hydrocarbons to more toxic metabolites. Contaminant effects on the immune system were also measured in both field-caught fish and in fish injected with contaminated sediment extract from the Duwamish Waterway. Results indicated suppression of the immune system in both assays. The final endpoint measured involved holding wild-caught fish in the lab for up to 80 days and results indicated that juvenile Chinook salmon from the Duwamish Waterway displayed significantly lower survival than specimens from non-urban comparison areas.

On December 1, 2000, it was proposed that the LDW be added to the National Priorities List (NPL), and on September 13, 2001, the LDW's listing as a NPL site became final (USEPA 2001). Concerns listed on the site narrative include sediments contaminated with semivolatile organic compounds, PCBs, inorganics and organotins (USEPA 2001). In 2002, a public health assessment was conducted and released for review and public comment by the Washington State Department of Health (2003). When the document was finalized in 2003, recommendations included that consumption of resident fish from the LDW should be limited to one 8 ounce meal per month due to concerns related to accumulation of PCBs in fish tissue. Salmon, which migrate through the LDW for short periods of time were not considered resident fish and thus were not included in this consumption advisory.

In August and September of 2004, additional marine sampling took place in the LDW for the purpose of updating the fish consumption advisory. In 2005, a report summarizing this data was released (Washington State Department of Health). The report found that PCB levels in fish were higher than historical levels and the recommendation was updated to suggest no consumption of resident LDW fish or shell fish. In 2007, a LDW fact sheet (Washington State Department of Health) was released which advised people to avoid eating any crab, shellfish or fish, with the exception of salmon, from the LDW. The fact sheet also provided recommendations on how to clean the salmon in preparation for cooking through fat removal, which could reduce the PCB exposure from salmon consumption by as much as 50%. Cleanup efforts have been extensive on the LDW since the site was added to the NPL. Some estimates put the total spent on

cleanup so far at more than $70 million, although continued study is needed as the source of much of the pollution is still in question (McClure 2007).

Collection of environmental samples

Sediment samples consisting of 0-15 cm depth were collected at seven sampling locations from a boat using a petite ponar grab sampler (WILDCO, Buffalo, NY). In addition, US EPA divers collected sediment cores consisting of 15-30 cm depth interval at seven sampling locations. Upon collection, the sediment samples were homogenized in stainless steel bowls and transferred to glass I-CHEM certified 1L sampling jars with Teflon lined lids (VWR, West Chester, PA). The samples were then shipped on ice overnight following chain of custody protocols to the laboratory at Texas A&M University or Baylor University, where they were stored at 4°C in the dark until further processing. Sediment samples from seven sampling locations were sent to Texas A&M University and were allocated for chemical analysis. In addition, sediment samples from five sampling locations (two replicates per locations) were sent to Baylor University and were allocated for aquatic toxicity bioassays. Sediment samples which were allocated for chemical analysis were oven-dried overnight at 60 ºC prior to extraction, then homogenized, ground in a mortar and pestle and passed through an 850 µm sieve.

Surface water samples were collected at each site using a Beta bottle sampling device (Wildlife Supply Co., Buffalo, NY) placed just above the sediment surface within the exposure zone of the deployed cages on the day of cage deployment and immediately following cage retrieval. In addition, ground water samples were collected with assistance from members of the US EPA Region 10 dive team. The collection procedure involves divers placing a minipiezometer approximately 50-60 cm into the sediment. Prior to deployment, 25 feet of polyethylene tubing (.25 inch inner diameter) is used to connect the minipiezometer to a centrifugal pump and then to a flow-through cup which allows measurement of water quality parameters during sample collection. The centrifugal pump was attached to a cordless drill, which was used to turn the centrifugal pump, creating suction. As water traveled through the flow-through cup, dissolved oxygen was monitored. When dissolved oxygen reached very low levels and

stabilized, ground water samples were collected. Both ground and surface water samples were stored in 1 L I-CHEM certified amber bottles with Teflon lids (VWR, West Chester, PA) and were shipped on ice overnight following chain of custody protocols to the laboratory at Texas A&M University. Following arrival, water samples were stored at 4 ºC until extraction.

Total organic carbon content (TOC) and sediment particle size were determined for each sediment sample. For TOC determinations, each sediment sample was homogenized and sub-sampled in three ~5g aliquots. Samples were then dried at 60°C for 48 hrs, pulverized using a Mini Bead-Beater to a uniform powder (Biospec Products Inc. 8), and re-dried at 60°C for 24 hrs. Samples were exposed to HCl to remove inorganic calcite fraction, and then TOC determined using a FLASH EA1112 Series CHN Soil Auto-Analyzer (Thermo Scientific Inc.). For sediment grain size analysis, wet sediment was placed into the Malvern Laser Particle Size Analyzer to generate grain size distribution. From this information the percent distribution of sand, silt, and clay was determined for each sample.

Within an eight week holding time recommended by US EPA (Ingersoll et al. 2000), whole sediment toxicity bioassays were initiated with each sample. Specifically, a 10-day toxicity study was conducted using *Hyalella azteca* as the test organism with survival and growth endpoints following US EPA recommendations for ambient toxicity testing of freshwater and estuarine sediments (Ingersoll et al. 2000). Briefly, sediments from each sampling site were homogenized, and 100 mL of sediment was placed in each of eight experimental replicates with 175 ml of overlying dechlorinated, activated carbon treated tap water (Ingersoll et al. 2000). Only six replicates were used for sites -003 and -004 because of limited sample volumes. Sand supplemented with 1% w/v of yeast-cerophyll-trout chow (YCT; a carbon source) was used for controls. After introducing samples to experimental units (day -1), test chambers were allowed to settle and equilibrate for 24 hours during which time overlying water was renewed. At the start of the study (day 0) general water quality measurements (e.g., dissolved oxygen (DO), salinity/conductivity, temperature, ammonia, pH) were taken following standard methods (American Public Health Association et al. 1999) and each test replicate received 1.0 ml of YCT food. Ten organisms were then added to each replicate.

Overlying water in experimental units was renewed daily and each replicate received 1 ml YCT daily. DO and ammonia was also monitored daily. Temperature was maintained at 23 ± 1°C for the study period with a 16:8 light:dark cycle. On day 10 surviving *H. azteca* were removed from experimental units, and placed in a drying oven (60°C) for 24 hours. Dry weight (growth) of surviving organisms were then determined using a Mettler-Toledo microbalance (Ingersoll et al. 2000).

Statistically significant toxicity differences among sites relative to controls ($\alpha = 0.05$) were determined with a t-test (Ingersoll et al. 2000). An ANOVA with Tukey-Kramer HSD was used to examine differences in sediment TOC among sites.

Sample extraction

For sediment samples, approximately 10 g of dried sample was extracted using methylene chloride in a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE). Liquid:liquid extractions were performed with methylene chloride according to US EPA standard extraction method 3510C (USEPA 1996b). Fish tissue was composited according to the sediment station where cages were placed. Compositing was necessary since analysis of individual fish was usually prevented by small fish masses and analytical detection limits. Therefore, average tissue PAH concentrations for each sampling station were determined. Fish tissue samples were first composited in a stainless steel blender according to exposure station (treatment group). Next, blended fish tissue composites were weighed wet and then lyophilized overnight under vacuum using a Labconco Free Zone 12 L Freeze Dry System (Labconco Corp., Kansas City, MO). Freeze-dried fish tissue composites were then re-weighed prior to extraction to determine the percent moisture of each composite. In addition, fish tissue composites were then ground into a powder-like consistency with a mortor and pestol to ensure optimal extraction.

Chemical analysis

Sediment extract and water extracts were analyzed for PAHs and other semivolatile organic compounds (SVOCs) using USEPA method 8270C (USEPA 1996c) and for total PCBs and PCB homologs following USEPA method 680 (Alford-Stevens et al.

November 1985). Analysis was performed using an Agilent 5975 gas chromatograph with a mass selective detector in selected ion monitoring mode. A 60 m x 0.25 mm ID x 0.25 mm film thickness column (Agilent Technologies, Palo Alto, CA) was utilized. The injection port was maintained at 300 °C and the transfer line at 280 °C. The temperature program was as follows: 60 °C for 6 minutes, increased at 12 °C/minute to 180 °C and then increased at 6 °C/minute to 310 °C and held for 11 min for a total run time of 47 minutes.

In situ *exposure with solid phase microextraction (SPME) fiber samplers*

Two types of solid phase microextraction fiber samplers were constructed by staff in Dr. Maruya's laboratory at the Southern California Coastal Water Research Project (SCCWRP) including porewater samplers, which are intended to be deployed in sediment and water column samplers, which are meant to be suspended in the water column. For construction of the SPME water column sampling unit, SCCWRP staff first cut 1/2" copper piping into 6" segments. Next, 4 rows of holes were drilled in the copper piping segments with a 19/64" drill bit 0.5 cm apart. Then the ends of the copper piping were sanded in order to insure proper fitting of 1/2" copper caps. A hole was then drilled in one of the caps using a 11/64" drill bit and the hole was then threaded with a 10-32 threader. This cap was used to hold the SPME fiber in place in the assembly. A section of copper screen was then cut to a length 1 cm less than the length of the casing and ~1 layer in thickness when rolled and inserted in the casing. The screen was then rolled and inserted into the casing and spread out to line the interior.

Constrction of the porewater sampler began with cutting 3/8" copper piping into 6" segments. Next, 4 rows of holes were drilled with a 15/64" drill bit 1.5 cm apart. The ends of the copper pipe were kept free of holes to prevent sealing problems with filter wrapping in a later step. The ends of the copper pipe were then sanded to insure proper fitting of ¼" copper caps. In addition, in some instances the ends of the copper piping were shaped with pliers if the piping was deformed during construction. This allowed caps to fit the piping properly. The caps holding SPME fibers were constructed to fit fairly loose to allow removal of cap without damaging fiber. Next, a hole was drilled in one cap using a 11/64" drill bit and the cap was then threaded with a 10-32 threader.

This cap was used to hold the SPME fiber in place in the assembly. The GF/F material was then cut into a single 142 mm GF/F filter, so it is no wider than the length of the copper piping (from bottom of cap to bottom of cap) and the 270 stainless steel mesh screen was cut into a section the same size as the GF/F filter. The GF/F filter and the stainless steel mesh screen were then carefully folded together around the copper piping, completely covering the drilled holes with the stainless steel mesh on the outside. The filter/mesh was tightened as to not allow any leakage from edges and then secured to the copper piping with .018 in. diameter stainless steel wire wrapped every 2-3 cm.

Solid phase microextraction (SPME) fibers which were used in both porewater and water column samplers were both prepared using the following procedures prior to assembly in the fabricated sampler casings. SPME fibers coated with 100 µm polydimethylsiloxane (PDMS) were purchased from Supelco (Bellefonte, PA, USA). The fibers were then pre-conditioned at 320°C under a stream of ultrahigh purity helium in a GC injection port for 1 h and kept in a sealed glass vial in a freezer before use. For in situ deployment, pre-conditioned SPME fibers were seated into a drilled copper end cap and protected by a cylindrical copper housing. For deployment in the water column, SPME fibers were encased in a perforated 15 cm L x 1.5 cm i.d. housing (Zeng et al. 2004). The sediment porewater configuration was slightly smaller (11 cm L x 0.75 cm i.d.) and included an exterior wrap of glass fiber filter (Whatman GF/F) and a single layer of 270 mesh (0.053 mm opening) T316 stainless steel screen held in place by No. 6 (0.406 mm dia) single strand stainless steel wire (Maruya et al. 2009). Prior to deployment, copper SPME housings were sonicated for 20 min each in dichloromethane:methanol (1:1) and hexane, dried at room temperature, and wrapped in aluminum foil. Following this process, SPME fibers were labeled with a sampler number using permanent marker and then screwed into both porewater and water column sampling devices. In addition, Teflon septas were added to aid in guidance of the fiber into and out of the casing without allowing the fiber to touch the sides and shatter. The samplers were then wrapped in foil, bagged in plastic bags and shipped over night on wet ice to the US EPA Manchester Lab in Port Orchord, Washington. Upon arrival, the samplers were kept in a -20° freezer until field deployment day. Figure 16 was provided by staff at the Southern California Coastal Water Research Project

(SCCWRP) and displays the SPME water column and porewater samplers. These specific illustrations are provided from the SCCWRP standard operating procedures for construction, deploying and retrieving SPME water column samplers at sea and the SCCWRP standard operating procedures for construction, deploying and retrieving SPME porewater samplers.

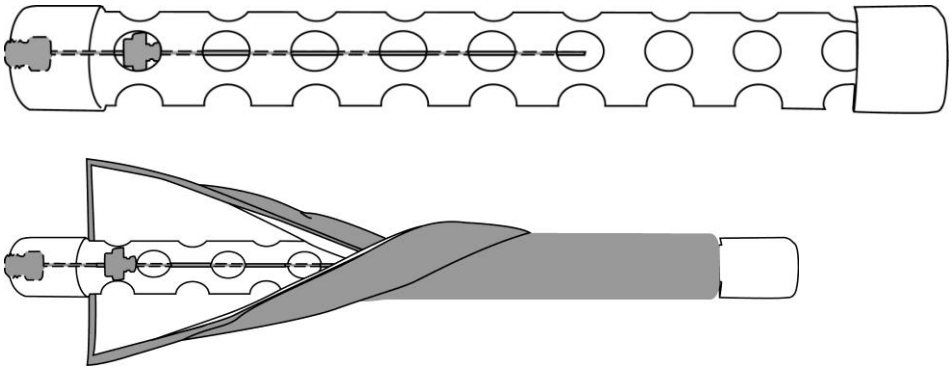

Figure 16. Investigators at the Southern Coastal California Water Research Project (SCCWRP) have developed two passive samplers which utilize Solid Phase Microextraction (SPME) fibers and can be field-deployed. While the SPME fibers are commercially available (Supelco), the outer protective copper casings must be fabricated. Water column (top) and porewater (bottom) SPME sampler illustrations which were provided by SCCWRP are displayed above.

The deployment process involved attachment of three water column SPME samplers to the side of a 45.7 x 30.5 x 20.3 cm plastic-coated, wire mesh pinfish trap cage (Model 1264; FRABILL, Inc., Jackson, WI) that has been modified to prevent fish escape. Three porewater SPME samplers were bundled using a thin nylon cord and the cord and were tied to the cage. These cages have been used in caged salmon exposure studies in previous years. Cages were deployed at five sampling stations in the historically polluted waterway (Figure 17) by slowly lowering the cage apparatus to the bottom of the water column.

Sampling stations were selected in consultation with staff at USEPA Region 10 as well as Washington Department of Ecology. Station K1 is located just offshore of Kellogg Island near (RM) 0.9 on the West side of the LDW. Kellogg Island currently represents the largest contiguous area of intertidal habitat in the LDW (Tanner 1991). Stations B2 and B3 are located between RM 3.4 and 3.5 approximately and both stations are located near the East bank of the LDW. Boeing plant 2, South Park Marina and the neighborhood of South Park are all located in close vicinity to the B2 and B3 sampling stations. Stations T4 and T5 are near RM 2.2 and are in an area which was recommended as early action area 2 (Windward Environmental LLC 2003b) out of 7 proposed early action areas. Early action area 2 consist of a small inlet which is approximately 80 feet wide at its mouth and is located on the West side of the LDW just past the 1st avenue South bridge (Good S. and Science Applications International Corporation 2007). Both of the sampling stations are located just before the mouth of the inlet near the edge of the main waterway. Historical industrial uses of the uplands near the area include marine operations and salvage, barrel reconditioning and vehicle storage and maintenance (Good S. and Science Applications International Corporation 2007). PCBs have been considered the main contaminant of concern at this site as previous studies at this site have revealed levels of PCBs in sediment as high as 5.2 ppm dry weight have been found and 6 surface sediment samples were found to exceed the Washington State sediment quality standards (Science Applications International Corporation 2007).

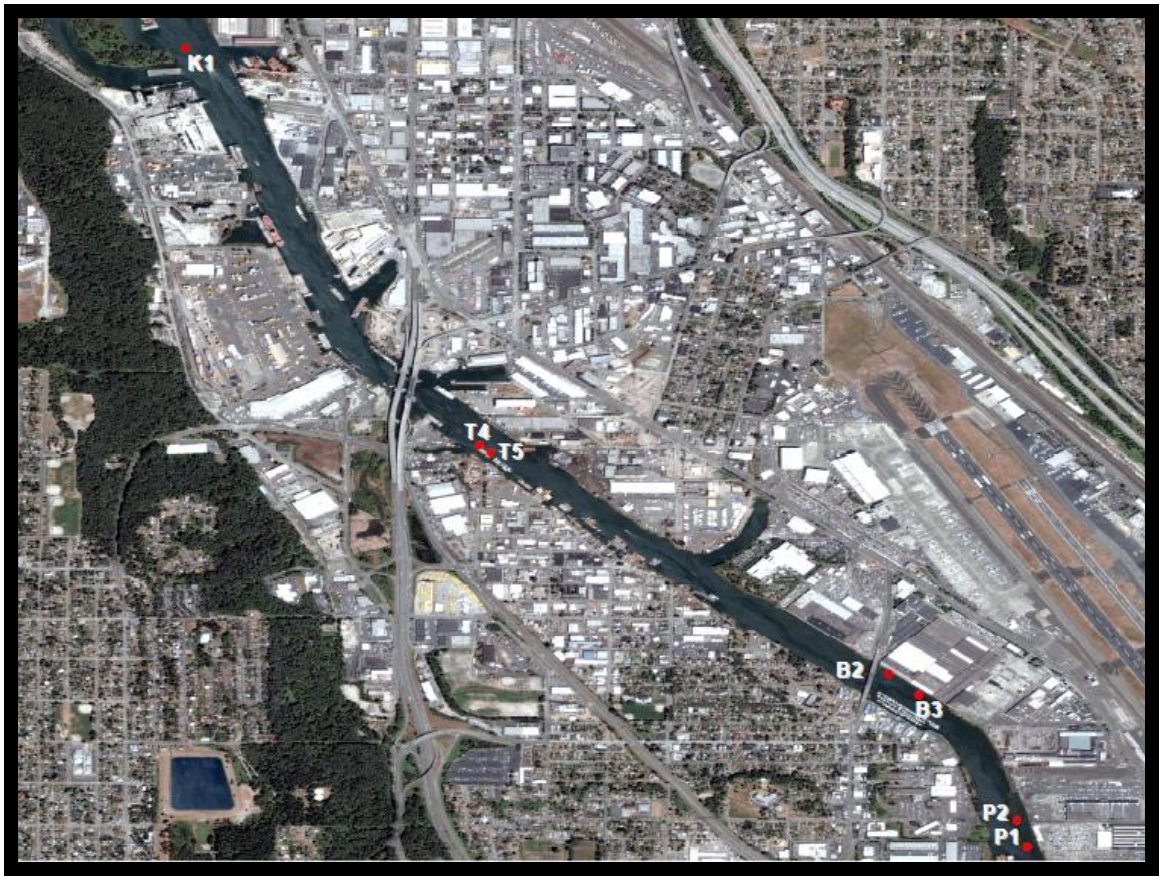

Figure 17. Displayed above are locations in the Lower Duwamish Waterway where samples of surface water and sediment were collected during the 2008 sampling event. In addition, at selected stations, SPME water column and porewater (buried at 5 cm depths in sediment) samplers were deployed and ground water samples were collected.

The B2 and B3 sampling locations are contained within an area that was recommended as early action area 4 (Windward Environmental LLC 2003b) out of 7 proposed early action areas on the LDW as the presence of PCBs, PAHs phthalates and metals has been previously demonstrated in the area (Windward Environmental LLC 2003a). The P2 and P1 sampling stations are located near RM 3.9 on the East side of the LDW. These sampling stations lie within an area that is frequently referred to as slip 6. Slip 6 is located between RM 3.9 and 4.4 on the East side of the LDW and land adjacent to slip 6 has been industrialized since the 1920s (Washington State Department of Ecology 2008). Historically, land near sampling stations P2 and P1 has been used in truck manufacturing, however more recently used as a vehicle storage lot (Washington State Department of Ecology 2008). PAHs and PCBs are among the contaminants of concern that previous studies on the site have documented as exceeding Washington State sediment quality standards (Good S. and Ecology and Environment Inc. 2008). Interestingly, PAHs in slip 6 surface sediments exceeded sediment quality standards in 8 samples, while PAHs in subsurface sediments exceeded sediment quality standards in 15 samples (Good S. and Ecology and Environment Inc. 2008). This would appear to indicate that the PAH plume on site may be more contaminated at depths below the sediment surface. In addition, PCBs in slip 6 sediment samples exceeded sediment quality standards in 8 samples, (5 of which were near sampling stations P2 and P1), and PCB levels in slip 6 sediments have been found up to 2.5 ppm dry weight (Good S. and Ecology and Environment Inc. 2008).

Weights were attached to the bottom of the cages to keep the cage location stationary. During cage placement, the porewater SPME sampler bundle was placed inside the cage, however upon cage deployment on the bottom of the polluted waterway, US EPA Region 10 divers buried the SPME porewater sampler bundle 5 cm into the sediment. Thirty days following placement of the cages, SPME samplers were retrieved from the sampling stations in the same order in which they were placed. Following retrieval, the SPME fiber is retracted into the protective sleeve and then exposed again momentarily. This step loosens any debris that is stuck on the fiber. The fiber is then rinsed briefly with deionized water and then the fiber is again retracted into the protective sleeve.

Field-exposed SPME fibers were analyzed using a Varian 3800 GC/Saturn 2000 ITMS (Varian, Walnut Creek, CA) with a 1079 split/splitless injector and an 8410 autosampler. The SPME syringe was manually injected into the injection port in splitless mode and the fiber thermally desorbed at 280°C for 6 min till split valve opening. The injector temperature was programmed from 100 to 280°C at ~100°C/min with a 20 minute hold time at the maximum temperature. Carrier gas was UHP helium with a flow rate of 1.0 mL/min. Chromatographic separation was achieved using a DB-5MS column (60m × 0.25mm × 0.25μm, J&W Scientific, Folsom, CA) temperature-programmed from 80°C (1 min hold) to 176°C at 8°C/min, followed by a ramp to 230°C at 1.5°C/min, and a final increase to 290°C at 5°C/min (29 minute hold). The temperatures of the ion trap, manifold and transfer line were 220, 120 and 280 °C, respectively. Mass spectra were acquired in the positive electron impact mode at 70 eV using the selected ion storage (SIS) mode.

Target analytes included 14 individual three to six ring PAH, 41 Cl2-Cl9 PCB congeners and 18 organochlorine pesticides (OCPs). A five point external calibration curve (25 to 2000 ng/mL) was generated to quantify the mass sorbed by the SPME fiber (Nf) for each target analyte. A mid-level calibration solution was analyzed periodically to monitor stability of instrument response. The dissolved aqueous concentration of the target chemical, Cw was calculated as:

$$C_w = \frac{C_f}{K_f} = \frac{n_f}{K_f V_f}$$

where Cf is the concentration of the chemical in the SPME fiber, Kf is the compound-specific SPME-water equilibrium partitioning coefficient (Maruya et al. 2009; Zeng et al. 2005b), nf is the mass of the chemical on the fiber, and Vf is volume of fiber coating (=0.612 μL for 100□m PDMS fibers).

Results

Sediments

One surface sediment sample was collected from a research boat using a petite ponar grab sampler at each station and one subsurface sediment sample (15-30 cm) was collected by US EPA Region 10 dive team members using a 30 cm core sampler. Upon return of the sediment core to the research boat, the 15-30 cm portion of the core sample was transferred into I-CHEM jars. Results of chemical analysis of surface sediment and subsurface sediment for PAHs (Figure 18), PCBs (Figure 19), and polybrominated phenyl ethers (PBDEs) are displayed below.

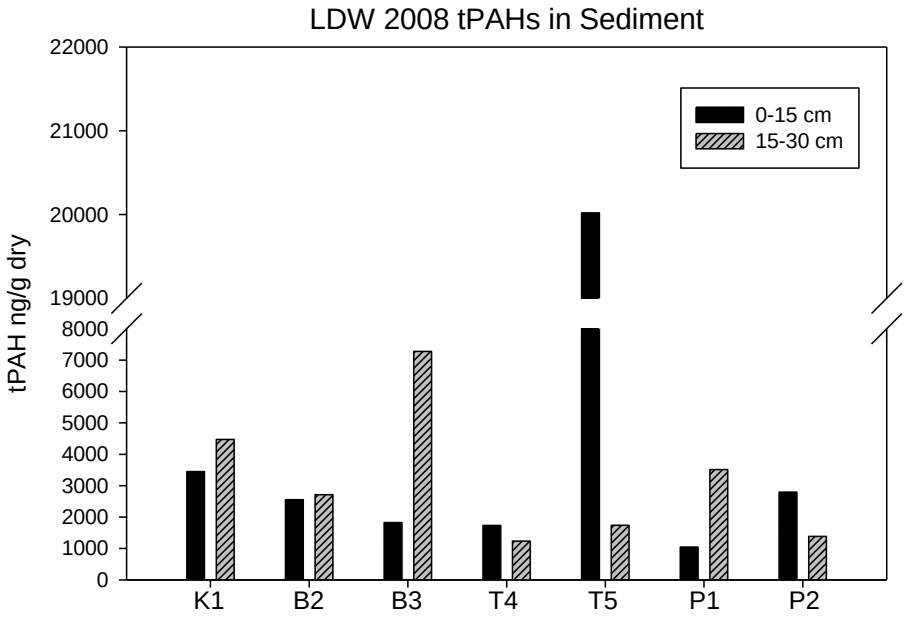

Figure 18. During the 2008 sampling event, surface (0-15 cm) and subsurface (15-30 cm) sediment samples were collected from various locations in the Lower Duwamish Waterway. Surface sediment samples were collected using a petite ponar grab sampler. Subsurface sediment samples were collected by USEPA Region 10 dive team members using core samplers. Levels of total PAHs detected by chemical analysis are displayed above.

Results from surface sediment samples indicate station T5 had the highest measured amount of PAHs at approximately 20 ppm. Also of note, many of the sampled stations displayed higher levels of PAH contamination in subsurface samples. This was the case at stations K1, B2, B3, and P1, possibly indicating that the contamination detected may be from historical pollution, which has been covered by new sediment more recently due to the LDW's net sediment gain over time.

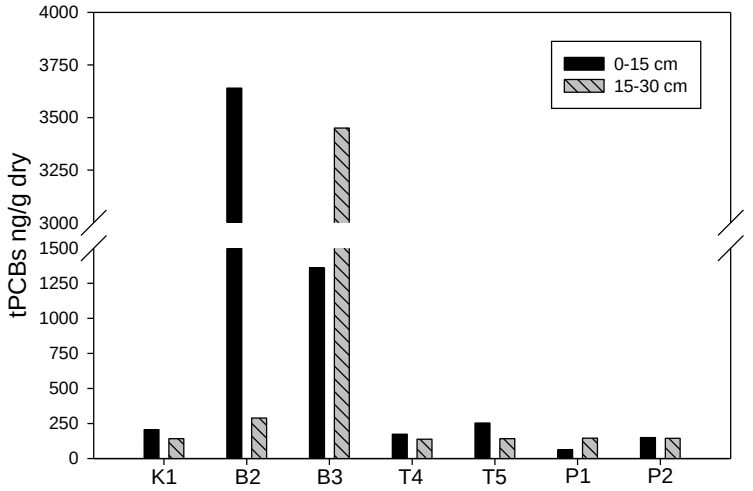

Figure 19. During the 2008 sampling event, surface (0-15 cm) and subsurface (15-30 cm) sediment samples were collected from various locations in the Lower Duwamish Waterway. Surface sediment samples were collected using a petite ponar grab sampler. Subsurface sediment samples were collected by USEPA Region 10 dive team members using core samplers. Levels of total PCBs detected by chemical analysis are displayed above.

Total PCB analysis was also performed on the surface and subsurface samples. As in the case of PAHs, levels of PCBs measured were sometimes higher in sediment samples measure from the 15-30 cm depth.

Levels of total PCBs were measured at levels well above project reporting limits at each of the sites sampled and at each of the depths sampled. The heterogeneous nature of sediment contamination in the LDW can be emphasized through examination of stations B2 and B3. Although the sampling stations are only approximately 1 mile apart, results from sampling at these locations varied greatly. Station B2 had more than 2.5 fold greater total PCBs in surface sediment than station B3, while levels of total PCBs measured in subsurface sediment samples at station B3 were approximately 15 fold greater than station B2.

At the request of Washington State Department of Ecology staff, LDW 2008 sediment samples were tested for the presence of PBDEs. In both surface and subsurface sediment samples, detectable levels of total PBDEs were characterized. Results of PBDE analysis are displayed in Figure 20. At three sampling stations (K1, B3, and P1), levels of total PBDEs were elevated in subsurface sediment samples compared to levels of total PBDEs detected in surface sediment samples from the same sampling station.

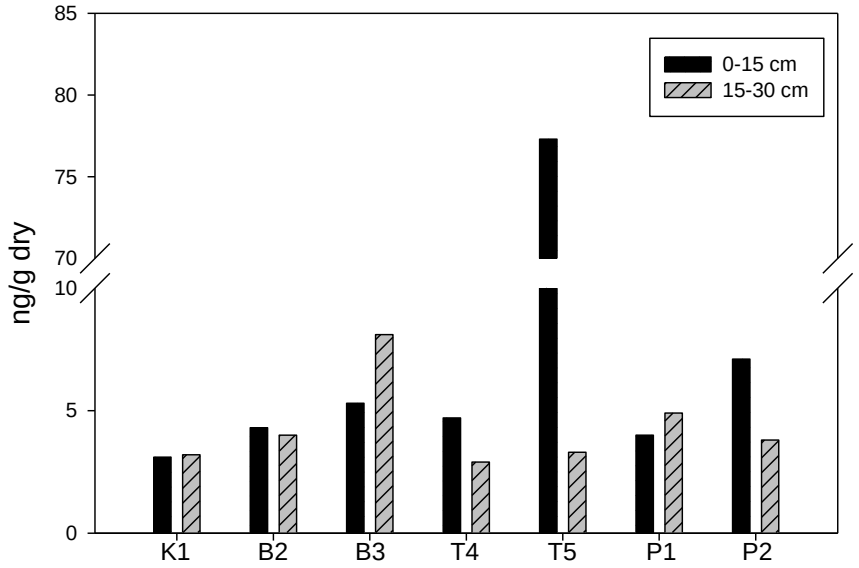

Figure 20. During the 2008 sampling event, surface (0-15 cm) and subsurface (15-30 cm) sediment samples were collected from various locations in the Lower Duwamish Waterway. Surface sediment samples were collected using a petite ponar grab sampler. Subsurface sediment samples were collected by USEPA Region 10 dive team members using core samplers. Levels of total PBDEs detected by chemical analysis are displayed above.

Results of total PBDE levels were comparable between all stations with the exception of sampling station T5. Levels of total PBDEs measured in the surface sediment sample collected at station T5 were more than 10 fold higher than any other surface sediment sample collected. Among subsurface sediment samples, measured levels of PBDEs were typically comparable among stations with the range being approximately 3 ppb to 8 ppb.

Sediment bioassays

In order to provide a measure of comparison for the SPME sampler analysis with a traditional well established method of toxicity assessment aquatic sediment toxicity bioassays were performed in Dr. Bryan Brooks' laboratory at Baylor University. Two surface samples were collected from each station for characterization using aquatic toxicity bioassays. All water quality measurements were within conditions prescribed by EPA recommendations for this *H. azteca* testing method (Ingersoll et al. 2000). For example, DO and pH remained consistent throughout the test: DO ranged from 5.5 – 6.5, pH ranged from 7.9 – 8.2. Although *H. azteca* has been considered predominantly a test organism for freshwater systems, it was selected based not only on widespread use for comparison purposes, but also on availability and budget limitations. Pilot test were performed using sediment samples from the LDW from previous study years to measure the salinity concentration in overlaying water, which the test were conducted in. Results of these pilot test indicated that the salinity concentrations were within a range that would not pose a threat to *H. azteca* survival.

Sediment TOC was performed and this analysis provided valuable data. Sediment TOC was significantly different among sites (Table 18, Figure 21), with values ranged from 0.94 to 2.76% between stations.

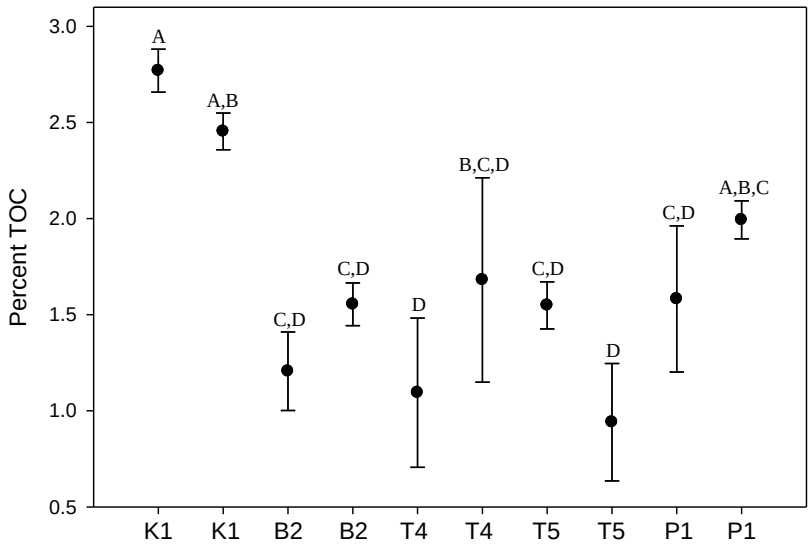

Figure 21. During the 2008 sampling event, surface (0-15 cm) sediment samples were collected using a petite ponar grab sampler from various locations in the Lower Duwamish Waterway. Analysis was performed to determine the percent of total organic carbon (TOC) that was present in the samples. Two samples were analyzed from each sampling location and results of this analysis are displayed above in the form of mean and standard deviation. Letters denote significant differences among samples.

Sediment at sampling station K1 was found to have the highest percent of total organic carbon. As sediment TOC is thought to affect bioavailability of contaminants, normalization of sediment contaminants to TOC is often performed to investigate potential associations between contamination in sediment and observed effects in bioassays. In some instances, if sediment TOC is not known, TOC is assumed to be 1%. Sediment TOC analysis performed on sediment samples from the LDW shows a relatively large variation and it is apparent that if sediment TOC analysis had not been performed, a 1% assumption may have severely underestimated the amount of bioavailable contaminants sorbed to sediment particles.

A comparison of sediment grain size was also performed and this data shows that the majority of all samples are made up of silt or clay particles (Table 18). A lower percent of sand (10-12%) was observed in two samples from sampling station K1 and this finding appeared to correspond to the higher TOC values observed in these samples.

The percentages of sand in the samples varied greatly from each site, ranging from 10.06 – 39.32. It should also be noted that the station K1 had the highest percentage of total organic carbon, the lowest percentage of sand at 10.06 and 12.91, highest percentage of silt at 84.07 and 80.81 and the highest percentage of clay at 5.87 and 6.28 respectively.

Table 18. During the 2008 sampling event, surface (0-15 cm) sediment samples were collected using a petite ponar grab sampler from various locations in the Lower Duwamish Waterway. Analysis was performed to determine TOC and bulk sediment characteristics of the samples. These results displayed below. Two samples were analyzed from each sampling location. Letters denote significant differences among samples in regard to TOC.

Site	% TOC	% TOC Standard Deviation	% Sand > 63 µm	% Silt 2 – 63 µm	% Clay (<2 µm)
K1	2.76[a]	0.11	10.06	84.07	5.87
K1	2.45[ab]	0.10	12.91	80.81	6.28
B2	1.21[cd]	0.20	23.17	72.97	3.86
B2	1.55[cd]	0.11	37.39	59.05	3.16
T4	1.09[d]	0.39	21.57	74.51	3.92
T4	1.68[bcd]	0.53	12.51	82.59	4.9
T5	1.55[cd]	0.12	32.86	63.76	3.38
T5	0.94[d]	0.31	39.32	57.84	2.84
P1	1.58[cd]	0.38	20.98	75.3	3.73
P1	1.99[abc]	0.10	26.01	70.44	3.55

The two main endpoints measured in the sediment aquatic toxicity bioassays included survival and growth of *H. azteca*. *H. azteca* control survival was 100% and survival for all sediment samples was high (77 – 100%) (Figure 22). However, survival in one of the two samples collected from station T4 was found to be significantly lower ($p<0.05$) than survival of controls.

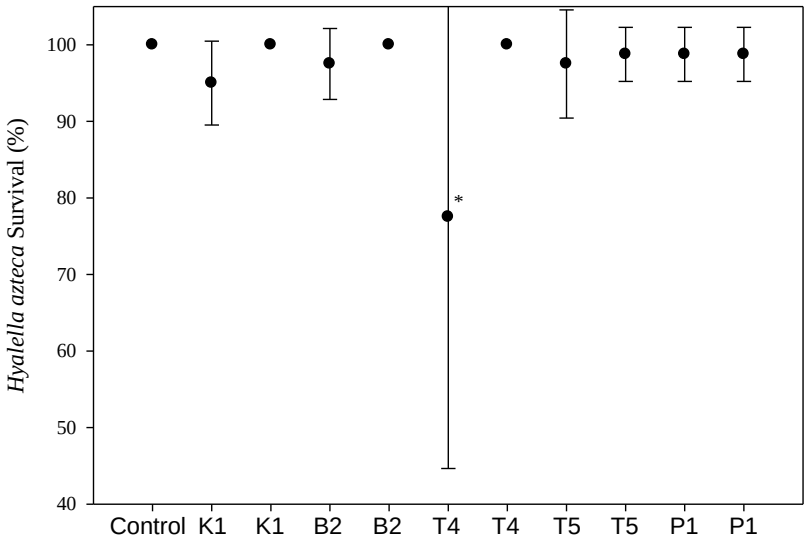

Figure 22. During the 2008 sampling event, surface (0-15 cm) sediment samples were collected using a petite ponar grab sampler from various locations in the Lower Duwamish Waterway. Sediment toxicity assays were then performed on the field-collected sediment samples in a laboratory setting using *Hyalella azteca*. Results for survival (± standard deviation) of *Hyalella azteca* following a 10 day exposure to whole sediments (collected in duplicate) are displayed above. * Significantly lower than control ($p < 0.05$).

It should however be noted that results were somewhat inconsistent as survival was 100% in the 2nd sediment sample collected from station T4. Further complicating matters, in the 1st sediment sample from station T4, where overall survival was 77%, a large amount of variation was found between the 8 replicates used to perform this analysis. In the majority of replicates most of the organisms survived, while in only a few replicates almost all of the organisms perished, thus resulting in a larger standard error of the mean which is displayed in Figure 22.

Measure growth of *H. azteca* is reported in Figure 23. Growth of organisms exposed to the 1st sediment sample collected from station T4 was significantly higher than controls, largely due to two of the eight replicates. In these replicates, fewer surviving individuals had less competition for resources, which likely resulted in the stimulated growth responses.

H. azteca growth was significantly decreased in both sediment samples collected from station K1 and one of the sediment samples collected from station B2 as compared to controls (Figure 23). In addition, with the exception of one of the sediment samples collected T4 and one of the sediment samples collected at station T5, all of the sediment samples tested displayed lower levels of growth than controls.

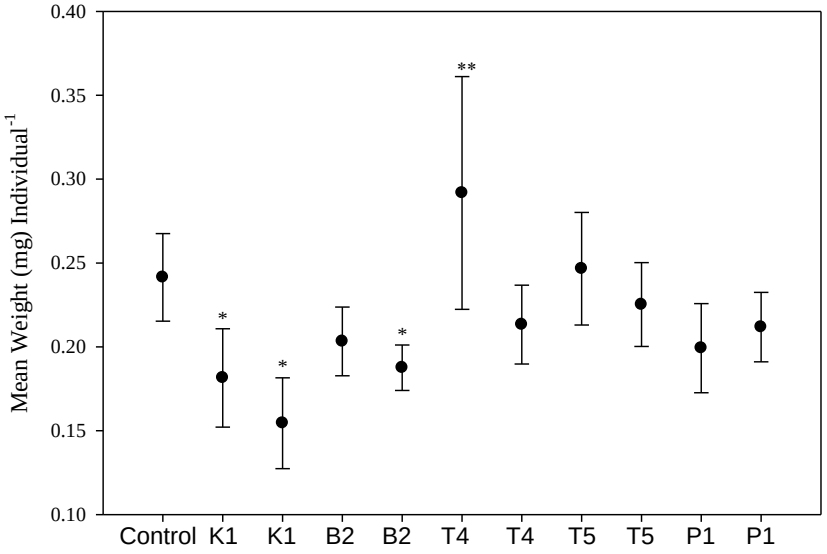

Figure 23. During the 2008 sampling event, surface (0-15 cm) sediment samples were collected using a petite ponar grab sampler from various locations in the Lower Duwamish Waterway. Sediment toxicity assays were then performed on the field-collected sediment samples in a laboratory setting using *Hyalella azteca*. Results for mean growth (as dry weight; ± standard deviation) of *Hyalella azteca* following a 10 day exposure to whole sediments (collected in duplicate) are displayed above. * Significantly lower than control ($p < 0.05$), **-significantly higher than control ($p < 0.05$).

Ground water

Ground water samples were collected at five stations by USEPA Region 10 divers. Results of the total PAH chemical analysis are displayed below in Figure 24. Variation between stations was observed as results range from approximately 50 ppt to 1.3 ppb. Interestingly, the highest level of total PAHs reported among the ground water samples was from sampling station K1. Station K1, despite having the highest percentage of TOC present, had the lowest reported concentration of total PAHs in surface sediment and was comparable to the lowest concentration reported in subsurface sediment. In addition, station K1 also reported the lowest concentration of total PAHs in surface water.

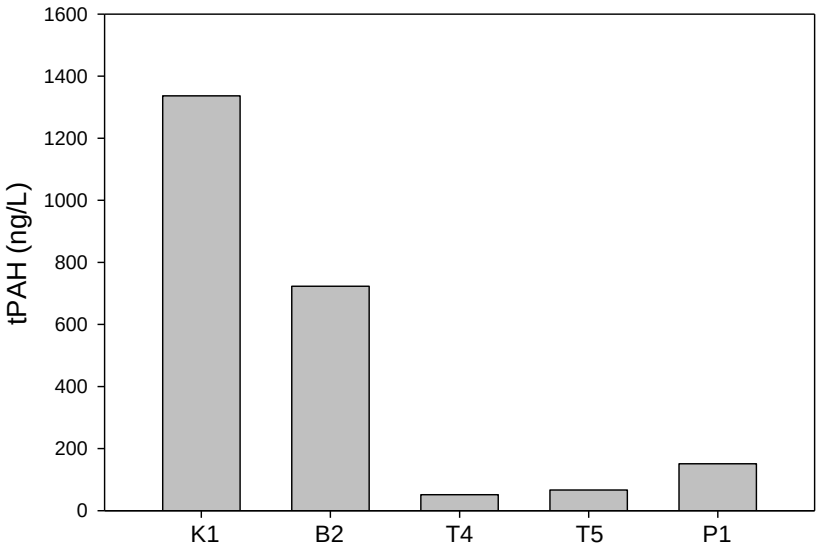

Figure 24. During the 2008 sampling event, ground water samples were collected by USEPA Region 10 dive team members from various locations in the Lower Duwamish Waterway. Levels of total PAHs which were detected by chemical analysis in the ground water samples are displayed above.

Surface water

Surface water samples collected in July 2008 revealed detectable levels of total PAHs in each sample tested with the total PAHs found ranging from approximately 50 ppt to 215 ppt. Results of the analysis are displayed in Figure 25. Surface water samples collected from sampling sites T4, B2, P2 and T5 had the highest measured levels of total PAHs. All samples were also analyzed for total PCBs and measurable quantities were not detected in any of the samples.

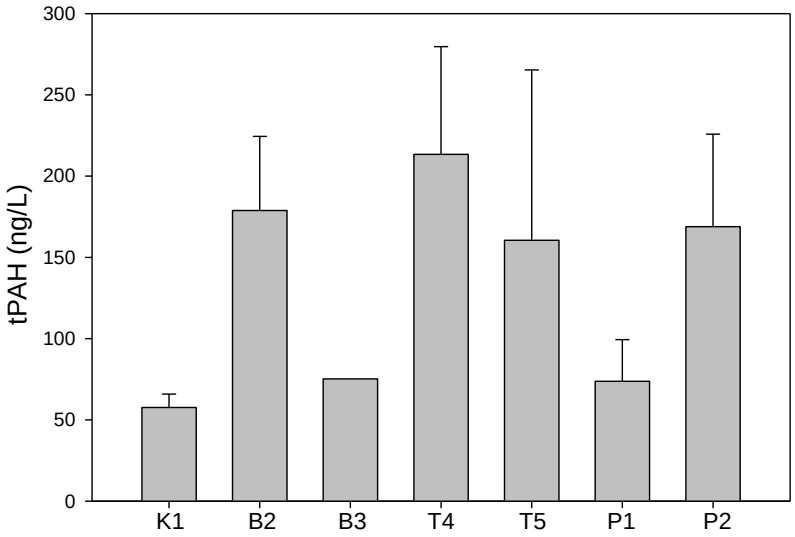

Figure 25. During the 2008 sampling event, surface water samples were collected using a beta bottle sampler just above the sediment surface from various locations in the Lower Duwamish Waterway. Mean levels of total PAHs that were detected in surface water samples and standard error of the mean are displayed above.

Compared with ground water samples collected in the same year (Fig. 24) three of sampling stations which displayed high levels of contamination in surface water, had very low levels of total PAHs present in ground water. Sampling site B2 was the only site sampled that had a high level of contamination present in both gound water and

surface water in regard to total PAH contamination. It should also be noted that sampling site K1 showed the highest groundwater PAH contamination, but the lowest level in surface water.

SPMEs

This study was part of a field trial for deployment of SPME samplers *in situ*. The original sampling plan called for deployment of SPME water column and porewater samplers in triplicate at 5 stations for a 30 day exposure period. In addition, at 2 of the sampling stations (stations K1 and T4) an additional set of SPME water column and porewater samplers were deployed for what was intended to be a 60 exposure period. This study design was intended to give an indication of whether or not the 30 day exposure period was long enough for the SPME samplers to reach equilibrium. As is often the case in field work however, due to factors beyond the control of the investigators, the study was not executed exactly as planned and adjustments had to be made in the field.

After 30 days of deployment in the LDW, USEPA Region 10 dive team members set out to retrieve the SPME samplers which were allocated for the 30 day exposure period. Despite exhaustive efforts, divers were only able to locate the SPMEs samplers from 4 of the 5 stations at which SPME samplers were deployed. At sampling station T4, where both a 30 day and 60 day set of SPME samplers were place, no samplers were found. As a result, a decision was made to go ahead and retrieve the additional set of SPME samplers at station K1 at day 30 instead of day 60 as originally planned. Possible explanations for the missing SPME samplers include that the samplers may have been caught in seining nets of fishermen, accidentally moved by boat traffic, or intentionally moved by man. During deployment, investigators attempted to place the samplers far enough away from shore in order for the samplers to be covered at all times, but close enough to the side of the waterway that boating traffic would not disturb the samplers. However, it is possible that at low tide, the SPME samplers may have become visible and were simply removed from the area as part of cleanup efforts. Results of the SPME samplers that USEPA Region 10 dive team members were able to retrieve are presented in Table 19.

Table 19. During the 2008 sampling event, following collection of sediment and surface water samples at various locations, water column and porewater (typically buried at a 5 cm depth in sediment) SPME samplers were deployed in the Lower Duwamish Waterway for 30 days. Mean levels of total PAHs (ng/L) detected in SPME fibers are displayed below. Standard error of the mean (SEM) is also presented. It is of note that although the porewater samplers were buried into the sediment during deployment, at retrieval, USEPA Region 10 dive team members found the SPME porewater samplers on top of the sediment surface.

	K1		T5		B2		P1	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Porewater SPME sampler	58.72	4.74	114.20	14.03	45.60	8.79	37.63	9.82
Water Column SPME sampler	32.95	2.23	109.26	21.88	28.47	12.97	27.70	2.10

SPME samplers were able successfully characterize contamination in the sediment and surface water of the LDW. At each station sampled, mean levels of total PAHs measured with porewater SPME samplers were elevated compared to water column SPME samplers deployed at the same sampling stations. Sampling station T5 showed the highest mean for both porewater and the water column at 114.20 and 109.26 respectively and showed the smallest range of variation between the two types of samplers. In addition, SPMEs were analyzed for PCBs and selected pesticides. Data are no shown because, in most instances, these compounds were detected but were present in quantities below project reporting limits.

Discussion

Comparison to SQGs

Numerous sediment quality guidelines (SQGs) are available to serve as screening tools to help estimate the liklihood of toxicity among aquatic organisms and to identify areas of concern. These tools are particularly useful as limited budgets often allow further study of only a small portion of the original study area. In Table 20, chemical analysis data from LDW surface sediments collected in 2008 are compared with selected SQGs. In addition, Table 21 compares chemical analysis data from LDW subsurface sediments collected in 2008 with selected SQGs.

The comparison of data presented above reveals several trends in the surface sediment data. The majority of PAHs related SQG exceedences were caused by HMW PAHs. Only one exceedence of a LMW PAH SQG was found (station T5) and in most cases LMW PAHs were not comparable to the SQG.

Table 20. Summary results for chemical analysis of surface sediment (0-15 cm) samples collected in the Lower Duwamish Waterway during July 2008 are displayed below. One sediment sample was collected from each station using a petite ponar grab sampler. Results of chemical analysis for PAHs and PCBs are displayed below. In addition, published sediment quality guidelines are also listed in order to facilitate comparison with results of the collected data. Underlined indicates one or more SQGs exceeded.

	LDW 2008 Sediment 0-15 cm depth (ppb dry wt)							Long et al. 1995 (ppb dry wt)		McDonald 1994 (ppb dry wt)	
Site/SQG	K1	T4	T5	B2	B3	P2	P1	ERL	ERM	TEL	PEL
LMW PAHs	171	78	399	103	52	81	20	552	3160	312	1442
HMW PAHs	1692	923	13598	1227	984	1699	489	1700	9600	655	6676
Total PAHs	3450	1735	20019	2554	1825	2796	1046	4022	44792	1684	16770
Total PCBs	206	173	253	3640	1360	150	62	22.7	180	21.6	189

Examination of the HMW PAH data revealed that every sampling station, with the exception of station P1, exceeded a minimum of one SQG. The SQG that was lowest, and most often exceeded, is the threshold exposure limit (TEL), which represents a level below which effects rarely occur. At the sampling stations where the PEL was exceeded but the concentration was less than the permissible exposure limit (PEL) effects would be expected to occur occasionally. Stations K1, T4, B2, B3 and P2 would fall into this category for the 2008 surface samples. The concentration present in surface sediment at station T5 would be expected to elicit effects frequently as both the TEL and PEL are exceeded. In addition, the effects range low (ERL) and effects range median (ERM) are also exceeded. At concentrations which fall below the ERL minimal effects are expected to occur. At concentrations between the ERL and ERM it is possible that effects may occur. Finally, at levels above the ERM, effects are probable. In addition to the exceedences related to PAH analysis, each of the samples exceeded a minimum of one total PCB SQG. In fact in most cases several PCB related SQGs were exceeded. Stations K1, T5, B2 and B3 exceeded each SQG listed, thus effects due to PCB toxicity at these stations may be likely to occur.

Results are displayed below for the comparison of subsurface sediment samples to SQGs. The data revealed a trend that is similar to the trend in the surface sediment data. In regard to PAH related toxicity, the majority of risk appears to be associated with HMW PAHs. Subsurface sediment LMW PAHs did not exceed or approach any SQGs listed.

Table 21. Summary results for chemical analysis of subsurface sediment (15-30 cm) samples collected in the Lower Duwamish Waterway during July 2008 are displayed below. One sediment sample was collected from each station by USEPA Region 10 dive team members using sediment cores. Results of chemical analysis for PAHs and PCBs are displayed below. In addition, published sediment quality guidelines are also listed in order to facilitate comparison with results of the collected data. Underlined indicates one or more SQGs exceeded.

	LDW 2008 Sediment 15-30 cm depth (ppb dry wt)							Long et al. 1995 (ppb dry wt)		McDonald 1994 (ppb dry wt)	
Site/SQG	K1	T4	T5	B2	B3	P2	P1	ERL	ERM	TEL	PEL
LMW PAHs	151	31	33	76	105	31	124	552	3160	312	1442
HMW PAHs	2438	651	1010	1446	4505	773	1690	1700	9600	655	6676
Total PAHs	4474	1232	1737	2711	7275	1383	3511	4022	44792	1684	16770
Total PCBs	141	138	141	289	3450	144	145	22.7	180	21.6	189

A minimum of one HMW PAH SQG was exceeded at each of the stations sampled with the exception of station T4. While the ERL and TEL were frequently exceeded, none of the values exceeded the ERM or PEL, thus PAH-related effects would only be expected to occur occasionally. PCB SQGs were again exceeded at each station sampled, however only stations B2 and B3 exceeded the ERM and PEL, so PCB-associated toxicity to aquatic organisms in subsurface sediment is more likely to occur at these two stations compared to the others.

In contrast to the SQGs used in the tables listed above, the Washington State Department of Ecology uses legally mandated SQGs. It is also of note that chemical analysis data must be normalized to OC prior to comparison with these numbers. Table 22 displays the results of this comparison. As sediment TOC analysis was only performed on surface sediments, subsurface sediments were not compared the TOC normalized SQGs.

Table 22. Summary results for chemical analysis of surface sediment (0-15 cm) samples collected in the Lower Duwamish Waterway during July 2008 are displayed below. One sediment sample was collected from each station using a petite ponar grab sampler. Results of chemical analysis for PAHs and PCBs have been normalized to organic carbon and are displayed below. In addition, Washington state sediment quality standards, which are reported normalized to organic carbon, are also listed in order to facilitate comparison with results of the collected data. Underlined indicates one or more SQGs exceeded.

	LDW 2008 Sediment 0-15 cm depth (ppm OC)					Washington Department of Ecology 1995 (ppm OC)	
Site/SQG	K1	B2	T4	T5	P1	Marine	Cleanup
LMW PAHs	7	8	6	32	1	370	780
HMW PAHs	65	89	67	<u>1088</u>	27	960	5300
Total PAHs	132	185	125	1608	59	--	--
Total PCBs	7	<u>163</u>	<u>12</u>	<u>19</u>	4	12	65

In regard to LMW PAHs, none of the sediment sampling stations exceed the Washington State Department of Ecology sediment standards. Among the HMW PAHs, station T5 exceeded the marine sediment quality standard, however even though station T5 was found to have the highest concentration of HMW PAHs, the level was roughly $1/5^{th}$ of the cleanup standard for HMW PAHs. Total PCB concentrations exceeded the marine sediment quality standard at three of the 5 locations for which TOC is known (stations B2, T4 and T5) however only station B2 with 163 ppm OCexceeded the cleanup level of 65 ppm OC.

Sediment PAH fingerprinting

PAH ratios were calculated to determine the possible source of the PAHs. Results indicate that the contamination in the Lower Duwamish Waterway likely comes from a variety of sources, as results did not consistently implicate one type of contamination. Neff et al. (2005) assert that a phenanthrene to anthracene (PH/AN) ratio of less than 5 is typical of pyrogenic assemblages, while a PH/AN ratio of greater than 5 is typical of petrogenic assemblages. In addition, pyrogenic assemblages typically have a fluoranthene to pyrene (FL/PY) ratio which approaches or exceeds 1, while FL/PY ratios which are substantially below 1 are typical of petrogenic assemblages (Neff et al. 2005).

The ratios calculated from dividing phenanthrene to anthracene and fluoranthene to pyrene in sediment samples collected during July 2008 show a distinct pyrogenic source. Table 23 notes the ratios for both 0 – 15 cm and 15 – 30 cm in depth. Sampling station P2 at both surface and subsurface depths and surface sediment sampling at station B2 indicate that the contamination likely came from multiple sources as ratios indicated PAHs of both pyrogenic and petrogenic origin**.**

Table 23. Ratios of the PAH isomers phenanthrene to anthracene (PH/AN) and fluoranthene to pyrene (FL/PY) in sediments collected from various locations in the Lower Duwamish Waterway in July 2008 are presented below. PH/AN ratios of greater than 5 are typically petrogenic and those less than 5 are typically pyrogenic. FL/PY ratios which are substantially below 1 are typically petrogenic assemblages and ratios that approach or exceed 1 are typically pygrogenic assemblages.

Site	Depth	PH/AN	FL/PY	Source
K1	0-15 cm	**2.25**	**1.01**	Pyrogenic
K1	15-30 cm	**2.88**	**0.87**	Pyrogenic
T4	0-15 cm	**3.66**	**1.44**	Pyrogenic
T4	15-30 cm	**2.36**	**0.89**	Pyrogenic
T5	0-15 cm	**2.91**	**0.89**	Pyrogenic
T5	15-30 cm	**1.21**	**0.98**	Pyrogenic
B2	0-15 cm	**2.04**	**0.80**	Pyrogenic/petrogenic
B2	15-30 cm	**1.29**	**1.07**	Pyrogenic
B3	0-15 cm	**1.49**	**1.01**	Pyrogenic
B3	15-30 cm	**1.53**	**1.43**	Pyrogenic
P2	0-15 cm	**5.35**	**1.52**	Petrogenic/pyrogenic
P2	15-30 cm	**5.03**	**1.87**	Petrogenic/pyrogenic
P1	0-15 cm	**2.82**	**1.60**	Pyrogenic
P1	15-30 cm	**2.57**	**0.91**	Pyrogenic

Regression sediment and sediment normalized to OC vs SPMEs

In order to examine the relationship between levels of PAHs present in environmental samples (sediment and surface water) and levels of total PAHs detected by SPME samplers exposed *in situ* in the Lower Duwamish Waterway, simple linear regression analysis was performed. Figure 26 depicts results of this analysis for total PAHs in sediment and total PAHs in sediment that has been normalized to organic carbon. From each sampling station, one sediment sample was collected at each station, while 3 SPME sediment samplers and 3 SPME water column samplers were deployed. In selected instances, SPME fibers shattered during the deployment or retrieval process and thus n = 2 for SPME samplers at a station.

The analysis reveals that a linear relationship does exist between levels of PAHs in sediment and levels of total PAHs detected on SPME samplers which were exposed *in situ* to the sampling locations in the Lower Duwamish Waterway, as linear regression analysis rejected the null hypothesis that the slope was equal to zero ($p < .005$). In addition, the correlation coefficient support this conclusion ($R^2 = .755$). In order to confirm the initial analysis, sediment chemical analysis data was normalized to organic carbon and the analysis was repeated. Results again indicated the presence of a linear relationship as the null hypothesis that the slope equals zero was rejected ($p < .005$) and a strong correlation coefficient continued to be reported ($R^2 = .709$). The prediction equations produced by the linear regression analysis are that porewater total SPMEs = 39.94 + 0.004*total PAHs detected in sediment and porewater total SPMEs = 44.60 + 0.0000435*total PAHs detected in sediment normalized to organic carbon.

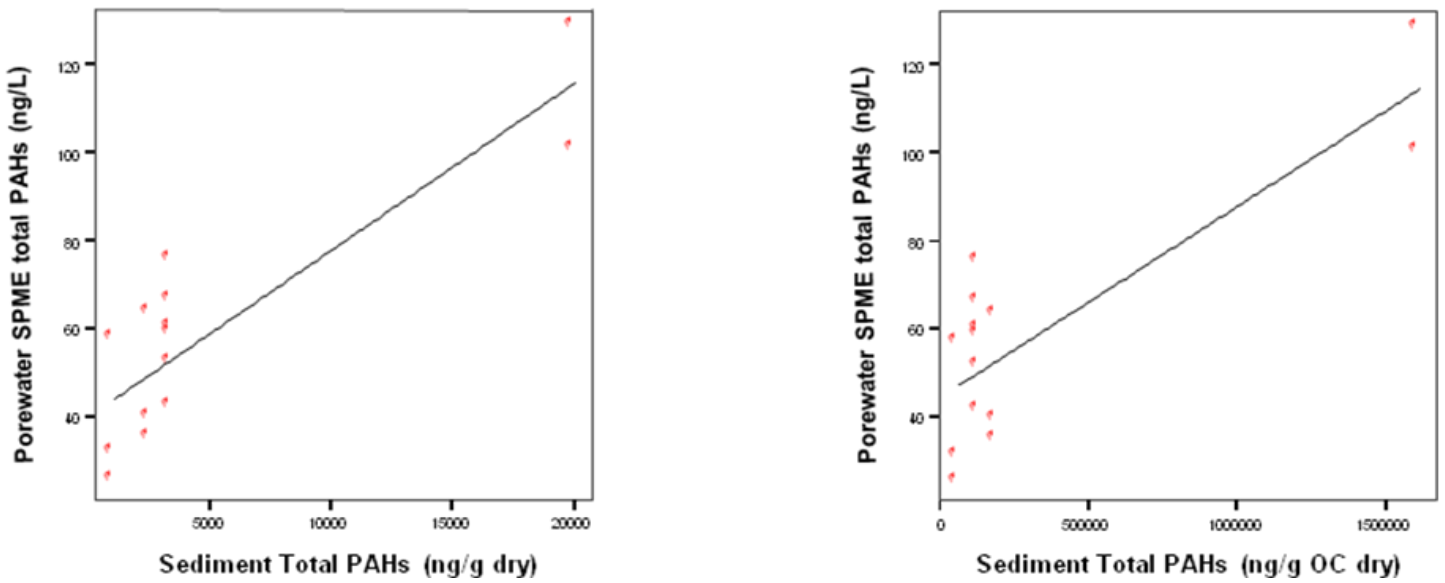

Figure 26. Sediment samples were collected from various locations in the Lower Duwamish Waterway during the 2008 sampling event. In addition, porewater SPME samplers were buried 5 cm into the sediment for 30 days at sampling locations. Plots of levels of total PAHs and total PAHs normalized to organic carbon that were detected in sediment samples versus total PAHs detected on porewater sampler SPME fibers.

Conclusions

Levels of PAHs and PCBs present in sediment (surface and subsurface), water (surface and ground), and *in situ* exposed SPME fiber samplers were determined through chemical analysis. In addition, aquatic toxicity bioassays were performed with sediment samples collected from the site of SPME sampler deployment.

In spite of levels of contaminants present in sediment that often exceeded sediment quality guidelines, results from aquatic toxicity bioassays using *Hyalella azteca* were mostly negative. In regard to survival, only station T4 demonstrated significantly reduced survival compared to controls and even this result was inconsistent as survival in a second replicate from station T4 was not affected. Growth was typically slightly reduced compared to controls, with the exceptions of both samples from station K1 and one of the two replicates from station B2, which demonstrated significantly reduced growth compared to controls.

The lack of response in the aquatic toxicity bioassays in most samples prevented any likelihood of correlation between levels of PAHs detected in SPME fiber samplers and response in the bioassays. Thus the data is not sufficient to evaluate the potential of SPMEs to measure the bioavailable portion of contaminants in sediment.

However, regression analysis was performed to evaluate the relationship between levels of PAHs present in environmental samples and levels of PAHs detected in the SPME fiber samplers. Results for the regression analysis of total PAHs detected in water column SPME samplers and total PAHs detected in surface water did not indicate a correlation, however a linear relationship was detected between levels of total PAHs detected in surface sediment and levels of total PAHs in SPME porewater samplers which were buried 5 cm into the sediment (R^2 = .755). Following normalization of the sediment data to total organic carbon, regression analysis was again performed and a strong correlation continued to be present (R^2 = .709). Although further testing is needed, these preliminary results indicate that SPME porewater samplers appear to provide an effective means of estimating the amount of PAHs present in sediment. These findings may particularly useful in future research as SPME fibers are relatively inexpensive, reusable, and substantially reduce the amount of solvent needed for analysis.

Disclaimer: the views and opinions expressed in this manuscript do not necessarily reflect those of the US Environmental Protection Agency. In addition, although EPA provided research support for this work, their effort was not a part of the negotiated risk assessment for the Lower Duwamish Waterway Superfund site.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

Summary of results

Accurate characterization of risk of adverse ecological health effects related to contaminated sediment presents a particularly difficult challenge. In addition to complications related to chemical-chemical interactions in complex mixtures that are often present in sediment, site managers must often attempt to characterize risk with a very limited budget for site-specific studies. The goal of this research was to provide information which could help increase the accuracy with which predictions of toxicity could be made at hazardous sites.

A calibration study was conducted using model PAHs, PCBs, a binary PAH mixture and a coal-tar mixture. This study was a collaborative effort between five university-based Superfund Research Programs (SRPs). Each program, with the help of funding through the NIEHS Superfund Research Program, has developed a chemical-class specific assay which is able to estimate toxicity of contaminants. This suite of bioassays expands the range of data typically obtained through the use of standard aquatic toxicity assays. Unlike chemical analysis, bioassays are able to measure interactions of mixture components. Results generated through use of this suite of bioassays will provide a more comprehensive characterization potential toxicity from complex mixtures which are present in contaminated sediment. The investigators participating in this pilot study feel that collaboration among NIEHS Superfund Research Programs, the EPA and other key stakeholders on a national level creates economies of scale and resources that can accomplish objectives unattainable by any separate entity. Results suggest that these bioassays provide more sensitive and selective information regarding complex mixture toxicity than chemical analysis data and aquatic toxicity bioassays alone. The data also suggest that bioassays provide valuable information that can be used to estimate the interactions of a complex mixture.

A series of caged *in situ* exposure studies have been conducted using juvenile Chinook salmon and Pacific staghorn sculpin in the Lower Duwamish Waterway. The exposure

study took place each summer during July and involved the placement of juvenile Chinook salmon (2004 – 2007), which were donated by NOAA, and Pacific staghorn sculpin (2004 only), which were caught by a private contractor, in cages at contaminated locations in the Lower Duwamish Waterway Superfund site. The study aimed to investigate the utility of several biomarkers in evaluating the relationship between contaminants present in environmental samples and responses in biomarkers following caged exposure. In addition to chemical analysis of environmental samples (sediment and surface water), biomarker analyses employed in the study included chemical analysis of fish tissue composites for PAHs and PCBs, DNA adduct analysis on both hepatic and gill tissue composites, and Western blot analysis of CYP1A1 expression in hepatic tissue.

Results from the first year of the study (2004) revealed that higher levels of DNA adducts were detected in gill tissue composites than in hepatic tissue composites regardless of which of the two species were used. In addition, levels of DNA adducts detected in both gill and hepatic tissue composites were higher in juvenile Chinook salmon than in Pacific staghorn sculpin. DNA adduct levels were also characterized in 2006 and 2007. In 2004 and 2006, at each station samples DNA adducts in exposed juvenile Chinook salmon were higher than levels of DNA adducts in reference fish. In several instances, the differences between DNA adduct levels in exposed fish and reference fish were found to be significant. However, in 2007, when the lowest levels of PAHs in environmental samples were recorded, DNA adduct levels in exposed fish were actually comparable or lower than DNA adduct levels in reference fish. DNA adducts do appear to provide an effective indicator of exposure, however a dose-response relationship was not present between adducts and levels of total PAHs in either sediment or surface water samples. DNA adduct analysis may have more utility as a biomonitoring tool if employed at sites where PAHs are the sole or predominant class of contaminants of concern present.

Similarly, levels of PAHs detected in fish tissue composites of total PAHs in juvenile Chinook salmon which were effective indicators of exposure as significantly higher levels of total PAHs were detected in exposed fish versus reference fish. No dose-

response relationship was present between environmental samples and levels of total PAHs in fish tissue of exposed fish likely due to rapid metabolism of PAHs in fish.

Finally, Western Blot analysis of did not appear to be an effective indicator of exposure in this exposure study protocol. The highest levels of CYP1A1 expression were actually recorded in analysis of hatchery fish which were sacrificed prior to exposure as a time = 0 treatment group. In another instance, levels of CYP1A1 expression detected were actually higher in fish that were caged in clean water at the hatchery than in fish that were caged at sampling stations B2 in the field.

The final study conducted as part of this dissertation research was concerned with evaluating the utility of using solid phase microextraction (SPME) fibers exposed *in situ* to evaluate contaminated sediment. The study used chemical analysis to characterize levels of PAHs and PCBs present in sediment (surface and subsurface), water (surface and ground), and *in situ* exposed SPME fiber samplers. Aquatic toxicity bioassays were also performed with sediment samples collected from the site of SPME sampler deployment with the intent of evaluating potential correlations between levels of contaminants detected in SPME fiber samplers and responses in standard aquatic toxicity bioassays.

Levels of PAHs and PCBs in sediment often exceeded sediment quality guidelines; however, results from aquatic toxicity bioassays using *Hyalella azteca* were mostly negative. The lack of response in sediment aquatic toxicity bioassays prevented any likelihood of correlation between levels of PAHs detected in SPME fiber samplers and response in the bioassays. However, regression analysis of total PAHs present in sediment and levels of PAHs detected in porewater SPME fiber samplers, which were placed 5 cm into the sediment for 30 days, revealed a strongly correlated linear relationship (R^2 = .755). Normalization of the sediment data to total organic carbon was performed to determine if the trend would remain present and the linear relationship was again confirmed (R^2 =.709). Investigators acknowledge that this is a small sample size and that further field testing is needed, however, these preliminary results appear to indicate that SPME porewater samplers provide an effective means of estimating the amount of bioavailable PAHs present in sediment.

Recommendations for further study include progression of the collaboration between university-based SRPs to the next phase of the project, which involves collection of sediment samples from a Superfund site and distribution of aliquots to each participating laboratory. The long term goal of the project is to provide a suite of tools and a process to evaluate mixtures that can be used to develop a supplemental line of evidence which can be integrated into ecological risk assessment by decision makers and site managers at regulatory agencies. Continued support by EPA scientist will be vital in making sure the end product of the collaboration is a tool that site managers would actually find helpful and employ in toxicity assessment.

DNA adduct analysis appears to be most useful when PAHs are the sole or predominant contaminant present. Future studies using a similar protocol might focus on collection or bile, in addition to performing DNA adduct analysis on hepatic and gill tissue. A comparison of FACs in bile and levels of DNA adducts present may be more likely to produce a correlation than any of the biomarkers used in the current study. In addition, if possible, chemical analysis of DNA adducts may help interpret results such as those produced in the current studies.

Last, further field testing of SPME porewater and water column samples is needed. Future studies could incorporate additional biological measures of comparison such as placement of mussels with the SMPE samples. This would allow comparison of PAHs that bioaccumulated in mussels with levels of PAHs which accumulated on the SPME fibers. Additional studies could also increase sample size and measure multiple time points in the field to give an indication if the SPME samplers had reached equilibrium. With further method development and characterization, SPME samplers have the potential to be a valuable tool in risk assessment of contaminated bodies of water.

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