The Effects of Polycyclic Aromatic Hydrocarbons in Fish from Puget Sound, Washington

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CONTENTS

Introduction ............................................................................................................................................ 874
Background ............................................................................................................................................ 874
Characteristics of Puget Sound ........................................................................................................ 874
Physical and Hydrologic Features ................................................................................................ 874
Puget Sound Fish Populations ..................................................................................................... 874
Demographics: Human Development ......................................................................................... 876
PAH Contamination in Puget Sound ............................................................................................. 877
Sources ................................................................................................................................ 877
Trends in PAH Concentrations ................................................................................................. 877
Co-occurrence with Other Contaminants ..................................................................................... 877
Exposure to PAHs .......................................................................................................................... 878
Bioavailability ............................................................................................................................... 878
Bioaccumulation/Food Web Transfer .......................................................................................... 878
Exposure Assessment Techniques .............................................................................................. 881
Bile Metabolites ......................................................................................................................... 881
CYP1A Activity ............................................................................................................................ 882
DNA Adducts .............................................................................................................................. 883
Bioindicators as Integrated Indicators of Toxicant Exposure and Response .............................. 885
PAH Exposure and Biological Effects in Puget Sound Fishes ......................................................... 886
General Approach to Ecotoxicological Investigations .................................................................. 886
Field Assessment of PAH Exposure in Puget Sound Fish ............................................................. 886
Bottomfish ................................................................................................................................ 886
Salmon .................................................................................................................................. 888
Physiological Effects in Puget Sound Fish ..................................................................................... 890
Liver Cancer and Related Toxicopathic Lesions ........................................................................ 890
Reproductive Effects .................................................................................................................. 891
Immunological Alterations ........................................................................................................ 893
Growth ................................................................................................................................ 895
Risk Characterization .................................................................................................................. 898
Risk Management ........................................................................................................................ 904
Puget Sound Damage Assessment Cases .................................................................................... 904
Elliott Bay ................................................................................................................................ 904
Commencement Bay .................................................................................................................. 905
Restoration Monitoring at PAH-Contaminated Sites ................................................................. 907
Conclusion ................................................................................................................................... 908
References .................................................................................................................................. 908
Introduction

Over the past 20 years, a number of studies have been conducted on the effects of polycyclic aromatic hydrocarbons (PAHs) on marine fish and other marine biota in Puget Sound. The types of studies include (1) sediment and water sampling to determine the concentrations, types, and distribution of PAHs present in the Sound; (2) studies of the uptake, metabolism, and bioaccumulation of PAHs in both fish and the invertebrates that serve as their prey; (3) studies on the biological effects of PAHs on marine organisms, including the role of PAHs in carcinogenesis in marine fish, as well as the impacts of these contaminants on mortality, growth, reproduction, and disease resistance; and (4) modeling efforts to quantify health risks associated with varying degrees of PAH exposure and to estimate the impact of PAH-related changes on survival and reproductive rates on fish populations. In this chapter, we outline what we have learned from these case studies about the risks posed to marine and estuarine fish from PAHs and discuss the application of this knowledge to the regulation and management of these common environmental contaminants.

Background

Characteristics of Puget Sound

Physical and Hydrologic Features

Puget Sound (Figure 22.1) is located in Washington State in the northwestern United States and is the southern-most glacially carved, fjord-like estuary on the west coast of North America (Thomson, 1994). It is a partially mixed estuary composed of several basins and interconnecting channels with an approximate length of 165 km and a highly variable width ranging up to about 10 km. The main basin has depths exceeding 200 m and extends 75 km from the entrance at Admiralty Inlet to The Narrows near the city of Tacoma. Because of Puget Sound’s narrow outlet and shelf at Admiralty Inlet, exchange with ocean water is relatively slow; the mean residence time for water in the central basin is about 120 to 140 days, but it can be much longer in isolated inlets and restricted deep basins (Kennish et al., 1998). Consequently, pollutants tend to be retained within the Sound (PSAT, 2002, 2004). Additionally, in a number of areas in Puget Sound, including sites adjacent to larger metropolitan areas, there is persistent water column stratification, with limited mixing of deep and surface layers. With such stratification, chemical contaminants are more likely to be contained within a smaller area and to remain more concentrated than would be the case if the water column were more fully mixed (PSAT, 2002, 2004). Moreover, sediment mass balance studies for Puget Sound show that the Sound is an efficient sediment trap (MacDonald and Crecelius, 1994). Puget Sound receives sediment particles from the river systems that drain the Cascade mountains, and, to a lesser extent, from shoreline erosion. Much of the sediment accumulates as fine-grained sediment in the central Basin (Baker, 1984). About 30% of Puget Sound is depositional, and sedimentation rates are estimated to be in range of 0.05 to 1.2 g/cm² per year (Carpenter et al., 1985). The prevalence of fine-grained, depositional sediments in Puget Sound acts to encourage the accumulation and retention of sediment-sorbed organic contaminants such as PAHs within the system.

Puget Sound Fish Populations

Puget Sound serves as the habitat for a number of recreationally and commercially important fish species, including Pacific salmon; forage fish such as Pacific herring, sand lance, and surf smelt; and 39 bottomfish stocks that have supported active fisheries in the past (Palsson, 1997; PSAT, 2002, 2004). Among the historically abundant bottomfish species are spiny dogfish, skates, spotted ratfish, Pacific cod, walleye pollock, Pacific whiting, rockfishes, lingcod, sablefish, greenlings, sculpins, wolf-eel, surfperches, English sole, rock sole, starry flounder, Dover sole, sand sole, and Pacific halibut. In addition to their commercial value, these Puget Sound stocks are of special scientific interest, because they are thought to be evolutionarily younger than related fish stocks along the Pacific coast and may have unique
characteristics that arose during their recolonization of Puget Sound after the last ice age (Rocha-Olivares et al., 1999; Seeb, 1998; Sotka et al., 2005). Much of Puget Sound and the Strait of Juan de Fuca was covered with ice during the maximum extent of the Wisconsin glaciation approximately 15,000 years ago, so genetic bottlenecks and drift could have occurred during recolonization of the Puget Sound fish populations.

Since the mid-1980s, populations of wild salmon, as well as forage fish and bottomfish, have seriously declined, either throughout Puget Sound or in selected embayments in the Puget Sound region (Bargmann, 1988; Palsson, 1997; Schmitt et al., 1994; West, 1997). Puget Sound Chinook and Hood Canal chum salmon have been listed as a threatened species under the Endangered Species Act (ESA) (McCall and Wainright, 2003; NMFS, 2003). Several marine fish stocks have also been reviewed for ESA listing, including Pacific hake, Pacific cod, Pacific herring, walleye pollock, and brown, quillback, and copper

FIGURE 22.1 Map of Puget Sound, Washington, showing locations where sediments and flatfish have been sampled in biomonitoring studies conducted by NOAA Fisheries Northwest Fisheries Science Center and the Washington State Department of Fish and Wildlife.
The Toxicology of Fishes

rockfish (Gustafson et al., 2000; Stout et al., 2001a,b). It was concluded that, although these species were not currently in sufficient danger of extinction to justify ESA listing, most of them met the International Union for the Conservation of Nature (IUCN) criteria for vulnerable species (Musick et al., 2001). The reasons for the declines of these fish stocks are not clear, but potential contributing factors include overharvesting, natural changes in environmental or climatic conditions, and various types of habitat degradation, including the discharge of toxic chemicals into the marine environment.

Demographics: Human Development

Over the past 100 years substantial urban and industrial development has occurred within the Puget Sound region, resulting in heavy inputs of chemical contaminants at selected sites, as well as significant loss or alteration of marine habitat (Levings and Thom, 1994). According to census data from the State of Washington (Puget Sound Regional Council, 2004, 2006; U.S. Census Bureau, 2006), between 1910 and 1990 the population of the counties bordering on Puget Sound (King, Kitsap, Snohomish, Pierce, Skagit, Island, Thurston, Whatcom, San Juan, Clallam, Jefferson, and Mason counties) increased nearly sixfold (Figure 22.2). Moreover, population growth and related urban and industrial development continued to increase in the Puget Sound region during the 1990s. Populations of Puget Sound counties grew from 13 to 40% between 1990 and 2000, with some of the of the highest increases in historically rural counties. Estimated 2005 populations are 2 to 11% above 2000 levels (U.S. Census Bureau, 2006). The estimated total population in the Puget Sound region area in 2005 was 4.2 million, with 76% residing in King, Pierce, and Snohomish counties, where major urban centers including Seattle, Bellevue, and Tacoma are located. Projected population increases in Puget Sound counties by 2025 range from 20 to 62% (PSAT, 2004). Each of these counties has a major river system and many small stream systems that empty into Puget Sound, and are sources of point and non-point source pollution. Population trends suggest that population growth and increased motor vehicle use in the Puget Sound region will continue, and the geographical area affected by urban development may expand beyond current population centers. These changes are likely to lead to increased and more widespread non-point source pollution from PAHs in the Puget Sound region.
PAH Contamination in Puget Sound

Sources

Aliphatic, aromatic, sulfur, and nitrogen-containing hydrocarbons have all been identified in sediments of Puget Sound. The majority of PAHs associated with sediments in Puget Sound as well as at other coastal urban sites originate from petroleum and combustion products (MacDonald and Crecelius, 1994; Varanasi et al., 1992). The geographic distribution of combustion PAHs in Puget Sound suggests that major sources are municipalities and industries that generate large quantities of PAHs, such as aluminum smelting, creosote, and oil refining. Atmospheric emissions from incineration and automobile emissions are other major sources of PAHs. PAHs are also introduced into marine systems through accidental spills of fuel oil, crude oil, and other petroleum products. The suite of PAHs detected in aquatic systems can generally be divided into two broad classes: the lower molecular weight compounds with one to three fused benzene rings (LAHs), which are mainly petrogenic or oil derived, and the higher molecular weight compounds with four to six rings (HAHs), which are mainly pyrogenic or combustion derived (Varanasi et al., 1992). Both classes tend to adsorb to organic or inorganic matter and become immobilized in sediments; however, relative to the LAHs, the HAHs are more hydrophobic and tend to remain more tightly sorbed to sediment, so they are more likely to be trapped in sediments and accumulate in depositional areas of the Sound.

Trends in PAH Concentrations

Studies of Puget Sound sediment cores (Carpenter and Peterson, 1989; Crecelius et al., 1985; MacDonald and Crecelius, 1994) show that at most sites, maximum PAH concentrations (e.g., ~8 to 12 mg/kg dry wt.) (MacDonald and Crecelius, 1994) occurred between 1945 and 1960, probably as a result of domestic coal burning, which increased until about 1950. Generally, total PAH concentrations appeared to decrease in surface sediments for the next 20 to 30 years, as coal has been gradually replaced by other fossil fuels that produce less PAH (Gschwend and Hites, 1981). Levels reported in sediment cores by MacDonald and Crecelius (1994) were about 4 to 5 mg/kg dry wt; however, this trend is countered by increasing fossil fuel use as a result of the increasing population, as well as increasing urbanization with its associated increased PAH-laden street and stormwater runoff. Consequently, PAH levels in sediments at a number of Puget Sound sites remain well above background concentrations. Recent surveys (EVS, 2003; Long et al., 2003) report sediment PAH levels above Washington State sediment quality standards at a number of sites in Puget Sound.* The majority of these sites were located in urban embayments, including Bellingham Bay, Sinclair Inlet, Everett Harbor, Elliott Bay, and Commencement Bay (EVS, 2003; Long et al., 2003). At some sites in Puget Sound, sediment PAH levels appear to be increasing. A recent study by Washington State Department of Ecology comparing surface sediment collected in 2000 to results from 1989 through 1996 at 10 long-term Puget Sound sites showed that PAH levels were significantly higher in samples collected in 2000 than they were historically at 5 of the 10 sampling stations (EVS, 2003; Long et al., 2003; PSAT, 2004). Total HAH levels were, on the average, 1.5 time higher than they were historically, while total LAH levels were 2.5 times higher.

Co-occurrence with Other Contaminants

Because PAHs are often found at urban sites near industrial discharges, they generally co-occur with a variety of other industrial pollutants, including polychlorinated biphenyls (PCBs), pesticides, and heavy metals, particularly in depositional zones that are distant from point sources. In Puget Sound, strong correlations are found between PAHs and PCBs in sediments from many of the major urban sites, such as the Duwamish Waterway, Elliott Bay, Commencement Bay, and Hylebos Waterway (EVS, 2003; Long et al., 2003). Other studies (Meador et al., 1994) have demonstrated high correlations among PAH compounds and toxic metals, including lead and copper. This feature can make it difficult to separate

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* Washington State screening level guidelines for HAHs and LAHs, respectively, are 960 mg/kg total organic carbon (TOC) and 370 mg/kg TOC, or 9.6–19.2 and 3.7–7.4 mg/kg dry wt sediment for typical TOC values of 1 to 2% in Puget Sound sediments (Washington State Department of Ecology, 1995).
the effects of PAHs from those of other compounds through field studies alone. Cause-and-effect relationships between PAHs and disease conditions in fish can be more definitively established through controlled exposure studies in the laboratory; however, even this approach does not fully address the possible modifying effects of other contaminants on the toxicology of PAHs in fish. The additive, synergistic, or antagonistic effects of various compounds in contaminant mixtures are not well understood, and this issue is of continuing concern in establishing exposure limits and sediment quality guidelines for marine organisms.

Exposure to PAHs

Bioavailability

The proportion of the total contaminant concentration that is available for uptake by organisms defines the bioavailable fraction. For neutral hydrophobic organic compounds such as PAHs, organic carbon is the main variable controlling bioavailability. Hydrophobicity, or the tendency to be water insoluble, is the primary determinant for partitioning behavior between water, sediment, and tissue (Burgess et al., 2003). As the hydrophobicity of PAHs increases, the ratio of water to sediment concentrations of a PAH will decline due to the tendency of the compound to avoid water and seek a nonpolar environment. The affinity for tissue also increases with hydrophobicity because of lipid in the organism.

The octanol–water partition coefficient ($K_{ow}$) is one physical parameter that can be used to predict the partitioning behavior exhibited by PAHs in the environment. A review of several studies was compiled in Meador et al. (1995a) for 24 of the more commonly measured PAHs to provide estimates of their $K_{ow}$ values. The range in $K_{ow}$ values is almost 4 orders of magnitude (about 5000-fold) from the least hydrophobic to the most hydrophobic compound on this list.

Another useful partition coefficient ($K_{oc}$) is the ratio of PAH concentrations in sediment organic carbon and water, which is determined by the concentration of PAH per gram of organic carbon in sediment divided by the concentration of PAH in water. This coefficient is useful for predicting the amount of waterborne PAH for a given sediment concentration under equilibrium conditions. It has been shown for many neutral hydrophobic compounds that the $K_{oc}$ is a good predictor of the $K_{ow}$. Several authors have developed equations that predict $K_{oc}$ values from the $K_{ow}$ for various hydrophobic compounds (Di Toro et al., 1991; Karickhoff, 1981; Means et al., 1980). These studies show that the $K_{oc}$ values range from $0.4*K_{ow}$ to $1.0*K_{ow}$.

A few studies have found differential partitioning and bioavailability, depending on the type of PAH and its history of association with sediment. Varanasi et al. (1985), for example, demonstrated that recently added (spiked) PAH in sediment was more bioavailable to organisms than the PAH in field-contaminated sediment; however, other studies have shown only about a twofold reduction in bioaccumulation by infaunal invertebrates exposed to sediment that had been aged several months with contaminants compared to sediment recently spiked with contaminants (Kukkonen and Landrum, 1998; Loonen et al., 1997). The source of the PAH is also a factor in determining bioavailability. Farrington et al. (1983) pointed out that PAHs from oil spills may be less strongly sorbed to sediment than pyrogenic PAHs and hence more available to organisms. This observation was supported by subsequent work showing that PAHs from field samples are tightly bound to sediment and only a few percent is available for equilibrium partitioning (McGroddy et al., 1996; Meador et al., 1995b). The lower water concentrations of PAHs in such sediments can produce much lower than predicted accumulation in organisms; however, it is not clear if species ingesting sediment with pyrogenic PAHs will also exhibit lower bioaccumulation (Meador et al., 1995b).

Bioaccumulation/Food Web Transfer

Bioavailability and organism physiology and behavior are the most important variables affecting the bioaccumulation of chemical contaminants, especially PAHs (Meador, 2003). Of the total environmental concentration, only the bioavailable fraction is available for assimilation into the organism. Unlike
metals and some ionizable organic compounds, the bioavailability of PAHs is affected by only a few environmental variables such as organic carbon and sediment surface area. Physiological factors, including lipid levels and the rates of uptake and elimination (metabolism, diffusion, and excretion), also determine contaminant tissue residues (Meador et al., 1995a). Behavioral patterns, such as organism site fidelity and variable rates of feeding, are very important for determining steady state tissue concentrations.

Bioaccumulation factors for PAHs and other hydrophobic organic compounds are generally expressed as the ratio of tissue to water (BCF) (Equation 22.1) or the sediment concentration ratio (BAF) (Equation 22.2). Currently, many researchers are interested in the biota–sediment accumulation factor (BSAF) (Equation 22.3), which is useful for reducing the variability observed in bioaccumulation. The BSAF is the lipid and organic carbon normalized bioaccumulation factor:
The Toxicology of Fishes

where \([\text{tissue}]\) and \([\text{sediment}]\) are in dry weight (parts per billion [ppb] or parts per million [ppm]), \(f_{oc}\) is the dry-weight fraction of organic carbon in sediment (g/g), and \(f_{lip}\) is the dry-weight fraction of lipid in tissue (g/g). Several factors, such as variable uptake and elimination rates, reduced bioavailability, and insufficient time for sediment-water partitioning or tissue steady state can affect each of these bioaccumulation factors.

Due to the propensity of hydrophobic compounds, such as PAHs, to partition into lipid, the \(K_{ow}\) has also been used as a surrogate measure to predict bioaccumulation. Several authors have developed equations to predict the BCF for a given compound based on its \(K_{ow}\) value (Lipnick, 1995; Mackay, 1982). The BAF is a useful measure of the amount of compound accumulated by organisms but is highly variable due to different sediment types and organism capabilities. The BSAF is a necessary refinement of the BAF that accounts for these differences and greatly reduces the observed variability. In general, the theoretical maximum BSAF is approximately one (Di Toro et al., 1991) and the empirical maximum values generally range from 2 to 4 (Boese et al., 1995; USEPA and USACE, 1991) for hydrophobic organic compounds at equilibrium in all phases. Because of the amounts of chemical expected in lipid and organic carbon, it is generally believed that hydrophobic organic compounds that are not metabolized will produce predictable levels of bioaccumulation. Although the BSAF is useful for characterizing bioaccumulation, adjustments have to be made for compounds that are metabolized and when conditions are not at equilibrium.

Equilibrium partitioning (EqP) theory is used to predict the amounts of hydrophobic organic compounds bioaccumulated in organisms (Di Toro et al., 1991; Pavlou and Weston, 1983). The basic premise for EqP is that when sediment and water are in equilibrium, the organism receives an equivalent exposure from each phase allowing predictions of the accumulated dose using either phase. The organismal lipid, total organic carbon (TOC) in sediment, and water can be considered as three phases that exhibit predictable concentrations at equilibrium due to equal chemical activity or fugacity. Because of this assumption, the route of exposure (e.g., water ventilation or prey/sediment ingestion) is immaterial because at equilibrium the concentration in each phase is a function of the thermodynamic properties, not the kinetics of accumulation. Also, because of the equal fugacity between phases, one phase (e.g., sediment) may be used to predict bioaccumulation from all phases, even though the organism may not interact directly with sediment. EqP has generally been successful in predicting sediment–water partitioning (\(K_{ow}\)) and bioaccumulation (BSAF) for the nonmetabolized, neutral hydrophobic organic compounds.

Because PAHs are so readily metabolized, predictive models such as the ones mentioned above are rarely accurate in determining PAH bioaccumulation. Potential bioaccumulation of PAHs can be determined with BSAF by setting the BSAF to the maximum (e.g., 4), rearranging the equation, and solving for tissue concentration. Other predictive models may include correlating the PAH metabolites measured in bile (bile FACs) with sediment concentrations or determining the half-life of PAHs in a given fish species and extrapolating the small amounts of parent PAH compounds that can be found in tissue. Stomach content analysis, coupled with uptake efficiency, could also be used to determine apparent bioaccumulation. Whatever methods are employed, it is crucial to be able to gauge the relative amounts of PAHs that are accumulated when attempting to assess exposure and effects at contaminated sites. This is especially true if the goal is to relate an exposure concentration with deleterious effects.

Despite the substantial uptake of PAHs by fish through exposure to food, water, and sediment, rapid metabolism of these compounds by fish precludes high levels in their tissues. Additionally, metabolism of PAHs by invertebrates is variable (James, 1989; Livingstone, 1991; Varanasi et al., 1989a) and can
have a major impact on the amounts that accumulate by fish. Biomagnification of PAHs, which is the increase of contaminants over two trophic levels or more, does not appear to occur in fish (Suedel et al., 1994). Benthic invertebrates in the diet are an important source of PAH exposure to marine fishes, as these compounds bioaccumulate in many invertebrate species. In one study (Varanasi et al., 1992), sediment from the Duwamish Waterway, a contaminated estuary in Puget Sound, was used to examine the uptake of PAHs by clams and amphipods. The HAHs were clearly accumulated by clams and amphipods, but accumulation of LAHs was not as great. Determining the cause of this pattern is complicated because of many factors that must be considered. Although the bioaccumulation factor will be higher in most invertebrates for the HAHs, some species will exhibit metabolism of these compounds, whereas others will not. The preferential retention of HAHs in invertebrates observed in laboratory exposure (Meador et al., 1995b; Varanasi et al., 1992) is also seen in free-living invertebrates and in the stomach contents (e.g., molluscs, arthropods, and annelids) of fish sampled from urban areas (Varanasi et al., 1989a).

Some studies of dietary uptake of PAHs in fish generally indicate low uptake efficiency. Experiments with rainbow trout demonstrated uptake efficiencies between 2 and 30%, depending on the PAH (Niimi and Dookhran, 1989), which was confirmed by another study (Niimi and Palazzo, 1986) with the same species fed herring oil spiked with PAHs, but these results were contrary to those obtained for nonmetabolized hydrophobic organic compounds such as PCBs (Madenjian et al., 1999). These studies on PAHs indicate that uptake efficiency generally declines with increasing chemical hydrophobicity, which may be due to a combination of slow kinetics and a short residency time in the gut. Because the role of metabolism is often not addressed in studies of dietary uptake efficiency, the parent compound plus metabolites must be determined. Recent work indicates uptake efficiencies of approximately 50% for organic compounds with a log $\log_{10} K_{ow}$ in the range of 3 to 6.5, which includes most PAHs (Arnot and Gobas, 2004). Additional research that includes uptake and elimination kinetics is needed to better assess uptake efficiency of PAHs for the different routes of uptake, especially the dietary route. These data will help greatly in predicting bioaccumulation from different environmental matrices.

Exposure Assessment Techniques

Because fish metabolize PAHs rapidly (Hellou and Payne, 1986; Roubal et al., 1977; Statham et al., 1978; Varanasi et al., 1989b), measurement of tissue PAH concentrations by standard analytical techniques is generally not a useful method for assessing exposure. Instead, alternative chemical and biological methods have been developed that have become essential tools for evaluating exposure to PAHs in marine fish. For any method, but in particular for a biochemical method, to be useful, it must undergo extensive validation to establish the interpretive limits for the data obtained. This section presents the underlying validation and field studies for three techniques for assessing PAH exposure (bile metabolites, DNA adducts, and CYP1A) that have been applied to Puget Sound fishes.

Bile Metabolites

As mentioned above, exposure to PAHs in fish generally should be assessed by measuring metabolites rather than the parent compounds. Two methods that employ reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection have been developed in our laboratory to screen for PAH metabolites in fish (Krahn et al., 1993). The first, a bile screening method, was developed to evaluate anthropogenic contamination by PAHs in fish sampled from urban estuaries (Krahn et al., 1984, 1986). At the present time, this method or variations of this method are widely used to assess PAH contamination in the environment (Aas and Klungsoyr, 1998; Beyer et al., 1996; Escartin and Porte, 1999a,b,c; Jonsson et al., 2003; Lin et al., 2001; McDonald et al., 1995). In addition, a method that estimates the concentrations of AC metabolites in fish tissues has been developed to address the issue of seafood contamination (Hufnagle et al., 1999; Krone et al., 1992).

In addition to estimating exposure to PAHs, HPLC chromatographic patterns can often provide information about the possible source of contamination, such as crude oil, diesel fuel, or pyrogenic contaminants; for example, the chromatographic patterns from bile of flatfish captured from Prince
William Sound after the Exxon Valdez spill are very dissimilar to those of fish exposed to contaminants from urban sites (Figure 22.3). The source of contamination suggested from the HPLC chromatogram can often be substantiated by examining the relative proportions of PAH metabolites determined by the gas chromatography–mass spectrophotometer (GC–MS) analysis of bile (Krahn et al., 1987, 1993). Bile of fish exposed to PAHs from urban sites typically contains high proportions of four- to six-ring PAHs from pyrogenic sources, whereas the bile of fish exposed to crude oil contains much larger proportions of metabolites of alkylated naphthalenes, phenanthrenes, and dibenzothiophenes than bile from urban fish.

Laboratory studies have shown that biliary fluorescent aromatic compound (FAC) levels increase in a dose-dependent manner with exposure to PAHs, and field studies have demonstrated strong and consistent correlations between biliary FAC concentrations in fish and sediment PAH concentrations at sites where the animals are collected (Figure 22.4). Biliary FAC concentrations, however, reflect relatively short-term exposure to PAHs. Concentrations typically increase very quickly with exposure, within a day, but decline to baseline levels within about 2 to 4 weeks of exposure (Anulacion et al., 1995; Collier and Varanasi, 1991).

CYP1A Activity

One of the earliest changes associated with exposure to contaminants is induction of cytochrome P450-associated enzymes (CYP) in the liver, especially CYP1A, which is largely responsible for metabolism of PAHs and a variety of other toxic compounds (Buhler and Williams, 1989; Goksøyr and Forlin, 1992). Although several methods are available to assess induction of CYP1A in fish, the most common methods are catalytic assays to measure the functional activity of the enzyme—for example, aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-O-deethylase (EROD) activities—and immunoquantitation of the CYP1A protein directly by methods such as an enzyme-linked immunosassay (ELISA). Three of these measures (AHH activity, EROD activity, and CYP1A quantitation by ELISA) were evaluated in Puget Sound flatfish species during a year-long field study (Collier et al., 1995). Each measure could detect significant between-site differences that were consistent with PAH concentrations in sediment where the fish were collected, but AHH activity measured by a standardized protocol showed the least amount of unexplained variability and was the measure most sensitive to site differences. For this reason, in our studies, we have primarily used measurement of hepatic AHH activity for monitoring of CYP1A induction in fish. We believe that this method is particularly useful for analysis of trends in contaminant exposure in fish (Collier et al., 1998a). Because CYP1A is inducible by a wide variety of organic chemical
contaminants, however, this measure should not be presumed to be diagnostic of PAH exposure in field situations, unless PAHs are the only likely organic contaminant present, such as following oil spills (Collier et al., 1996).

### DNA Adducts

Covalent binding of carcinogenic PAHs to DNA (DNA adducts) in liver, an initial molecular step in the chemical hepatocarcinogenesis model (Farber and Sarma, 1987), is observed in several species of fish exposed to benzo(a)pyrene (BaP) and related PAHs (Collier et al., 1993; Ericson et al., 1999; Sikka et al., 1991; Varanasi et al., 1989c). A very sensitive technique for determining levels of DNA adducts in fish tissues is the 32P-postlabeling (PPL) method, which was developed in Dr. Kurt Randerath’s laboratory in the early 1980s (Gupta et al., 1982) and has evolved substantially since then (Reichert et al., 1999). Currently, the 32P-postlabeling technique is the most sensitive method for the detection of a wide range of bulky, hydrophobic compounds bound to DNA. For hydrophobic, aromatic DNA adducts, such as PAH–DNA adducts, this method can detect 1 adduct in 10⁹ to 10¹⁰ (Gupta, 1985; Reddy and Randerath, 1986). The versatility and high sensitivity of the assay has led to the broad use of the 32P-postlabeling assay in studies with mammals and fish for assessing exposure to environmental genotoxins (Balch et al., 1995; Dunn et al., 1987; Liu et al., 1991; Poginsky et al., 1990; Ray et al., 1991; Stein et al., 1992; van der Oost et al., 1994; Varanasi et al., 1989d) and to specific genotoxic compounds, such as BaP and 7H-dibenzo(c,g)carbazole (Ericson et al., 1999; Randerath et al., 1984; Schurdak et al., 1987; Sikka et al., 1991; Stein et al., 1993; Varanasi et al., 1989c; Watson et al., 1998).

In 1987, we initiated studies using the 32P-postlabeling assay to evaluate exposure of marine fish to environmental carcinogens. These studies have shown that the levels of hepatic DNA adducts in wild fish positively correlate with the concentrations of PAHs present in marine sediments (Collier et al., 1993; Stein et al., 1992) (Figure 22.5). Moreover, laboratory studies with model PAHs and sediment extracts have shown that PAH–DNA adducts formed are persistent and have chromatographic characteristics similar to DNA adducts detected in wild fish (French et al., 1996; Stein et al., 1993; Varanasi et al., 1989d) (Figure 22.6). The study by French et al. (1996) of English sole exposed to a gradient of contaminated sediments showed that the levels of hepatic DNA adducts increased in both a time- and a dose-dependent manner. These findings suggest that the levels of hepatic DNA adducts found in fish tissues could function as indices of cumulative exposure to potentially genotoxic environmental contaminants, such as carcinogenic PAHs. The use of DNA adducts as an exposure index has several important features. First, it is a quantifiable measure of the biologically effective dose reaching a critical target site.

![Figure 22.5](image-url)
and thus is a useful epidemiological/epizootiological tool for detecting exposure to genotoxins. Second, DNA adduct levels integrate multiple toxicokinetic factors (i.e., uptake, metabolism, detoxication, excretion, and covalent binding of reactive metabolites to target tissues). Third, the DNA adduct profiles and levels can be used in identifying species differences in exposure and processing of genotoxic compounds.

Hepatic DNA adducts are currently being used as a marker of exposure to potentially genotoxic contaminants in environmental monitoring of Puget Sound and have been applied in national monitoring programs, such as the National Benthic Surveillance Project of the National Oceanographic and Atmospheric Administration (NOAA) National Status and Trends (NS&T) Program and in the Bioeffects Surveys of NOAA's Coastal Ocean Program. The International Council for the Exploration of the Sea (ICES) has recently published a methods manual on the ³²P-postlabeling assay to encourage its use in marine biomonitoring studies (Reichert et al., 1999).

Other investigators have also detected DNA damage in English sole from Puget Sound sites with different assessment techniques. Using GC–MS with selected ion monitoring (GC–MS/SIM) and Fourier-transform infrared (FT–IR) spectroscopy, Malins and colleagues (Malins and Gunselman, 1994; Malins et al., 1996, 1997) have observed hydroxy-radical-induced DNA damage in liver tissue of English sole from contaminated sites in Puget Sound (e.g., the Duwamish Waterway). Common types of damage included hydroxyl-radical-induced ring-opening products (e.g., 2,6-diamino-4-hydroxy-5-formimidopyrimidine) and 8-hydroxy adducts of adenine and guanine (e.g., 8-hydroxyguanine). These mutagenic base modifications were statistically correlated with an increased incidence of preneoplastic or degenerative liver lesions (e.g., basophilic foci, hepatocellular karyomegaly, megalocytic hepatitis, hyalin droplet formation, and apoptosis) in English sole (Malins et al., 1996).

Related forms of oxidative damage have been detected in juvenile salmonids exposed to dietary PAHs in the laboratory (Bravo, 2005). Juvenile rainbow trout (*Oncorhyncus mykiss*) fed a diet containing a mixture of 10 common HAHs at 40 or 400 mg/kg, 160 mg/kg BaP, or 160 mg/kg benzo(EP)pyrene had induced CYP1A1 in liver and kidney and increased oxidative damage as indicated by DNA strands breaks measured in blood by comet assay, protein nitration measured in kidney by immunohistochemistry,
and lipid peroxidation measured in kidney by F2-isoprostanes. Fish from the highest dose PAH treatment group yielded the strongest effect. After 50 days of exposure to PAHs, fish responses began to moderate but were still significantly different from control. Reactive oxygen species generated in the kidney and liver likely contributed to the damage observed in kidney and blood. The head kidney is thought to be the primary immune organ in fish (Wester et al., 1994), so oxidative stress in this organ might contribute to decreased animal fitness through increased disease susceptibility (Livingstone et al., 2001).

**Bioindicators as Integrated Indicators of Toxicant Exposure and Response**

In ecotoxicological field studies, we typically employ several of the techniques described above to provide an integrated picture of PAH exposure. All three indicators (bile metabolites, CYP1A induction, and DNA adducts) are strongly correlated with environmental exposure measures, such as concentrations of PAHs in sediments or food, and also show significant correlation with each other. Each of the exposure indicators, however, has its own specificity, sensitivity, and time response; for example, bile FAC concentrations and AHH activity respond relatively quickly to PAH exposure but decline fairly rapidly when exposure ceases, with half-lives of 2 to 4 weeks (Anulacion et al., 1995). In contrast, hydrophobic DNA adducts are relatively stable, with a much longer half life (Anulacion et al., 1995; Stein et al., 1993), so they are better indicators of relatively long-term, cumulative exposure to genotoxic PAHs. The indicators also vary in their specificity; both DNA adducts and biliary FAC concentrations respond specifically to PAH exposure, while CYP1A may be induced in response to either PAHs or coplanar PCBs, dioxins, and related compounds. Moreover, studies indicate that species may differ in their range of response to these indices. For example, in one study with several species of Puget Sound flatfish, AHH and EROD activity in rock sole was substantially higher and more uniform over a range of sediment PAH and PCB concentrations than was the case for either English sole or starry flounder. Consequently, a suite of measurements used concurrently can enhance the ability to identify fish populations affected by exposure to chemical contaminants (Stein et al., 1992).

Biochemical indicators of exposure can also be very useful in epizootiological analyses of field data on PAH exposure and disease conditions in fish. Risk factors for certain diseases or other forms of biological injury can be generated by correlating biochemical indicators of PAH exposure with the disease occurrence, thus allowing the use of a relatively simple biomarker in predicting risk. Additionally, because these parameters can be measured in individual fish, factors such as age and sex can be taken into account in risk analyses. Several examples of such analyses are discussed in the section on PAH exposure and biological effects in Puget Sound fish.

These biochemical parameters can also yield important information on the uptake and metabolism of PAHs and provide insight into mechanisms of toxicant action and pathogenesis of toxicopathic hepatic lesions and other disease conditions in fish. As an example, immunohistochemical localization of CYP1A activity, in combination with quantitation of PAH–DNA adducts, has recently been applied to investigate the role of resistance to cytotoxicity in liver neoplasia in English sole (Myers et al., 1998a). Immunohistochemical studies of English sole from PAH-contaminated sites show a consistent reduction in expression of CYP1A in hepatic neoplasms and most preneoplastic foci of cellular alteration. The reduction in CYP1A expression is accompanied by a significant and nearly parallel reduction in DNA adduct level as compared to non-neoplastic liver tissue. These findings are consistent with the hypothesis, developed from studies with mice and rats, that neoplastic hepatocytes possess a resistant phenotype in which there is a reduced capacity to activate PAHs and related compounds to toxic and carcinogenic intermediates (Roomi et al., 1985).

Similarly, the presence of PAH–DNA adducts in tissues of PAH-exposed marine fish suggests the potential for genomic alterations, including oncogene activation. Our laboratory has now cloned and sequenced the entire K-ras b cDNA from liver of English sole (Peck-Miller et al., 1998). The percent identity between the predicted amino acid sequence of English sole and human K-ras b was 97%, whereas the percent identity between the English sole gene and rainbow trout or *Rivulus* K-ras b was 98%. Areas of amino-acid sequence conservation include codons 12, 13, and 61, the positions in which mutations are observed in ras cellular oncogenes in other species. Analysis of K-ras mutations was performed on a variety of necrotic, preneoplastic, and neoplastic lesions in livers from 13 English sole
collected from contaminated waterways in Puget Sound; however, despite reports of K-ras mutations in hepatic tumors from other fish (Chang et al., 1991; McMahon et al., 1990; Wirgen et al., 1989), no mutations in codons 12, 13, or 61 were found in hepatic neoplasms and related lesions from English sole by direct DNA sequencing of polymerase chain reaction-amplified genomic DNA. The results suggest that K-ras has a role in liver carcinogenesis that varies according to the fish species or carcinogen. Future studies of the etiology of chemically induced cancer in wild English sole should consider mutations in other cancer-related genes, such as p53, which is involved in the control of apoptosis, or programmed cell death (Fridman and Lowe, 2003), as well as Ha-ras and N-ras, which may have oncogenic activities similar to those of K-ras (Crespo and Leon, 2000). Forms of all three of these genes have been identified in other fish species (Bhaskaran et al., 1999; Brzuzan et al., 2006; Busch et al., 2004; Cachot et al., 2004; Franklin et al., 2000; Rotchell et al., 2001).

### PAH Exposure and Biological Effects in Puget Sound Fishes

#### General Approach to Ecotoxicological Investigations

We have addressed the impact of PAHs and related industrial pollutants on benthic fish in Puget Sound through a broad interdisciplinary approach. This research has involved exposure assessment tightly linked to the detection of effects at several levels of biological organization. Exposure assessment is typically carried out by measurement of PAHs in sediments at sites where fish are collected, as well as in stomach contents of target fish species, and through measurement of PAH metabolites in fish bile. Animals are also examined for early biochemical changes, such as CYP1A induction and DNA adducts, which can often be linked in a dose-responsive fashion to PAH exposure, both in the field and in the laboratory through controlled experiments. Additionally, fish are examined for effects of contaminants on critical life processes, such as growth, reproduction, and immunocompetence and disease susceptibility, which may then be related potential changes in vital rates and fish abundance. Our primary target species in Puget Sound is English sole (Parophrys vetulus). This pleuronectid species was selected because it is widespread, and in early studies (Malins et al., 1984, 1985) it appeared to be particularly sensitive to the effects of chemical contaminants. We have sampled English sole at a range of both urban and non-urban embayments throughout Puget Sound (Figure 22.1). These sites vary widely in sediment PAH concentrations (Figure 22.7). In addition to English sole, we have examined PAH exposure and effects in several other Puget Sound bottomfish (e.g., rock sole and starry flounder), as well as in several species of juvenile salmon that use estuaries during their migration from fresh to saltwater.

#### Field Assessment of PAH Exposure in Puget Sound Fish

**Bottomfish**

Field assessments of PAH exposure in English sole and other bottomfish have generally included measurement of biliary FACs, CYP1A induction, and DNA adducts. This suite of indicators is useful in evaluating both long- and short-term exposure to PAHs, with the caveat that CYP1A induction, while responsive to PAH exposure, is not diagnostic of PAH exposure. Of the three indicators, biliary FACs are most purely measures of recent exposure, while the others are indicators, at least to some degree, of biological or biochemical response. Bile screening for PAH metabolites was originally developed as a tool to rapidly estimate concentrations of metabolites resulting from the uptake and transformation of PAHs by English sole (Krahn et al., 1984, 1986). Since that time, the technique has been applied in a number of studies with Puget Sound fish (Collier et al., 1998a,b; Krahn et al., 1987; Myers et al., 1994, 1995, 1998b,c, 2000, 2005; O’Neill et al., 1999; Stein et al., 1992), demonstrating clearly that bottomfish from urban and industrialized areas in Puget Sound take up PAHs. Average biliary FAC concentrations of English sole from representative sites are shown in Figure 22.8. In studies within Puget Sound and in other areas of the United States (Collier et al., 1993; Johnson et al., 1993; Myers et al., 1994; Stehr et al., 1997, 2004), strong and consistent correlations have been found between biliary FAC levels in sole and other bottomfish and sediment PAH concentrations.
Fish from contaminated areas show not only elevated levels of FACs in bile but also other physiological changes that are indicators of biological responses to contaminant exposure, including increased AHH activity and covalent binding of PAHs to DNA in liver. Both of these types of biochemical alteration are observed in English sole, starry flounder (Platichthys stellatus), and rock sole (Pleuronectes bilineata) from areas in Puget Sound with elevated sediment PAH concentrations (Collier and Varanasi, 1991; Collier et al., 1995; Stein et al., 1992) (Figure 22.9). Moreover, both hepatic AHH activity and levels of DNA adducts in liver, determined by 32P-postlabeling, are significantly correlated with sediment PAH concentrations at sites where fish are collected, and with biliary FAC concentrations in the same fish (Stein et
Laboratory studies suggest that English sole is comparatively less sensitive to CYP1A induction following PCB exposure than to other bottomfish species (Collier and Varanasi, 1991). This finding raises the possibility that the extensive CYP1A induction in English sole from Puget Sound is due primarily to PAH exposure; however, this hypothesis remains unproven. The correlation of FACs with hepatic DNA adducts is consistent with laboratory studies in which PAHs extracted from sediment and model PAHs produced adduct patterns in exposed sole similar to those found in wild sole (Reichert et al., 1998).

**Salmon**

In addition to benthic fish, such as English sole, which reside in bottom sediments, transient species such as outmigrant juvenile salmon show detectable exposure to PAHs as they migrate through urban estuaries (McCain et al., 1990; Olson et al., 2006; Stehr et al., 2000; Stein et al., 1995). McCain et al.
(1990) found significantly higher AH concentrations in stomach contents as well as higher levels of FACs in bile in juvenile Chinook salmon from the Duwamish Waterway compared to juvenile Chinook from the relatively uncontaminated Nisqually Estuary or from either of the hatcheries from which fish in these two river systems are released. Since this initial study, PAH exposure has been examined in juvenile Chinook, Coho, and chum salmon from hatcheries and their respective estuaries of five river systems of Puget Sound: the Green-Duwamish, the Puyallup-Hylebos/Commencement Bay, the Nisqually, the Snohomish, and the Skokomish (Olson et al., 2006; Stehr et al., 2000; Stein et al., 1995).

As in the earlier study, salmon collected from the Duwamish and Commencement Bay/Hylebos Waterway estuaries adjacent to Seattle and Tacoma showed elevated levels of FACs in bile in comparison to fish from hatcheries or from the less-urbanized Nisqually and Skokomish systems. Additionally, elevated AHH activity and elevated levels of DNA adducts in liver were detected in salmon migrating through the urban estuaries (Figure 22.10). In comparison to English sole collected from the same waterways, FAC concentrations in bile of salmon were similar. In contrast, levels of DNA adducts in liver of salmon, whose residence time in the estuary is relatively short, were much lower than levels in English sole (Collier et al., 1998b; Stehr et al., 2000). This is consistent with data from dose–response experiments showing that biliary FAC concentrations are a good indicator of relative short-term exposure to PAHs, while DNA adducts are a better measure of chronic, long-term exposure.

**FIGURE 22.10** (A) Biliary fluorescent aromatic compounds (FACs) (ng BaP equiv per mg protein); (B) hepatic aryl hydrocarbon hydroxylase (AHH) activity (pmol/min*mg protein); and (C) hepatic DNA adducts (nmol/mol bases) in juvenile salmon from estuaries and hatcheries sampled from Puget Sound, Washington. The bars represent the mean ± standard error. The sample size (n) equals the number of tissue composites that were analyzed. CB, Commencement Bay; DW, Duwamish Waterway; NE, Nisqually River Estuary. (Data are from Stein et al., 1995; figure reprinted from Johnson, L.L. et al., Can. Tech. Rep. Fish. Aquat. Sci., 1948, 304–329, 1994. With permission.)
Liver cancer (and the occurrence of a spectrum of other toxicopathic lesions involved in the pathogenesis of liver cancer) is one of the most dramatic and best-documented effects of contaminants on English sole in Puget Sound (Myers et al., 1987, 1990, 1994, 1998b,c, 2003; O’Neill et al., 1999; PSAT 2002, 2004). In general, the prevalence of these liver lesions increases with increased urbanization (Figure 22.11). Typically, between 25 and 40% of English sole sampled from urban embayments such as Elliott Bay and Commencement Bay exhibit neoplastic, preneoplastic, or unique degenerative liver lesions, as compared to 3 to 8% of fish from non-urban and moderately urbanized sites.

In statistical analyses of field data (Landahl et al., 1990; Myers et al., 1990, 1994, 1998b,c, 2003; Rhodes et al., 1987), exposure to PAHs has been identified as the major risk factor for neoplasms and related liver lesions in English sole, although certain chlorinated hydrocarbons, including PCBs and pesticides, may also play a role in lesion progression through their actions as tumor promoters. Multiple field studies in bottomfish species, including English sole, have consistently shown strong statistical correlations between both site sediment PAH concentrations and prevalences of toxicopathic hepatic lesions (Myers et al., 1998b,c). A cause-and-effect relationship between PAHs and toxicopathic liver lesions in English sole has been confirmed by induction of degenerative, proliferative, and preneoplastic lesions, identical to those observed in field-collected fish, in sole exposed in the laboratory to model carcinogenic PAHs such as BaP and to extracts of sediments from PAH-contaminated sites (e.g., Eagle Harbor) in Puget Sound (Schiewe et al., 1991).

Field studies have also examined the association between rapidly responding biomarkers of PAH exposure (bile FAC levels, CYP1A induction, and DNA adduct formation) and toxicopathic hepatic lesion prevalences in wild fish. Relationships between liver disease risk and these biomarkers of PAH exposure were clarified in a multi-season study targeting subadult, reproductively immature English sole from nine sites in Puget Sound representing a broad gradient for sediment contaminants (Myers et al., 1998b). This study expanded upon information provided in companion studies examining links between...
liver histopathology and a more limited set of biochemical markers in subadult English sole, rock sole, and starry flounder at all seasonal sampling points (Myers et al., 1992) and relationships among the various biochemical markers of contaminant exposure in English sole, rock sole and starry flounder in specimens of these species sampled at a single time point (Stein et al., 1992).

Although neoplasms were rarely detected in these subadult fish, whose average age was less than 2 years, higher prevalences of the earlier-occurring lesions were found in young sole from contaminated sites. Prevalences for any of the earlier-occurring lesions were significantly higher at all of the moderately to severely contaminated sites (Duwamish Waterway, inside Eagle Harbor, inside Everett Harbor, and Commencement Bay). Confirming earlier results in adult English sole (Myers et al. 1990, 1994, 1998c), biliary FAC levels were significant risk factors for prevalences of these early lesion categories in logistic regression analyses. More interestingly, the study also identified induction of hepatic AHH activity as a significant risk factor for prevalences of all of the early lesion categories, and DNA adduct level as a significant risk factor for nuclear pleomorphism/hepatic megalocytosis as well as the inclusive early lesion category.

With the acquisition of additional data on lesion occurrence, DNA adduct levels, and AHH activity in wild fish, it has been possible to establish hepatic DNA adducts as a significant risk factor for hepatic lesion occurrence on an individual fish basis. In stepwise logistic regression analyses that accounted for the potential influences of fish age and sex on the risk of lesion occurrence, hepatic DNA adduct level was identified as a positively significant ($p \leq 0.05$) risk factor for the occurrence of hepatocellular nuclear pleomorphism and hepatic megalocytosis in English sole, starry flounder, and rock sole and for preneoplastic foci of cellular alteration in English sole (Myers et al., 1998b,c). These findings give further credence to the role of PAH exposure in the etiology of these lesions and suggest that xenobiotic–DNA adduct formation is integral to the early initiation phase of hepatocarcinogenesis, as well as the induction of these more prevalent lesions known to occur prior to the development of hepatic neoplasms in fish and rodent models of hepatocarcinogenesis. Moreover, they support the utility of certain non-neoplastic hepatic lesions as early indicators of biological damage in subadult and adult fish environmentally exposed to xenobiotics. Overall, the collective available evidence shows a strong causal relationship between PAH exposure and hepatic neoplasms and neoplasia-related liver lesions in English sole (Myers et al., 2003).

**Reproductive Effects**

In addition to toxicopathic liver disease, English sole residing in contaminated areas in Puget Sound also suffer from various types of reproductive impairment. Field studies conducted in the late 1980s (Johnson et al., 1988) showed that female English sole from areas with high concentrations of PAHs in sediment were less likely to enter vitellogenesis and had lower plasma concentrations of the female reproductive hormone 17β-estradiol than sole with low levels of contaminant exposure (Figure 22.12). At minimally to moderately contaminated sites within Puget Sound such as Port Susan and Sinclair Inlet, approximately 80 to 90% of adult females underwent gonadal development, while at the Duwamish Waterway and Eagle Harbor the percentage declined to 40 to 60%. A 1994 field study of English sole from the Hylebos Waterway in Commencement Bay, a site with high sediment concentrations of CHs and PAHs, yielded similar results (Johnson et al., 1999). Approximately 55% of adult female sole from the Hylebos Waterway were vitellogenic, in comparison to 80% of adult sole from Colvos Passage, a nearby reference site. As in the earlier study, exposure to PAHs was a major risk factor for inhibited ovarian development in adult sole. English sole from PAH-contaminated areas also display increased ovarian atresia, particularly of primary oocytes (Johnson et al., 1988, 1997). Increased atresia is associated with a trend toward reduced egg production and increased egg weight, although the magnitude of the effect is not great. Similarly, in gravid female dolly varden and yellowfin sole sampled following the *Exxon Valdez* oil spill, plasma estradiol concentrations were depressed in fish with high biliary FAC levels (Sol et al., 2000).

Results of these field studies are supported by laboratory experiments showing that pretreatment of gravid female English sole with extracts of contaminated sediment or crude oil containing high levels of PAHs decreased levels of endogenous estradiol (Johnson et al., 1995; Stein et al., 1991). Related experiments suggest that exposure to BaP or PAH-contaminated sediment may suppress estradiol-induced vitellogenin production in English sole (Anulacion et al., 1997).
FIGURE 22.12 Reproductive success of English sole from urban and non-urban sites in Puget Sound. Shown in the figure are (A) the percentage of adult female sole entering vitellogenesis; (B) the percentage of adult female sole successfully spawning following induction with luteinizing hormone releasing hormone analog; and (C) the percentages of fertile eggs (solid) and normal larvae (diagonal lines) produced by those females that spawned successfully, following fertilization with pooled sperm from reference male English sole. The number of animals sampled at each site is indicated in parentheses. Values significantly different from those at the reference sites (Port Susan for 1985–1987 studies and Colvos Passage for 1994 study) are indicated by asterisks (*). (Data are from Casillas et al., 1991; Collier et al., 1997; Johnson et al., 1988. Figure is reprinted from Johnson, L.L. et al., *J. Sea Res.*, 39, 125–137, 1998. With permission.)
Studies also suggest that English sole from contaminated areas that do successfully enter vitellogenesis may experience inhibited spawning ability and reduced viability of eggs and larvae. When gravid English sole from Port Susan, Sinclair Inlet, the Duwamish Waterway, and Eagle Harbor were brought into the laboratory and artificially induced to spawn, spawning success was significantly lower in fish from Eagle Harbor and the Duwamish Waterway, where sediment PAH concentrations are high (Casillas et al., 1991). Exposure to PAHs in the water column (e.g., fluoranthene at 0.075 to 7.5 mg of PAH per L seawater) caused larvae to become disoriented and to exhibit signs of narcosis, with mortality at higher concentrations (Eddy et al., 1993).

Although effects of PAHs on reproduction have not been studied as extensively in male as in female English sole, evidence suggests that males may also be susceptible to PAH-related reproductive dysfunction. Preliminary studies indicate that, although testicular development in male sole from PAH contaminated sites is relatively normal, plasma concentrations of 11-ketotestosterone and testosterone are reduced in fish with particularly high concentrations of PAH metabolites in bile (Sol et al., 1998).

The impacts of PAHs on the reproduction and development of wild Puget Sound salmon have not been well characterized, although some laboratory exposure studies have shown developmental abnormalities in Pacific Northwest salmon species exposed to PAHs (Ostrander et al., 1988, 1989). Also, the effects of PAHs on early development were investigated extensively in salmon and other fish after the 1989 Exxon Valdez oil spill in Prince William Sound, Alaska. Following the Exxon Valdez spill, field and laboratory studies in several species, including Pacific herring (Clupea pallasi) and pink salmon (Oncorhynchus gorbuscha), demonstrated a common syndrome of oil-induced embryolarval toxicity characterized by pericardial and yolk sac edema, jaw reductions, and curvature of the body axis (Carls et al., 1999; Couillard, 2002; Heintz et al., 1999; Marty et al., 1997; Pollino and Holdway, 2002), generally resulting in embryo death. Delayed mortality also occurred in fish with no external malformations, as indicated by the reduced oceanic survival of pink salmon exposed to weathered crude oil as embryos and released as smolts (Heintz et al., 2000). Although the mechanisms leading to PAH-associated malformations and sublethal effects during fish development were not fully understood, it was assumed that toxicity was mediated largely through PAH interactions with the aryl hydrocarbon receptor (AhR) (Nebert et al., 2004; Schmidt and Bradfield, 1996), producing a syndrome similar to that associated with exposure to other potent AhR ligands such as dioxin (Peterson et al., 1993). More recently, Incardona et al. (2004, 2005) have found that, although the dioxin-like syndrome may occur with exposure to certain high-molecular-weight PAHs such as pyrene, exposure to the lower molecular weight tricyclic PAHs that are the most common components of weathered crude oil results in an alternative toxic response, cardiotoxicity, which is AhR independent. This cardiovascular dysfunction appears to be a major cause of deformities associated with exposure to petrogenic PAHs such as those in weathered crude oil. These studies raise the possibility that lower exposure concentrations, within the range often found in the environment, may cause subtle cardiovascular effects in fish that otherwise appear normal. This might explain, for example, the reduced marine survival of pink salmon exposed as embryos to lower levels of weathered Alaska North Slope crude oil from the Exxon Valdez spill (Heintz et al., 2000). Similar deformities could also be affecting survival of Pacific herring and Pacific Northwest salmon in Puget Sound, but this has not yet been confirmed with field studies. This work highlights the importance of sublethal, potentially long-term effects of PAHs that enter the environment through oil spills, as well as other sources (Peterson et al., 2003).

**Immunological Alterations**

Several studies conducted over the last ten years suggest that fish from PAH-contaminated sites in Puget Sound may have reduced immune function and increased susceptibility to infectious disease. To date, the most extensive research on the effects of PAHs on immune function has been conducted with juvenile salmon. As discussed earlier, juvenile Chinook salmon from polluted Waterways in Puget Sound, such as the Duwamish and Hylebos Waterways, show exposure to PAHs as demonstrated by elevated concentrations of PAH metabolites in bile, increased levels of DNA adducts in liver, elevated PAH concentrations in stomach contents, and induction of hepatic CYP1A activity (McCain et al., 1990; Stehr et al., 2000; Stein et al., 1995). In a series of field and laboratory studies, Arkoosh and co-workers (Arkoosh and Collier, 2002; Arkoosh et al., 1991, 1994, 1998, 2001) demonstrated that the immunocompetence
of juvenile salmon from the Duwamish Waterway was suppressed when compared to salmon from a nonurban estuary or hatcheries (Figure 22.13). Leucocytes of salmon from the urban estuary were unable to generate a secondary (or memory) \textit{in vitro} B-cell immune response following exposure to either the T-cell-independent antigen trinitrophenylated lipopolysaccharide (TNP-LPS) or the T-cell-dependent antigen TNP-keyhole limpet hemocyanin (TNP-KLH), suggesting that PAH exposure suppresses immunological memory (Arkoosh et al., 1991).

\textbf{FIGURE 22.13} The mean number ($\pm$ sd) of primary and secondary plaque forming cells/culture to an antigen for juvenile Chinook salmon from the releasing hatcheries, a nonurban estuary and an urban estuary. (Adapted from Arkoosh, M.R. et al., \textit{Fish Shellfish Immunol.}, 1, 261–277, 1991.)

\textbf{FIGURE 22.14} Percent cumulative mortality of juvenile Chinook salmon from an urban and nonurban estuary and their corresponding hatcheries four days after exposure to the marine pathogen \textit{V. anguillarum}. (Adapted from Arkoosh, M.R. et al., \textit{Trans. Am. Fish. Soc.}, 127, 360–374, 1998.)
The Effects of Polycyclic Aromatic Hydrocarbons in Fish from Puget Sound, Washington

895

To determine if salmon from a contaminated environment are also more susceptible to an infectious disease, we collected juvenile fall-run Chinook salmon from an urban estuary and from a nonurban estuary and the respective releasing hatcheries upstream from these estuaries, and we exposed them in the laboratory to the marine bacterial pathogen *Listonella anguillarum*. We found that juvenile Chinook salmon from the contaminated estuary were more susceptible to *L. anguillarum*-induced mortality than were fish from the corresponding hatchery upstream from the estuary, which were not exposed to contaminants. In contrast, juvenile fall Chinook salmon from a nonurban estuary showed no increase in susceptibility to *L. anguillarum*-induced mortality compared to fish from the corresponding hatchery (Figure 22.14). These disease challenge studies indicated that juvenile Chinook salmon with contaminant-associated immunodysfunction were also more susceptible to one of their natural pathogens (Arkoosh et al., 1998). Follow-up laboratory exposure studies with sediment extracts and contaminant model mixtures determined that contaminants, apart from other estuarine variables specifically associated with the Duwamish and Hylebos Waterways, could independently suppress immune function and increase disease susceptibility in juvenile Chinook salmon (Arkoosh et al., 1994, 2001). Because these fish were exposed to mixtures of contaminants, there was some uncertainty regarding the relative contributions of PAHs and other chemicals present in sediment (e.g., PCBs) to the observed reductions in disease resistance. In a recent disease challenge study with *L. anguillarum*, Palm et al. (2003) found little indication of reduced disease resistance in juvenile Chinook salmon exposed to dietary PAHs at environmentally relevant concentrations; however, these studies were conducted in freshwater, in spite of the fact that *L. anguillarum* is a saltwater pathogen, so the results may not reflect the virulence of *L. anguillarum* in the natural environment.

To better characterize the effects of PAHs on immunocompetence, we exposed juvenile rainbow trout (*Oncorhynchus mykiss*) to PAHs in their diet at environmentally relevant concentrations, similar to those found in stomach contents of Chinook salmon (*Oncorhynchus tshawytscha*) from contaminated estuaries in Puget Sound (Arkoosh et al., 1998), and examined changes in disease resistance and expression of immune-regulating genes (Bravo, 2005). Like salmon in earlier experiments, these fish exhibited higher susceptibility to the pathogen, *Aeromonas salmonicida*, than fish fed the control diet. When we profiled gene expression in head kidney using microarrays, we found that over 20 immunologically relevant genes were differentially expressed after pathogen challenge. Transcripts from five immune genes—interleukin 8 (IL-8), transport associated protein 1 (TAP1), NF-κB essential modulator (NEMO), recombination activating gene 2 (RAG 2), and a major histocompatibility complex II (MHC II) gene—were also measured by RT-PCR at the time points examined by microarray. These genes participate in innate and adaptive immunity and some are key regulators of immune response such as NF-κB modulator (Mann et al., 2001). Moreover, they have been previously been described as important components of resistance of salmon to *A. salmonicida* (Gerwick et al., 2002; Vanya et al., 2005). All selected genes were significantly down regulated by 2 days post-challenge, suggesting that PAH exposure decreases transcription of genes involved in the immune response.

Evidence also suggests altered immune function in English sole exposed to PAHs in the field, PAH-contaminated sediment, or PAH-contaminated sediment extracts from Eagle Harbor (Arkoosh et al., 1996; Clemons et al., 2000) (Figure 22.15 and Figure 22.16). Eagle Harbor, the site of a former creosote plant, is located at Bainbridge Island in Puget Sound and has sediments characterized by high levels of PAHs. In sole exposed to PAHs, the leukoproliferative (mitogenic) response and macrophage production of cytotoxic reactive oxygen intermediates were augmented. In other species, increased production of reactive oxygen species by macrophages has been associated with an increase in peroxidative damage of kidney and gill tissues (Bravo, 2005; Fatima et al., 2000), but the effects of augmented reactive oxygen intermediate production and leukoproliferative response on English sole’s immune function are unknown. Studies are currently underway to better establish the linkage between these changes and disease susceptibility in English sole.

**Growth**

Studies over several years with juvenile Chinook salmon from the Duwamish and Hylebos Waterways suggest that exposure to PAHs may suppress growth in this species (Casillas et al., 1995, 1998a). Growth was monitored in juvenile salmon collected from these sites and held in the laboratory for 90 days, and it was found that growth rates for the fish from urban estuaries were lower than those for fish from the
corresponding hatcheries or from nonurban estuaries. Furthermore, concentrations of plasma hormones involved in the regulation of growth in fish, such as thyroxine (T₄), triiodothyronine (T₃), and insulin-like growth factor (IGF), were altered in salmon from urban estuaries in comparison with hormone levels in hatchery or non-urban fish (Casillas et al., 1995, 1998a, unpublished data). These findings suggest that exposure to PAHs and other organic contaminants may interfere with the endocrine modulation of growth in juvenile salmon, resulting in impaired overall growth.

Laboratory exposure experiments with model compounds and sediment extracts from contaminated Puget Sound sites also indicate that exposure to PAHs may suppress growth or alter the metabolism of
juvenile salmon (Casillas et al., 1998a,b; Meador et al., 2006). There is some uncertainty in these and other studies regarding the concentrations of PAHs required to suppress growth of juvenile salmon if fish are exposed to PAHs alone. In studies by Casillas et al. (1998a,b), fish exposed to PAHs alone at concentrations comparable to those present in the Hylebos Waterway did not exhibit consistent reductions in growth in all treatment groups, although growth was reduced consistently in fish exposed to sediment extracts containing PAHs in combination with PCBs and other contaminants. In a feeding study, Palm et al. (2003) found no effects on the growth of juvenile Chinook salmon exposed to PAHs for 28 days at concentrations as high as 252 mg/kg dry wt. (or approximately 50 mg/kg wet wt.) feed; however, the lipid content of the diet was substantially higher than would be expected in a natural diet, and this may have made the detection of alterations in growth rate more difficult. More recently, Meador et al. (2006) conducted a dietary feeding study in which juvenile Chinook salmon were dosed with PAH at concentrations in feed ranging from 1 to 1171 mg/kg dry wt. (or approximately 0.2 to 234 mg/kg wet wt.), a range encompassing PAH concentrations measured in stomach contents of juvenile salmon from Pacific Northwest estuaries (Johnson et al., 2007; Stehr et al., 2000; Stein et al., 1995; Varanasi et al., 1993). Significant differences in mean fish weight did not appear except in fish at the two highest doses, but at the lowest dose (38 mg/kg dry wt.) variability in fish weight increased significantly. Significant changes were also observed in plasma chemistry and fatty acid profiles of the fish at doses in the range of 122 to 324 mg/kg dry wt.

These studies indicate effects of PAHs on fish growth and energy balance but also suggest that other compounds present in contaminated Puget Sound estuaries, such PCBs, are contributing significantly to growth reductions that have been observed in field collected fish; however, more work is needed to determine the relative importance of various compounds in generating this effect. The stage of development at which fish are exposed to PAHs may also be important in determining effects, as more definitive reductions in growth have been observed in pink salmon exposed to low levels of crude-oil associated PAHs during embryonic development, possibly as a result of impaired cardiac function (Heintz et al., 2000; Incardona et al., 2004, 2005).

English sole growth also appears to be affected by exposure to PAHs. Data from two laboratory studies (Kubin, 1997; Rice et al., 2000) show reduced growth in juvenile English sole exposed to PAHs through contaminated sediments or diet. Kubin (1997; see also Johnson et al., 1998, 2002) exposed juvenile English sole to sediments contaminated with PAHs at concentrations of approximately 5 mg/kg, 2.5 mg/kg, and <5 μg/kg dry wt. for 6 months.* For the first 3 months, growth rates were similar for all treatments (1.0 to 1.1% per day for weight and 0.36 to 0.38% per day for length), but during the next 3 months growth rates were significantly lower in the high-exposure group. The percent change in weight was 0.35% per day in fish exposed to 5 mg/kg of PAHs, as compared to 0.43% in control fish; the percent change in length was 0.13% per day in exposed fish as compared to 0.16% per day in control fish. The fish exposed to sediments with total PAH concentrations of 2.5 mg/kg dry wt. showed no significant decrease in growth rate relative to control fish. Actual threshold effect concentrations could be lower, however, because uptake of PAHs was from sediment and water only, and in the natural environment substantial exposure would also occur through the diet via ingestion of invertebrate prey species residing in contaminated sediment.

A study by Rice et al. (2000) confirmed both the effect of PAHs on growth of juvenile English sole and the importance of dietary exposure. The findings showed significantly reduced weight in juvenile English sole fed polychaete worms reared on sediments containing 3 to 4 mg/kg dry wt. PAH after an exposure period of only 28 days. The percent change in weight was markedly less (0.05 to 0.1% per day) in exposed fish, as compared to control fish (1.1 to 1.2% per day). These effects have not yet been corroborated in wild populations of English sole, but if they do occur they could impact sole populations by reducing fecundity or altering the time required to reach sexual maturity (Brandt et al., 1992). Slow growth rates have also been associated with increased juvenile mortality in several fish species (McGurk, 1996).

* Sediment PAH concentrations for this study were determined using the HPLC/PDA screening system of Krahn et al. (1991), which provides an estimate of PAH concentration by measurement of fluorescent aromatic compounds (FACs) in sediments. To validate the screening method, total PAH concentrations in sediment samples from a variety of urban and non-urban sites along the U.S. west coast were analyzed using both HPLC/fluorescence and GC–MS (Krahn et al., 1988).
Ecological risk assessment and characterization are critical components of our research program on the effects of PAHs on marine and estuarine fish. Suter (1993) defined risk assessment as “the process of assigning magnitudes and probabilities to adverse effects of human activities or natural catastrophes.” This process involves identifying hazards, such as releases of toxic chemicals to surface waters that support fisheries, and uses measurement, testing, and mathematical or statistical models to quantify the relationship between perceived hazards and subsequent adverse health effects. The standard paradigm for ecological risk assessment (NRC, 1983) includes four major steps: (1) hazard definition, which involves the choice of endpoints for the assessment (e.g., mortality, cancer risk, reductions in fecundity) and the target species of interest, environmental description, and source terms; (2) exposure assessment and effects assessment; (3) risk characterization; and (4) risk management.

For the case study illustrated in this chapter, PAHs in the marine environment are the hazard of interest, the primary target species is English sole, and exposure and effects assessment data have already been presented for several endpoints, including hepatic lesions, reproductive dysfunction, and growth. This section on risk characterization describes our efforts to use these data to estimate the types of adverse health effects that would be expected to result from a given environmental exposure to PAHs. Uncertainties in the analysis are discussed, but risk management options are not examined in detail.

The analytical methods we used in the effects assessment and risk characterization steps are primarily logistic regression techniques. These methods calculate the risk of an adverse health effect (e.g., the risk of cancer) associated with a given PAH exposure in an individual fish or fish subpopulation (e.g., fish collected from a particular sampling site). We have used this approach in several studies with English sole and other Pacific Coast bottomfish to statistically relate lesion prevalences to biological risk factors and measures of contaminant exposure (Johnson et al., 1993; Myers et al., 1994; Stehr et al., 1997, 1998, 2004). Through stepwise logistic regression techniques, it is possible to determine the influence of contaminant exposure-related risk factors on hepatic disease risk while simultaneously accounting for the influence of biological risk factors such as age and sex. This technique is commonly used on binomial or proportional data in epidemiological and epizootiological studies (Breslow and Day, 1980).

### TABLE 22.1
Associations between PAH Concentrations in Sediments, Stomach Contents, and Bile with Site-Specific Prevalences of Selected Categories of Idiopathic Liver Lesions in English Sole, as Determined by Logistic Regression (p < 0.05)a

<table>
<thead>
<tr>
<th>Chemical Class/Compartment</th>
<th>n</th>
<th>Neoplasms</th>
<th>FCA</th>
<th>SDN</th>
<th>Prolif</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΣLAHs Sediment</td>
<td>22</td>
<td>0.020/54</td>
<td>0.001/52</td>
<td>0.001/45</td>
<td>0.001/68</td>
<td>NS</td>
</tr>
<tr>
<td>Stomach contents</td>
<td>9</td>
<td>NS</td>
<td>0.001/65</td>
<td>0.001/75</td>
<td>0.001/73</td>
<td>NS</td>
</tr>
<tr>
<td>Bile (FACs-L)</td>
<td>20</td>
<td>0.032/15</td>
<td>0.001/26</td>
<td>0.001/35</td>
<td>0.002/21</td>
<td>NS</td>
</tr>
<tr>
<td>ΣHAHs Sediment</td>
<td>22</td>
<td>0.004/48</td>
<td>0.001/40</td>
<td>0.001/38</td>
<td>0.003/35</td>
<td>NS</td>
</tr>
<tr>
<td>Stomach contents</td>
<td>9</td>
<td>NS</td>
<td>0.001/65</td>
<td>0.001/75</td>
<td>0.001/73</td>
<td>NS</td>
</tr>
<tr>
<td>Bile (FACs-L)</td>
<td>20</td>
<td>NS</td>
<td>0.01/13</td>
<td>0.001/22</td>
<td>0.001/22</td>
<td>NS</td>
</tr>
</tbody>
</table>

a Analyses were performed while adjusting for mean age and sex ratio (female:male). Table indicates p-value, and percent of total variance in lesions prevalence explained by risk factor (reduction in scaled deviance).

Abbreviations: FACs-H, aromatic compounds fluorescing at benzo(a)pyrene wavelengths; FACs-L, aromatic compounds fluorescing at naphthalene wavelengths; FCA, foci of cellular alteration; HAHs, high-molecular-weight polycyclic aromatic hydrocarbons; LAHs, low-molecular-weight polycyclic aromatic hydrocarbons; n, number of sites; NS, not significant; Prolif, proliferative lesions; SDN, specific degenerative/necrotic lesions.

Analyses of a number of different datasets (Myers et al., 2003) have consistently identified PAH exposure as a significant risk factor for the development of hepatic lesions in English sole, as well as other marine bottomfish species. Measures of exposure utilized in these analyses include sediment PAH concentrations, PAH concentrations in stomach contents, biliary FAC concentrations, hepatic AHH activity, and levels of DNA adducts in liver. Of the hepatic lesions observed in bottomfish sampled in these studies, preneoplastic focal lesions, proliferative lesions, specific degeneration and necrosis, and hydropic vacuolation were most commonly associated with exposure to and uptake and metabolism of PAHs. In these analyses, we determined the significance of the relationships between prevalences of lesions at particular sampling sites and discrete risk factors such as levels of PAHs in sediments and fish tissues, while adjusting for mean fish age and sex ratio, with each sampling event for a species at a site treated as an independent occurrence. Separate analyses for each contaminant class or chemical-associated risk factor (e.g., PAHs in stomach contents, bile FACs) were performed, with results expressed as the proportion of variation in lesion prevalence attributable to significant risk factors. For example, the data in Table 22.1 show that 35% of the variation in site-specific prevalences for specific degeneration and necrosis in English sole can be explained by mean levels of bile FACs in fish from those sampling sites. Similar logistic regression analyses (Anderson et al., 1980; Schleselman, 1982) that examine the risk of lesion occurrence associated with a PAH exposure parameter (e.g., DNA adducts as measure of longer term PAH exposure) in individual fish (while accounting for fish age) are capable of generating data and dose–response curves that estimate the relative risk (as expressed by the odds ratio) of liver lesion occurrence attributable to that PAH-exposure parameter. As an example, the odds of an English sole from Eagle Harbor exhibiting specific degeneration or necrosis (also called hepatocellular nuclear pleomorphism/hepatic megalocytosis in pathological terminology) or preneoplastic focal lesions increase by 1.05 and 1.03 times, respectively, for each unit increase in hepatic DNA adduct level (Figure 22.17).

More recent studies have focused on linking adverse fish health effects with sediment PAH concentrations to supply risk characterization information in a form that is more directly applicable to the development of sediment quality guidelines. By applying segmented regression techniques to data from field surveys conducted over the past 15 years in Puget Sound and the west coast of the United States, we estimated the threshold sediment concentrations of PAHs at which increased toxicopathic liver disease and levels of PAH–DNA adducts are initially observed (Horness et al., 1998; Johnson et al., 2002).
Hockey-stick regression, the specific model applied for these analyses, is one of a number of standard dose–response models (Gad and Weil, 1991), and has been used in a variety of epidemiological and toxicological studies (Cox et al., 1989; Gordon and Fogelson, 1993; Hammer et al., 1974). The model consists of two linear segments whose blade-and-handle shape resembles a hockey stick (Yanagimoto and Yamamoto, 1979). The lower segment is assigned a slope of zero to represent a constant low-level background effect. The upper segment is defined as a linear function with a positive slope that represents a dose–response relationship above a threshold that is estimated by the point of intersection of the two segments. An advantage of this approach is that quantitative assessments of uncertainty are provided in the form of confidence intervals for the threshold values.

Many risk analysis models used in epidemiology typically assume that DNA damage and cancer induction are non-threshold phenomenon, and our choice of a threshold model for this exercise is not meant to imply that a true threshold exists in the process of carcinogenesis in English sole. Rather, the model was chosen for pragmatic reasons, to facilitate our identification of exposure levels at which statistically detectable and biologically relevant increases in the endpoints would be expected to occur in wild fish populations. The application of a threshold model is supported by the fact that, for most carcinogens and mutagens, repair processes and compensatory mechanisms exist that can counteract their effects at low levels of exposure, even though one molecule of a carcinogen could theoretically induce an initiated cell, leaving no latitude for a threshold. Based on a similar rationale, the application of a threshold approach for regulating exposure to some carcinogens has been suggested for human health risk management (Butterworth and Bogdanffy, 1999; Gaylor et al., 1999; Lutz, 1998).

We have used this model to relate sediment PAH concentrations to PAH–DNA adducts levels and to prevalences of the four most common toxicopathic hepatic lesion types found in English sole: neoplasms; preneoplastic foci of cellular alteration (FCA), which are thought to be precursors of neoplasms; specific degeneration/necrosis (SDN), a degenerative lesion manifesting cytotoxicity associated with exposure to PAHs; and non-neoplastic proliferative lesions, such as hyperplasia of bile ducts (Figure 22.18 and Figure 22.19) (Johnson et al., 2002). Threshold sediment PAH concentrations for toxicopathic liver lesions in English sole ranged from 54 to 2800 ng/g dry wt. For DNA adducts, the threshold effect estimate was 290 ng/g dry wt., with a 90% confidence interval of 6 to 1380 ng/g dry wt. A threshold in this range is supported by a laboratory study (French et al., 1996) in which exposure to sediments contaminated with 1200 ng/g dry wt. PAH resulted in DNA adduct concentrations in English sole liver of 15 to 20 adducts per mol nucleotides, in comparison with 5 to 6 adducts per mol nucleotides in fish exposed to sediments containing 20 ng/g dry wt. PAH.
Although the data are insufficient to statistically determine precise thresholds for other effects in English sole, the available information suggests that impacts on growth and reproduction begin to occur at sediment PAH concentration in a similar range; for example, in Figure 22.20 the prevalences of several

![Figure 22.19](image)

**FIGURE 22.19** Hockey-stick regressions of hepatic lesion prevalence in English sole vs. total polycyclic aromatic hydrocarbons (PAHs) in bottom sediment in ng/g dry wt (ppb) for (A) neoplasms (Neo); (B) foci of cellular alteration (FCA); (C) specific degenerative/necrosis (SDN) lesions; (D) proliferative (Prolif) lesions; and (E) Neo, FCA, or SDN (any lesion). Threshold concentrations are indicated by arrows. Shaded gray bar represents the 90% confidence interval. No lower confidence limit was found for the FCA threshold estimate. \( n = 29. \) (From Horness, B. et al., *Environ. Toxicol. Chem.*, 17, 162–172, 1998. With permission.)
types of reproductive impairment observed in adult female English sole (Casillas et al., 1991; Johnson et al., 1988, 1999) are plotted against sediment PAH concentrations at the sites where fish were collected in Puget Sound. The plots indicate that spawning failure and egg infertility begin to increase above background levels at sediment PAH concentrations between 250 and 1600 ng/g, while the proportion of sole failing to undergo ovarian maturation begins to increase at concentrations between 1600 and 10,000 ng/g. If we estimate the effect thresholds by taking the geometric mean of these two points, this yields an effect threshold of 4000 ng/g for inhibition of ovarian development and a threshold of 630 ng/g for all other reproductive endpoints. Exposure–response relationships for reproductive endpoints at sediment PAH concentrations above the threshold levels were examined by fitting the upper segment of the hockey-stick regression, substituting estimated threshold and background effect levels into the model. The proportion of sole that failed to mature, the proportion that failed to spawn, and the proportion of eggs spawned that were infertile were all significantly correlated with increasing sediment PAH concentration. Although more data would be needed to calculate sediment threshold concentrations and confidence intervals for the reproductive endpoints, the results suggest that these types of reproductive impairment are found at sediment PAH concentrations similar to those associated with the development of hepatic lesions. Currently, we do not have sufficient data on growth rates of field-collected English sole to apply the hockey-stick regression approach to this endpoint; however, preliminary data from laboratory growth studies with English sole (Kubin, 1997; Rice et al., 2000) suggest that significant effects on fish growth are first observed at sediment PAH concentrations between 2000 and 4000 ng/g, consistent with exposure levels associated with liver disease and reproductive impairment.

To estimate the level of effects experienced by English sole exposed to sediments with different sediment PAH concentrations, expected prevalences of liver lesions and reproductive abnormalities were calculated using the regression equations from the hockey-stick analyses for \( \Sigma PAH \) concentrations.
ranging from 50 - 100,000 ng/g. As illustrated in Table 2, liver lesion prevalences, as well as levels of other detrimental effects in English sole, were generally close to levels characteristic of fish from uncontaminated sites at sediment PAH concentrations below 1000 ng/g. At higher concentrations, the proportion of animals affected and the number of adverse effects observed increases. The degree of increase is modeled by the upper arm of the hockey-stick regression, which can be used to estimate the probability of effects to English sole at various sediment PAH concentrations (Table 22.2). At 5000 ng/g, for example, levels of hepatic DNA adducts would be approximately 10-fold the levels found in fish from uncontaminated reference sites, about 30% of the population is predicted to have some form of toxicopathic liver disease, and the number of fish failing to spawn would increase from about 12% to over 35%. At PAH concentrations of 10,000 ng/g, DNA adducts levels would have increased by 12- to 13-fold, 50% of the sole would be expected to have liver disease, nearly 30% of the females would show inhibition of gonadal growth, and over 40% would show inhibition of spawning. This type of information, in combination with data on contaminant effects on other indigenous species, can be used in estimating the loss of productivity or ecosystem services due to PAH contamination at impacted sites.

Although the current analysis provides guidance on approximate sediment PAH concentrations associated with injury in English sole, some variables that could influence exposure–response relationships are not fully accounted for in the current treatment of the data; for example, the current analysis adjusts

**TABLE 22.2**
Estimated Effect Levels Associated with Increasing Sediment PAH Concentration for Selected Liver Lesions and Indicators of Reproductive Function in English Sole

<table>
<thead>
<tr>
<th>PAH (ppb dry wt.)</th>
<th>Neoplasm (Prevalence)</th>
<th>FCA (Prevalence)</th>
<th>SDN (Prevalence)</th>
<th>Proliferative Lesion (Prevalence)</th>
<th>Any Lesion (Prevalence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>100</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>1000</td>
<td>0.00</td>
<td>0.06</td>
<td>0.01</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>2000</td>
<td>0.00</td>
<td>0.07</td>
<td>0.12</td>
<td>0.11</td>
<td>0.18</td>
</tr>
<tr>
<td>3000</td>
<td>0.01</td>
<td>0.08</td>
<td>0.20</td>
<td>0.13</td>
<td>0.24</td>
</tr>
<tr>
<td>5000</td>
<td>0.03</td>
<td>0.09</td>
<td>0.27</td>
<td>0.14</td>
<td>0.31</td>
</tr>
<tr>
<td>10000</td>
<td>0.06</td>
<td>0.10</td>
<td>0.38</td>
<td>0.17</td>
<td>0.40</td>
</tr>
<tr>
<td>100000</td>
<td>0.16</td>
<td>0.14</td>
<td>0.75</td>
<td>0.26</td>
<td>0.71</td>
</tr>
</tbody>
</table>

**Reproductive Indicators**

<table>
<thead>
<tr>
<th>PAH (ppb dry wt.)</th>
<th>Inhibited Gonadal Development (Prevalence)</th>
<th>Inhibited Spawning (Prevalence)</th>
<th>Infertile Eggs (Proportion of Eggs Spawned)</th>
<th>DNA Damage (Adducts per mol Nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.15</td>
<td>0.12</td>
<td>0.38</td>
<td>5</td>
</tr>
<tr>
<td>100</td>
<td>0.15</td>
<td>0.12</td>
<td>0.38</td>
<td>5</td>
</tr>
<tr>
<td>1000</td>
<td>0.15</td>
<td>0.17</td>
<td>0.42</td>
<td>25</td>
</tr>
<tr>
<td>2000</td>
<td>0.15</td>
<td>0.25</td>
<td>0.48</td>
<td>36</td>
</tr>
<tr>
<td>3000</td>
<td>0.15</td>
<td>0.30</td>
<td>0.51</td>
<td>43</td>
</tr>
<tr>
<td>5000</td>
<td>0.18</td>
<td>0.35</td>
<td>0.55</td>
<td>51</td>
</tr>
<tr>
<td>10000</td>
<td>0.27</td>
<td>0.43</td>
<td>0.61</td>
<td>63</td>
</tr>
<tr>
<td>100000</td>
<td>0.58</td>
<td>0.69</td>
<td>0.80</td>
<td>100</td>
</tr>
</tbody>
</table>

**Note:** For all liver lesions, inhibited gonadal development, and inhibited spawning in English sole, the effect level is the proportion of fish estimated to be affected at the indicated sediment PAH concentration; for infertile eggs, the effect level is the proportion of eggs produced by an individual female that are estimated to be unfertile. Effect levels for liver lesions were calculated with hockey-stick regression. For reproductive indicators, effect levels at the sampling sites where PAH concentrations were lowest were used to estimate background effect levels (i.e., effect levels at PAH concentrations below 5000 ppb for inhibited gonadal development and below 1000 ppb for inhibited spawning and infertile eggs).

**Source:** Adapted from Johnson, L.L. et al., *Aquat. Conserv.*, 12, 517–538, 2002.
for fish age only in a very basic manner by excluding very young fish and sites where the majority of fish collected were subadults. Also, the thresholds are based on data from urban sites contaminated with a combination of petrogenic and pyrogenic PAHs, and their applicability to estuarine environments where the suite of PAHs present differs substantially from those typically present at industrialized urban sites is not known. Another factor that is not accounted for is the presence of other contaminants along with PAHs at sites where English sole were collected. Although the correlations between PAH exposure and the endpoints we measured in this study are statistically valid and well supported by other scientific evidence, sediments at the sites included in the analysis contain a variety of other compounds that are promoters of carcinogenesis or are reproductive toxicants. These compounds could act either additively or synergistically with PAHs to produce the observed health impacts. Their presence could alter disease prevalence, and they are likely an important factor contributing to variability in response among fish populations at different sampling sites. The potential of interactive effects among co-occurring compounds is an area that warrants additional research because it does introduce uncertainty in the estimation of sediment threshold values.

Overall, these analyses suggest that several important health effects, including selected degenerative liver lesions, spawning inhibition, and reduced egg viability, can be observed in English sole residing at sites where PAH concentrations are approximately 1 to 2 mg/kg and above. Moreover, the proportion of animals affected and the number of adverse effects observed steadily increase as sediment PAH concentrations increase. Additionally, the data suggest that liver lesions could be used as a surrogate to indicate that resident fish are at risk for additional adverse impacts, such as impaired reproduction and growth. With further development of such data, including testing with fish species other than English sole, these relationships could be used to help assess the likely degree of injury to marine resources at various sediment PAH concentrations.

Risk Management

The information generated by our laboratory on the risks posed to marine fishes by exposure to PAHs is being used in environmental management decisions in a variety of settings. One of the most notable has been our involvement in NOAA’s Natural Resources Damage Assessment (NRDA) process. Under the Clean Water Act and the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), U.S. federal or state officials, acting as trustees for natural resources, can seek compensation from responsible parties for damage caused by releases of toxic materials. Such cases require that a damage assessment be performed to determine what injuries need to be remediated and what compensation is necessary. NOAA, in conjunction with other trustees of marine resources, has pursued damage assessment cases at several sites in Puget Sound, including Elliott Bay in Seattle and Commencement Bay in Tacoma, using PAH-associated health effects on fish as evidence of resource damage. We have also been involved in monitoring efforts to assess the effectiveness of cleanup and restoration activities at Superfund sites in Puget Sound, such as Eagle Harbor (Myers et al., 1995, 2000, 2003, 2005). The following summarizes the salient aspects of a few representative cases.

Puget Sound Damage Assessment Cases

Elliott Bay

In 1990, the United States filed suit against the City of Seattle and Metro (now the King County Department of Metropolitan Services) to recover damages for alleged injuries to natural resources caused by the release of hazardous metals and organic chemicals from the City and Metro combined sewer overflows and storm drains discharging into Elliott Bay and the lower Duwamish River. The parties negotiated a cooperative agreement to work together to restore the natural resources in Elliott Bay and the lower Duwamish River. Under this agreement, the City and Metro provided approximately $24 million in funding, real estate, and in-kind services for sediment remediation, habitat restoration, and source control efforts in Elliott Bay. The information on PAH exposure and associated health effects in
benthic fish in Elliott Bay and the Duwamish, reviewed in the previous section, played an important role in the damage assessment process, because these data established that there had been releases of a series of hazardous substances into Elliott Bay and that public-trust natural resources were injured by the releases. The long-term nature of these studies, the thorough documentation of PAH exposure and biological injury, the consistency of association between PAH exposure and detrimental health effects, and the cause-and-effect relationships established in the laboratory for PAHs and endpoints measured in the field all contributed to the strength of utility of these data.

Commencement Bay

In 1991, the Commencement Bay Natural Resource Trustees (NOAA, U.S. Fish and Wildlife Service, Bureau of Indian Affairs, Washington Department of Ecology, Washington Department of Fish and Wildlife, Washington Department of Natural Resources, and Puyallup and Muckleshoot Indian Tribes) initiated a NRDA process in Commencement Bay, the harbor for Tacoma in southern Puget Sound. A wide range of industrial and commercial concerns, including pulp and lumber mills, marinas, chemical manufacturing plants, and facilities involved in concrete production, oil refining, and food processing, are located along the waterways connected to the Bay. The release of hazardous substances from industries along the waterways and contamination of bottom sediments in the Bay and its waterways posed a potential hazard to marine fish and salmonids that used these areas as rearing and feeding habitat. In fact, because of high contaminant levels in the area, part of Commencement Bay was designated a Superfund site in 1983.

As part of the Commencement Bay Natural Resource Damage Assessment, the Trustees authorized a series of resource injury assessment studies in fish from the Hylebos Waterway, the most heavily contaminated portion of the Commencement Bay site. Fish injury studies were modeled largely on our earlier research at other contaminated sites in Puget Sound, such as the Duwamish Waterway and Eagle Harbor, and included assessments of biological markers of contaminant exposure and injury in juvenile Chinook and chum salmon, toxicopathic conditions in flatfish, and contaminant-induced reproductive dysfunction in English sole (Collier et al., 1998a,b). These investigations demonstrated that flatfish from the Hylebos Waterway exhibited toxicopathic liver disease and reproductive abnormalities similar to those reported from the same species from comparably contaminated sites elsewhere in Puget Sound (Collier et al., 1998a,b; Johnson et al., 1999). They also established exposure to and uptake of PAHs in juvenile Chinook and chum salmon from the Hylebos Waterway (Stehr et al., 2000).

A second set of investigations was conducted to better characterize the uptake and biochemical responses of juvenile Chinook salmon to several classes of contaminants present in the Hylebos Waterway and to determine the effects of these contaminants on growth rate and disease resistance (Arkoosh et al., 2000; Casillas et al., 1998a,b). These studies established that injection of fish with extracts of Hylebos sediment or specific classes of compounds present in Hylebos sediment (e.g., PAHs, PCBs, and hexachlorobutadiene) led to statistically significant reductions in growth and increased mortality in disease challenge experiments. More recently, dietary exposure studies (Bravo, 2005; Meador et al., 2006) have been conducted with juvenile Chinook salmon to better characterize dose–response relationships between contaminants present in the Hylebos and specific injuries and exposure biomarkers in a more realistic exposure regimen.

As the damage assessment has progressed, the Trustees have entered into partial or full settlements of claims with several parties. These settlements provided funds, property, and in-kind services for restoration projects, and restoration activities have been carried out at a number of nearshore and intertidal sites in Commencement Bay (CBNRT, 2001, 2002). In developing these projects, the Trustees had to determine whether and to what extent it was necessary to remediate existing contamination to ensure an acceptable likelihood of success for the proposed restoration project. Washington State sediment management standards (Washington State Department of Ecology, 1995) provided some guidelines for sediment remediation, but these were based primarily on a suite of bioassays with benthic invertebrates, so were not directly applicable to salmonids or bottomfish. Indeed, under the sediment management standards, the sediment quality criteria for PAHs, which are considered to be the concentrations that will result in no adverse acute or chronic health effects in either biological resources or humans, were
set at a level substantially above the sediment PAH thresholds concentrations we associated with onset of liver disease and other adverse health effects in English sole: ~1 to 5 mg/kg dry wt. total PAH or, for sediment with the 2% organic carbon (OC) content typical of Puget Sound sediments from urban embayments, 50 to 250 mg total PAH per kg OC (Johnson et al., 2002). In contrast, the sediment quality criteria for LAHs* and HAHs† were 370 mg/kg OC and 960 mg/kg OC, respectively. The minimum cleanup levels, which are considered allowable within zones where sediments have been impacted from existing or past discharges, were even higher: 780 and 5300 mg/kg OC (Washington State Department of Ecology, 1995). This discrepancy raised some questions as to whether current standards were adequately protective of marine and estuarine fish.

Concern for the safety of marine resources in Commencement Bay prompted the Trustees to recommended more stringent standards for sediment remediation than those in state regulations. Based on the information generated by studies on sediment PAH concentrations associated with liver disease and reproductive impairment in English sole, they proposed a sediment cleanup goal of 2 mg/kg dry wt. total PAHs (or 100 mg total PAH per kg organic carbon) as the default sediment cleanup goal for active NRDA restoration projects in Commencement Bay. The application of this goal is currently restricted to proposed sites for restoration projects within Commencement Bay, but it poses a challenge to current sediment quality standards for PAHs and sediment quality assessment methods in a wide range of projects and situations. The issue is likely to become increasingly important in the future, with the listing under the Endangered Species Act of Chinook salmon that utilize urban waterways during their migration from fresh to saltwater. Under the Endangered Species Act, adverse health effects to individuals are considered as harm to listed species, as these types of injury could impair the ability of threatened or endangered populations to recover.

Beginning in 2002, the Trustees authorized a set of monitoring studies to determine whether juvenile salmonids and other estuarine fish were utilizing the restored sites and to collect data on contaminant concentrations in fish at the restoration sites (CBNRT, 2001; Olson et al., 2006; Ridolfi and Adolfson, 2003). Target species for contaminant monitoring were juvenile salmon, particularly juvenile fall Chinook, and a resident fish species, Pacific staghorn sculpin. The sculpin were chosen as the target species in lieu of English sole, because the number of sole present at the restoration sites was very limited.

Fish habitat use monitoring (Olson et al., 2006; Ridolfi and Adolfson, 2003) indicated that fish were present at all of the sites, and several supported significant numbers of juvenile salmonids; however, the study also revealed the presence of PAH contamination in fish bile, fish prey, and sediments from all restoration sites (Olson et al., 2006). Concentrations of PAHs in sediments at most of the restoration sites were comparable to levels observed at Commencement Bay sites used as reference areas for the Hylebos Waterway Damage Assessment sediment evaluation studies (EVS, 1996) and were substantially cleaner than sediments in the heavily industrialized sections of the Hylebos Waterway (EVS, 1996). Total PAH concentrations in sediments from a few of the sites, however, were in the range of 8 to 15 mg/kg dry wt., comparable to concentrations measured at some of the more contaminated sites in the Hylebos Waterway as part of the Damage Assessment Study (Collier et al., 1998a,b; EVS, 1996). Similarly, PAH concentrations in salmon bile and stomach contents were lower than those measured in salmon from the Hylebos Waterway during Damage Assessment at most of the restoration sites (Stehr et al., 2000), but concentrations of PAHs in the stomach contents of juvenile salmon from one site were elevated, and bile metabolite levels were above those typically found in salmon from non-urban sites (Johnson et al., 2007). Like juvenile salmon, staghorn sculpin showed exposure to PAHs, based on the presence of PAC metabolites in bile. Bile metabolite levels in sculpin were lower than those measured in English sole and juvenile salmon collected from the Hylebos Waterway during the Fish Injury Study (Collier et al., 1998a,b; Johnson et al., 1999; Stehr et al., 2000) but still above levels typically found in fish from non-urban sites (Brown et al., 1998).

These studies highlight the importance of prerestoration monitoring for contaminants, especially for projects conducted at urban sites. Although the restoration projects generally provided improved habitat

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* LAHs represent the sum of the following compounds: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene.

† HAHs represent the sum of the following compounds: fluoranthene, pyrene, benz[a]anthracene, chrysene, total benzofluoranthenes, benzo(a)pyrene, indeno(1,2,3-c,d)pyrene, dibenzo(a,h)anthracene, and benzo(g,h,i)perylene.
opportunities for salmon and marine fish within Commencement Bay, in a few cases, enhancements were conducted on habitats that were as contaminated as those that formed the basis of the Hylebos Waterway Damage Assessment case, and existing sediment remediation activities had not yet effectively reduced exposure levels to baseline conditions.

**Restoration Monitoring at PAH-Contaminated Sites**

The presence of PAH-contaminated sediments in marine environments is widely recognized as a threat to both the ecological and economic viability of coastal regions. A variety of approaches are being used to reduce the environmental risks associated with PAH-contaminated sediments, including source control followed by natural recovery, physical removal or containment of sediments, and *in situ* bioremediation. The effectiveness of these techniques in removing or sequestering contaminants is often carefully monitored, but relatively few studies have been conducted to determine whether or not such treatments reduce contaminant exposure and biological effects in resident fishes. Since 1993, we have had the opportunity to conduct such a study at the Wyckoff/Eagle Harbor Superfund site near Bainbridge Island in Puget Sound.

Eagle Harbor is located near Bainbridge Island in Central Puget Sound, Washington. The 500-acre harbor is heavily used by recreational boaters and for ferry transport to and from Seattle. The mouth of the Harbor was the site of the former Wyckoff Company wood-treatment facility, which operated from 1903 to 1988. As a result of the activities at this plant, marine sediments in the eastern portion of Eagle Harbor are heavily contaminated with creosote-derived PAHs. Studies conducted in the 1980s showed that exposure to PAHs and related compounds were associated with liver cancer, related toxicopathic lesions, and reproductive abnormalities in English sole residing in Eagle Harbor (Johnson et al., 1998; Myers et al., 1998c). Further studies expanded the target species to include starry flounder and rock sole and incorporated additional biological markers of PAH exposure and effect, including hepatic CYP1A expression, biliary FACs, and hepatic DNA adducts, measured by 32P-postlabeling. Hepatic lesion prevalences and other biochemical measures in these three species from Eagle Harbor are among the highest found among Puget Sound sites (Myers et al., 1998b,c; Stein et al., 1992).

Because of its high level of sediment contamination and associated injury to marine life, Eagle Harbor/Wyckoff was designated an EPA Superfund site in 1987. Since that time, several major cleanup projects have been carried out at the site. From 1993 to 1994, a sediment cap of sandy fill material was installed in the East Harbor to contain PAH-contaminated sediments, which was amended and expanded upon in 2000 and 2001. Additionally, the Wyckoff facility has been demolished, and groundwater remediation and soil cleanup efforts are underway. Since completion of the sediment cap installation in the East Harbor in 1994, we have monitored PAH exposure and various effect indicators in English sole and other flatfish species in Eagle Harbor to assess their response following sediment remediation.

Even before the cap was put into place, PAH exposure showed some reduction in Eagle Harbor flatfish in comparison to historical values. This decrease was likely the result of source-control measures enacted at the site between 1988 and 1993. Initially, placement of the cap did not have a clear effect on PAH concentrations in English sole stomach contents or in biliary FAC levels in English sole bile. These data suggested that English sole, because of their preference for fine-grained sediment, were occupying the PAH-contaminated areas peripheral to the cap and not using the remediated habitat. With time, however, PAH exposure levels in English sole from Eagle Harbor have declined significantly. Data collected through 2004 following capping in 1993 are showing significant reductions in indicators of PAH exposure (PAH metabolites in bile, PAH–DNA adducts), as well as dramatic and highly significant reductions in prevalences of and relative risks for liver neoplasms and related lesions associated with PAH exposure (Myers et al., 2003, 2005). Stated in terms of estimated relative risk, the risk of lesion occurrence has been reduced by 0.9813 times for each month since the capping process was begun. The decline in risk of lesion occurrence is especially marked from the fourth year since capping was begun (December 1997); since that sampling point, relative risks have been in the vicinity of 0.1 (as compared to 1.0 at the beginning of capping), and age-adjusted relative risks of toxicopathic hepatic lesion occurrence have stayed consistently low (0.05 to 0.36) for the last 8 years. Overall, this study demonstrates that with a good understanding of cause-and-effect relationships between contaminants and biological health effects and consistent long-term monitoring we can show biological responses in resident organisms to contaminant control and remediation measures.
Conclusion

Field and laboratory data on flatfish in Puget Sound indicate that exposure to PAHs is associated with increases in disease and alterations in growth and reproductive function that could potentially reduce the productivity of fish subpopulations residing at contaminated sites. Studies also suggest that even short-term exposure to PAHs may be associated with reduced growth and altered immune function in anadromous fish species that utilize contaminated estuaries in Puget Sound. Similar biological effects have also been observed in fish in urban coastal waters throughout Europe and the United States, suggesting that marine pollution and other forms of habitat degradation may be a widespread threat to fish species, particularly those that rely on nearshore coastal areas for nursery and feeding grounds. The studies with English sole substantiate the value of multidisciplinary studies that combine field and laboratory experiments with quantitative modeling techniques in evaluating the ecological risk to fish stocks of exposure to chemical pollution. The application of such an approach to other fish species should yield much interesting information on species differences in sensitivity and the factors that predispose certain species or stocks to pollution-related impairment, and they improve our ability to protect these stocks from contaminant-related health injury. Although the effects of PAHs on the health of Puget Sound fish have been recognized for over two decades, the problem has not diminished, except in a few urban areas (Seattle Waterfront in Elliott Bay and Thea Foss Waterway in Commencement Bay) where recent data from the Puget Sound Ambient Monitoring Program indicate significant decreases in toxicopathic liver lesion risk since 1998 (PSAT, 2004). Because of changes in demography and urban growth in the Puget Sound Areas, PAHs still pose significant ecological risks to resident bottomfish.

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