

# Significance of cytochrome P450 system responses and levels of bile fluorescent aromatic compounds in marine wildlife following oil spills

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## Abstract

The relationships among cytochrome P450 induction in marine wildlife species, levels of fluorescent aromatic compounds (FAC) in their bile, the chemical composition of the inducing compounds, the significance of the exposure pathway, and any resulting injury, as a consequence of exposure to crude oil following a spill, are reviewed. Fish collected after oil spills often show increases in cytochrome P450 system activity, cytochrome P4501A (CYP1A) and bile fluorescent aromatic compounds (FAC), that are correlated with exposure to polycyclic aromatic hydrocarbons (PAH) in the oil. There is also some evidence for increases in bile FAC and induction of cytochrome P450 in marine birds and mammals after oil spills.

However, when observed, increases in these exposure indicators are transitory and generally decrease to background levels within one year after the exposure. Laboratory studies have shown induction of cytochrome P450 systems occurs after exposure of fish to crude oil in water, sediment or food. Most of the PAH found in crude oil (dominantly 2- and 3-ring PAH) are not strong inducers of cytochrome P450. Exposure to the 4-ring chrysenes or the photooxidized products of the PAH may account for the cytochrome P450 responses in fish collected from oil-spill sites. The contribution of non-spill background PAH, particularly combustion-derived (pyrogenic) PAH, to bile FAC and cytochrome P450 system responses can be confounding and needs to be considered when evaluating oil spill effects. The ubiquity of pyrogenic PAH makes it important to fully characterize all sources of PAH, including PAH from natural resources, e.g. retene, in oil spill studies. In addition, such parameters as species, sex, age, ambient temperature and season need to be taken into account. While increases in fish bile FAC and cytochrome P450 system responses, can together, be sensitive general indicators of PAH exposure after an oil spill, there is little unequivocal evidence to suggest a linkage to higher order biological effects, e.g. toxicity, lesions, reproductive failure.

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## 1. Introduction

This paper reviews the use of bile fluorescent aromatic compound (FAC) analysis and cytochrome P450 system responses in marine wildlife to assess the impact and subsequent recovery of marine ecosystems from a crude oil spill. Both techniques have been and continue

to be used as indicators of exposure to the polycyclic aromatic hydrocarbons (PAH) contained in crude oil. Some studies have attempted to correlate the magnitude of the response to injury of the exposed species. However, there are many confounding factors that influence these exposure indicators. We consider those factors and highlight cautions that should be invoked when interpreting results from field investigations.

The first step in the metabolism of petroleum hydrocarbons by marine vertebrates and invertebrates is

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oxidation, catalyzed by the cytochrome P450 monooxygenase system which, by increasing the hydrophilicity of the hydrocarbons, facilitates their elimination via urine and/or bile. The cytochrome P450 monooxygenase system is a coupled electron transport system in the endoplasmic reticulum of the cell where cytochrome P450 binds and activates oxygen and the generated reactive oxygen species is inserted into the petroleum hydrocarbon (Mansuy, 1998). A number of studies found that fish collected from oil-spill sites had increased cytochrome P450 monooxygenase activities, e.g. arylhydrocarbon hydroxylase (AHH) or ethoxyresorufin O-deethylation (EROD) (Collier et al., 1996; Kirby et al., 1999; Kurelec et al., 1977; Lindström-Seppä, 1988; Payne et al., 1984; Spies, 1989).

Exposure to certain polycyclic aromatic hydrocarbons (PAH) found in crude oil is thought to be the cause of the observed increases in cytochrome P450 monooxygenase activities. Table 1 lists individual PAH which have been tested with fish or fish cell cultures to determine if they induce the cytochrome P450 system. Strong induction was observed with certain 4–6 ring aromatics, e.g. benzo(a)pyrene, which are in low concentrations in most crude oils (Aas et al., 2000; Burns et al., 1997; Sauer et al., 1993).

Cytochrome P450 systems responses to PAH exposure in marine animals are also functions of the age, gender and stage of sexual development of the species, by

the tissue selected (liver, kidney, gill, intestine, blood) and by external environmental factors, such as season and water temperature (Andersson and Koivusaan, 1985; Goksoyr and Förllin, 1992; Stagg et al., 1995). These factors needed to be considered when planning a oil spill field program where P450 system responses will be monitored.

Exposure of fish to the PAH in crude oil also results in increases in bile fluorescent aromatic compounds (FAC) (Aas et al., 2000; Gagnon and Holdway, 2000; Krahn et al., 1986a). When the exposure pathway is via the foodchain, PAH are absorbed by the gut, transported to the liver, converted to water-soluble polar metabolites and excreted in bile (e.g. Varanasi et al., 1995; Lee et al., 1972b; Lee, 2002). Concentrations of these bile metabolites are measured directly through high-pressure liquid chromatography (HPLC) (Krahn et al., 1984) or by gas chromatography–mass spectrometry (GCMS) techniques (Krahn et al., 1992). Bile metabolite concentrations are also measured indirectly through fluorescence analysis over specified excitation and emission wavelengths. Laboratory studies have shown a positive correlation between exposure dose and FAC response (Collier and Varanasi, 1991). Those studies further show that post-exposure depuration occurred in a matter of several weeks indicating that elevated bile FAC reflects relatively recent exposure to PAH. The correlation of elevated bile FAC with CYP1A in field collected fish is the basis for attributing the CYP1A response to PAH exposure (Collier et al., 1996; Collier and Varanasi, 1991; Varanasi et al., 1995; Huggett et al., 2003).

While an oil spill contributes PAH to the marine environment, other sources of PAH are commonly present. Sites with high concentrations of pyrogenic PAH, derived from past and current combustion processes and other anthropogenic sources such as creosote use, often have fish with increased cytochrome P450, bile FAC and liver neoplasia (Collier et al., 1992; Krahn et al., 1986a). Crude oil hydrocarbons with primarily 2- and 3-ringed PAH are quite different with respect to the type of bile FAC, cytochrome P450 system responses and higher order biological effects than pyrogenic hydrocarbons with primarily 4- to 6-ringed unsubstituted PAH (Neff, 2002). CYP1A-inducing compounds have been identified in sediment samples taken from coastal regions of the United States, where no oil spills have taken place (Anderson et al., 1999, 2005). Pyrogenic PAH and total PAH (TPAH) have correlated with the observed CYP1A induction in fish, but there are often multiple sources of the PAH and other inducing compounds, e.g. dioxins, furans, coplanar polychlorinated biphenyls, present. Consequently, attribution of CYP1A induction and elevated bile FAC in fish to a specific exposure source requires careful evaluation of all potential sources.

Table 1  
Induction/non-induction of cytochrome P4501A/EROD in fish/fish cells by different polycyclic aromatic hydrocarbons

PAH analyte	Relative induction
Benzene	0
Naphthalene	0
Phenanthrene	0
Anthracene	0
Pyrene	0/+
Perylene	0
Acenaphthylene	0
Acenaphthene	0
Fluorene	0
Fluoranthene	0/+
Triphenylene	+
Benzo(e)pyrene	+
Benz(a)anthracene	++
Chrysene	++/+++
Benzo(b)fluorene	++
Benzo(b)fluoranthene	+++
Indeno[1,2,3-cd]pyrene	+++
Benzo(a)pyrene	++++
Dibenzo[a,h]anthracene	++++
Dibenzo[a,i]pyrene	++++
Benzo[k]fluoranthene	++++

Data taken from Barron et al. (2004), Fent and Bättscher (2000), Bols et al. (1999) and Van der Weiden et al. (1994).

Notations used for induction: 0 = lack of induction; + = very weak induction; ++ = weak induction; +++ = strong induction; ++++ = very strong induction.

## 2. Responses of fish cytochrome P450 to PAH exposure

Exposure of fish to certain PAH induces cytochrome P4501A1 (CYP1A1) which is a member of the CYP1A subfamily (Goksøyr and Förlin, 1992). The steps involved in the induction of CYP1A1 include the activation and transcription of the CYP1A1 gene, translation of CYP1A1 messenger RNA to produce CYP1A1 protein and post-translational modification to give the catalytically active enzyme (Goksøyr, 1995). The induction of the CYP1A subfamily occurs via the high affinity binding of an aromatic hydrocarbon to an intracellular receptor complex (the Ah receptor), translocation of the inducer-receptor complex to the nucleus, and transcriptional activation of the genes in the Ah battery (Hahn, 1998). Induction stimulates the rate of gene transcription, which results in increased levels of P450 messenger RNA and increased synthesis of P450 protein.

In addition to the PAH, there are a large number of compounds found in the marine environment which have been shown to induce CYP1A in fish including certain dibenzofurans, dioxins, polychlorinated biphenyls, pesticides, herbicides and paper mill effluents (Goksøyr and Förlin, 1992; Livingstone, 1993). There are also naturally occurring compounds in the diet that can cause increases in CYP1A. For example, the levels of CYP1A in salmon fed food containing astaxanthin, the primary natural carotenoid pigment in many marine animals, were higher than for salmon fed food not containing this pigment (Goksøyr and Husøy, 1998). Retene (7-isopropyl-1-methylphenanthrene) a naturally occurring PAH, formed from tree resins through anaerobic degradation in sediments (Vehniainen et al., 2003) or through combustion (Tanner, 2003) has been shown to be a strong inducer of CYP1A in fish (Oikari et al., 2002) and human cells (Jones et al., 2001).

Two approaches for the study of cytochrome P450 responses after oil spill exposure are the use of antibodies and/or enzyme assays. Antibodies have been produced against CYP1A proteins in fish, allowing immunodetection of CYP1A in various fish tissues or quantification by enzyme linked immunosorbent assays (ELISA) (Goksøyr and Förlin, 1992; Goksøyr et al., 1991a) after oil exposure. Immunoquantification of P4501A, using polyclonal or monoclonal antibodies to fish P4501A, includes both western blotting and ELISA techniques.

Two different assays (arylhydrocarbon hydroxylase, AHH; ethoxyresorufin O-deethylation, EROD) for cytochrome P450 monooxygenase activity have been used. The AHH assay uses benzo(a)pyrene as a substrate for the assay and most studies report AHH fish liver activity in units of 3-hydroxybenzo(a)pyrene-formed/min/mg protein using procedures described by Nebert and Gelboin (1968). The EROD assay involves the fluorescence detection of the resorufin product and the EROD activ-

ity is reported as nmol resorufin/min/mg protein, often following modifications of the procedures described by Burke and Mayer (1974). Most investigators report the results in units per microsomal protein but some investigators use post-mitochondrial extract protein rather than microsomal protein so their results would be in units per mg of post-mitochondrial protein. There is some evidence that EROD uses CYP1A and thus many studies use EROD activity as surrogate for CYP1A responses (Goksøyr and Förlin, 1992).

## 3. Responses of fish cytochrome P450 monooxygenase systems to oil exposure

### 3.1. Oil spills

Livers of fish collected from oil spills with spill volumes varying from 0.06 to  $87 \times 10^6$  L have increased cytochrome P450 monooxygenase activities when compared with fish collected from reference sites (Table 2). A number of studies showed elevated CYP1A in fish collected from sites with high PAH concentrations in the sediments, however at most sites the PAH were predominantly pyrogenic (Bucheli and Fent, 1995; Stegeman et al., 1988; Van Veld et al., 1990; Collier et al., 1992; Stein et al., 1992). Increased CYP1A levels were detected in fish collected during two oil spill investigations, *Exxon Valdez* and *Braer*, and from a natural oil seep (Carls et al., 1996; Ritchie and O'Sullivan, 1994; Roy et al., 2003).

Most of the work on fish collected from oil-spill sites found transitory increases in EROD or AHH activities that generally reached a peak within 2–3 months after the spill, followed by a return to background activities often within one year or less (Table 2). For example, perch (*Perca fluviatilis*) collected from spill and reference sites in the Gulf of Bothnia, Finland had liver AHH activities of 20 pmol/min/mg of protein and 10 pmol/min/mg of protein, respectively (Lindström-Seppä, 1988). Generally, AHH activities in fish collected from spilled sites had returned to background activities four months after the spill. EROD activities on liver extracts from salmon collected 11 and 51 days after the *Braer* oil spill in the North Sea ranged from 200 to 275 pmol/min/mg protein. EROD activities subsequently decreased to 150 pmol/min/mg protein between days 66 and 76 and to 50 pmol/min/mg protein by day 114 (Ritchie and O'Sullivan, 1994).

A number of fish species were assayed for AHH activities at different times following the *Exxon Valdez* oil spill (EVOS) (Table 2). The AHH activities of Dolly Varden trout, *Salmo malma*, decreased from 240 to 90 pmol of metabolized benzo(a)pyrene/mg protein in fish sampled at Snug Harbor, an embayment that was heavily oiled in 1989, approximately 120 and 460 days

Table 2  
Aryl hydrocarbon hydroxylase (AHH) and/or ethoxyresorufin O-deethylation (EROD) activities in fish liver collected from oil-spill sites

Spill and reference sites	Oil type and quantity	Fish	Time after spill (days)	AHH act. (pmol/min/mg protein)	EROD act. (pmol/min/mg protein)	Reference
N. Adriatic Sea	Crude oil (0.06–0.6 × 10 <sup>6</sup> L)	<i>B. pava</i>	30	800 <sup>a</sup>	–	Kurelec et al. (1977).
			90	250 <sup>a</sup>	–	Zahn et al. (1996)
Ref. site	–	<i>B. pava</i>	–	100 <sup>a</sup>	–	
Baie Verte, Newfoundland	Fuel oil (0.08 × 10 <sup>6</sup> L)	<i>P. americanus</i>	90	58	–	Payne et al. (1984)
Ref. site	–	–	–	50	–	Payne et al. (1984)
Gulf of Bothnia, Finalnd	Fuel oil (0.3 × 10 <sup>6</sup> L)	<i>P. fluviatilis</i>	120	20	–	Lindström-Seppä (1988)
Gulf of Bothnia, Finalnd	Fuel oil (0.3 × 10 <sup>6</sup> L)	<i>P. fluviatilis</i>	240	10		
Ref. site	–	<i>P. fluviatilis</i>	–	10		
San Francisco	San Joaquin	<i>L. armatus</i>	60–90	4.5–5.0	1.3–1.6	Spies (1989)
Ref. site	Valley crude (1.5 × 10 <sup>6</sup> L)	<i>L. armatus</i>	–	3	0.3	Spies (1989)
PWS, Alaska, Snug Harbor	North Slope crude (75 × 10 <sup>6</sup> L)	<i>P. bilineatus</i>	≈120	230 ± 170 <sup>b</sup>	–	Collier et al. (1996)
			≈460	250 ± 150 <sup>b</sup>	–	Collier et al. (1996)
PWS, Alaska, Snug Harbor	North Slope crude (75 × 10 <sup>6</sup> L)	<i>P. bilineatus</i>	≈760	200 ± 70 <sup>b</sup>	–	Collier et al. (1996)
PWS, Alaska, Snug Harbor	North Slope crude (75 × 10 <sup>6</sup> L)	( <i>S. malma</i> )	≈120	240 ± 50 <sup>b</sup>	–	Collier et al. (1996)
PWS, Alaska, Snug Harbor	North Slope crude (75 × 10 <sup>6</sup> L)		≈240	90 ± 10 <sup>b</sup>	–	
Alaska, Tonsina Bay	North Slope crude (75 × 10 <sup>6</sup> L)	<i>P. bilineatus</i>	≈120	160 ± 50 <sup>b</sup>	–	Collier et al. (1996)
Alaska, Tonsina Bay	North Slope crude (75 × 10 <sup>6</sup> L)	<i>P. bilineatus</i>	≈460	140 ± 30 <sup>b</sup>	–	Collier et al. (1996)
Alaska, Tonsina Bay	North Slope crude (75 × 10 <sup>6</sup> L)	<i>S. malma</i>	≈120	240 ± 30 <sup>b</sup>	–	Collier et al. (1996)
Alaska, Tonsina Bay	North Slope crude (75 × 10 <sup>6</sup> L)	<i>S. malma</i>	≈460	60 ± 10 <sup>b</sup>	–	Collier et al. (1996)
Alaska, Kukak Bay	–	<i>P. bilineatus</i>	≈120	210 ± 80 <sup>b</sup>	–	Collier et al. (1996)
Ref. site	–	<i>S. malma</i>	≈120	60 ± 10 <sup>b</sup>	–	Collier et al. (1996)
	–	<i>S. malma</i>	≈460	40 ± 5 <sup>b</sup>	–	Collier et al. (1996)
North Sea	Forties crude, Fuel oil (87 × 10 <sup>6</sup> L)	<i>L. limanda</i>	90	–	4910, 3920	Kirby et al. (1999), Law et al. (1997)
Ref. site-offshore	–	<i>L. limanda</i>	–	–	3900, 3800	Kirby et al. (1999), Law et al. (1997)
North Sea	–	<i>P. platessa</i>	90	–	780, 840	Kirby et al. (1999), Law et al. (1997)
Ref. site-offshore	–	<i>P. platessa</i>	–	–	840	Kirby et al. (1999), Law et al. (1997)

Fish used from the various sites included staghorn sculpin (*Leptocottus armatus*), perch (*Perca fluviatilis*), blenny (*Blennius pavo*), flounder (*Pseudopleuronectes americanus*), dab (*Limanda limanda*), plaice (*Pleuronectes platessa*), rock sole (*Pleuronectes bilineatus*) and Dolly Varden (*Salvelinus malma*). Liver tissues were used AHH activity is in terms of pmol of 3-hydroxybenzo(a)pyrene formed/min/mg microsomal protein. EROD activity is in terms of nmol resorufin formed/min/mg microsomal protein. Abbreviation: Ref = reference; PWS = Prince William Sound.

<sup>a</sup> Arbitrary fluorescence units.

<sup>b</sup> nmol of metabolites of benzo(a)pyrene/min/mg of microsomal protein.

after the spill, respectively (Collier et al., 1996). However, the AHH activities of rock soles, *Pleuronectes bilineatus*, at the same site did not decrease significantly at 120, 460 and 769 days after the spill (200–250 pmol of metabolized benzo(a)pyrene/min/mg protein) (Collier et al., 1996). Dolly Varden trout collected from Tonsina Bay, a site on the Kenai Peninsula oiled by the *Exxon Valdez* spill in 1989, showed a significant decrease between 120 and 460 days after the spill ( $240 \pm 30$  and  $60 \pm 10$  nmol of benzo(a)pyrene metabolites formed/min/mg microsomal protein for 120 and 460 days, respectively). Rock sole again did not show significant difference between the two collecting periods ( $160 \pm 50$  and  $140 \pm 30$  nmol of benzo(a)pyrene metabolites formed/min/mg microsomal protein for 120 and 460 days, respectively) (Collier et al., 1996). The AHH activities of Dolly Varden trout and rock soles from Kukak Bay, an unoiled reference site, were 60 and 210 pmol of metabolized benzo(a)pyrene/mg protein, respectively (Collier et al., 1996). Thus, there was no difference between rock sole AHH activities for samples analyzed from oiled and unoiled sites. The response differences between rock sole and Dolly Varden trout could be due to differences in biochemical responses of these species, as well as differences in PAH uptake from sediment, water or food.

One difficulty encountered in interpreting AHH data from the *Exxon Valdez* spill zone was the contribution of PAH from other hydrocarbon sources including discharges related to the fishing fleet and other boating activity, combustion products from chronically contaminated historical industrial sites, natural oil seeps, eroding organic shales, etc., to the observed AHH activities at the different sites. Different sea floor sites in Prince William Sound have PAH distributions and concentrations that vary depending upon the relative contributions of the various petrogenic and pyrogenic sources in the region, including oil residues from EVOS (Bence and Burns, 1995; Bence et al., 1996; Page et al., 1995, 1996, 1999, 2002). Page et al. (1999) found high concentrations of pyrogenic PAH (up to 5000 ng/g dry wt) in Snug Harbor subtidal sediments, which raises questions about the source of PAH causing the AHH responses observed in Dolly Varden from this embayment.

There are also reports of fish collected from oil-spill sites showing no difference from reference fish with respect to cytochrome P450 systems. EROD activities in livers from dab (*Limanda limanda*) and plaice (*Pleuronectes platessa*) from two stations near the *Sea Empress* spill were not different from fish collected from several reference stations, including an offshore site (Kirby et al., 1999). Payne et al. (1984) found no increase in AHH activity (50–60 pmol/min/mg protein) in liver from fish collected from a number 2 fuel oil-spill site in Newfoundland.

Several studies have noted that petroleum PAH concentrations in the sediment did not correlate with fish EROD activities. There was no relationship between EROD activities and sediment hydrocarbon concentrations in fish collected from different sites after the *Braer* spill in the North Sea (Ritchie and O'Sullivan, 1994) and after EVOS in Prince William Sound (PWS), Alaska (Jewett et al., 2002). In PWS it was found that copper and quillback rockfish (*S. caurinus* and *S. maliger*) caught in embayments had EROD activities that correlated positively with the pyrogenic PAH indicator ratio (fluoranthene + pyrene)/C2-4 alkylphenanthrenes (Huggett et al., 2003; Page et al., 2004). No sediment total PAH (TPAH) correlation with EROD was observed in these fish, species that characteristically exhibit strong site fidelity (Matthews, 1990a,b).

AHH and EROD activities have also been determined on fish collected at different distances from oil platforms. It is hypothesized that petroleum concentrations in water near such platforms would stay elevated due to chronic inputs in contrast to an oil spill where water concentrations of petroleum components rapidly decrease. While Davies et al. (1984) and Lange et al. (1992) found higher AHH or EROD activities in fish near oil platforms in the North Sea relative to fish from more distant locations, McDonald et al. (1996) found no significant differences in AHH or EROD activities in 16 species of fish collected from sites close to and distant from oil drilling platforms in the Gulf of Mexico. Stagg et al. (1995) found no clear relationship between fish EROD activity and sediment hydrocarbon concentrations at stations around oil platforms on the North Sea. McDonald et al. (1996) suggested that the relatively low sediment total PAH (TPAH) concentrations (mean TPAH concentration = 361 ng/g sediment; maximum TPAH concentration = 2300 ng/g) at sites near oil platforms in the Gulf of Mexico did not result in increases of fish AHH or EROD activities. Based on other studies they concluded that fish showing increased AHH activities were associated with sediments having TPAH concentrations in excess of 3  $\mu\text{g/g}$ . Most of the reported sites had pyrogenic-type PAH, i.e. dominated by 4- to 6-ring PAH. The high molecular PAH known to increase AHH activities are in relatively low concentrations in crude oil and it seems likely that TPAH concentrations much higher than 3  $\mu\text{g/g}$  sediments would be required to induce fish-liver AHH in sediments with predominantly petrogenic hydrocarbons. It is noteworthy that the 3  $\mu\text{g/g}$  TPAH threshold of effect suggested by McDonald et al. (1996) is similar to the 4.022  $\mu\text{g/g}$  sediment TPAH "effects range-low" (ER-L) which has a 10% probability of adverse effects (Long et al., 1995).

Fish collected from urban sites having high concentrations of pyrogenic PAH have AHH activities that correlate well with sediment TPAH concentrations (Eufemia et al., 1997; Stein et al., 1992).

### 3.2. Laboratory exposure to oil in water, food or sediment

After an oil spill, petroleum components can enter fish via water, sediment or food. Laboratory studies have followed changes in cytochrome P450 monooxygenase activities after fish were exposed to petroleum in water, sediment or food. It is not clear which PAH found in crude oil are responsible for the fish cytochrome P450 system responses to oil in the different matrices. As noted in Table 1 the highest response by fish cytochrome P450 systems is to certain 4–6 ring PAH, e.g. chrysenes. However, this class of PAH has very low solubility in water. Depending on the particular oil, it is possible that there are high enough concentrations of the 4-ringed chrysenes to cause the observed cytochrome P450 responses. While the 3-ringed PAH found in oil do not induce EROD activity (Hawkins et al., 2002; Jung et al., 2001), quinones, formed as

photo-oxidation products of anthracene have been shown to induce EROD activity (Schirmer et al., 2001). Thus, it may be that under oil spill conditions certain PAH photooxidation products could contribute to observed responses of fish cytochrome P450 systems. These photooxidation products are water-soluble and degradable, properties that are consistent with the observed rapid increases, followed by decreases, of the fish cytochrome P450 systems following an oil spill.

#### 3.2.1. Exposure via water

For water studies, fish have generally been exposed to what is referred to as the water-soluble fraction (WSF) of crude oil or a water-accommodated fraction (oil-in-water emulsion), where oil is vigorously mixed with sea water and fish are exposed to this oil in water mix (Table 3). There are often questions regarding the relationship between oil-in-water mixes used in the laboratory and those observed in the water after an oil spill.

Table 3  
AHH and EROD activities in fish exposed to oil in water

Fish	Oil and Conc.	Exposure time (days)	Depuration time (days)	AHH (pmol/min/mg)	EROD (pmol/min/mg)	Reference
<i>S. salar</i>	Bass Strait crude (250 µg/L)	4	–	–	100	Gagnon and Holdway (1998)
<i>S. salar</i>	Bass Strait crude (250 µg/L)	4	15	–	30	
<i>S. salar</i>	Control	–	–	–	30	Gagnon and Holdway (1998)
<i>G. morhua</i>	North Sea crude	30	–	–	30	Aas et al. (2000)
<i>G. morhua</i>	North Sea crude	30	7	–	6	Aas et al. (2000)
<i>G. morhua</i>	Control	–	–	–	3	Aas et al. (2000)
<i>G. morhua</i>	Statfjord crude	49	–	–	25 ± 10	Goksøyr et al. (1991b)
<i>G. morhua</i>	Statfjord crude	49	2	–	16 ± 6	Goksøyr et al. (1991b)
<i>G. morhua</i>	Control	–	–	–	14 ± 9	Goksøyr et al. (1991b)
<i>C. carpio</i>	Diesel oil (50 µg/L)	12	–	500 <sup>a</sup>	–	Britvic et al. (1993)
<i>C. carpio</i>	Diesel oil (50 µg/L)	12	15	40 <sup>a</sup>	–	Britvic et al. (1993)
<i>C. carpio</i>	Control	–	–	40 <sup>a</sup>	–	Britvic et al. (1993)
<i>S. fontinalis</i>	Kuwait crude (930 µg/L)	2	–	100 <sup>b</sup>	–	Vandermeulen (1990)
<i>S. fontinalis</i>	Kuwait crude (600 µg/L)	4	–	220 <sup>b</sup>	–	Vandermeulen (1990)
<i>S. fontinalis</i>	Control	–	–	5 <sup>b</sup>	–	Vandermeulen (1990)
<i>O. kisutch</i>	Cook Inlet crude (950 µg/L)	30	–	19 <sup>a</sup>	–	Collodi et al. (1984)
<i>O. kisutch</i>	Cook Inlet crude (950 µg/L)	30	15	8 <sup>a</sup>	–	Collodi et al. (1984)
<i>O. kisutch</i>	Control	–	–	7 <sup>a</sup>	–	Collodi et al. (1984)
<i>O. kisutch</i>	Cook Inlet crude (400 µg/L)	2	–	80	–	Thomas et al. (1989)
<i>O. kisutch</i>	Control	–	–	10	–	Thomas et al. (1989)
<i>C. pallasii</i>	North Slope crude (3 µg/L—PAH)	8	–	25	–	Thomas et al. (1997)
<i>C. pallasii</i>	North Slope crude	8	–	25	–	Thomas et al. (1997)
<i>C. pallasii</i>	Control	–	–	3	–	Thomas et al. (1997)
<i>P. flesus</i>	Diesel oil (40 µg/L)	120	–	15.7 ± 7.0	–	Addison and Edwards (1988)
<i>P. flesus</i>	Control	–	–	7.8 ± 6.7	–	Addison and Edwards (1988)
<i>P. flesus</i>	Control	–	–	10	–	Addison and Edwards (1988)

Fish used for the various exposure studies included Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), carp (*Cyprinus carpio*), flounder (*Platichthys flesus*), coho salmon (*Oncorhynchus kisutch*), brook trout (*Salvelinus fontinalis*) herring (*Clupea pallasii*) and cod (*Gadus morhua*). AHH activity, unless listed differently, was in pmol of 3-hydroxybenzo(a)pyrene formed/min/mg protein. EROD activity was in pmol of resorufin formed/min/mg protein.

<sup>a</sup> Arbitrary fluorescence units.

<sup>b</sup> pmol of 3-hydroxybenzo(a)pyrene formed/min/mg protein nitrogen.

Oil in water after a spill will obviously vary with environmental conditions, and these will also govern the amount of oil droplets in the water. Short and Harris (1996) reported PAH concentrations between 1.3 and 6.2 µg/L in near-surface (1 m and 5 m depths) seawater adjacent to oiled beaches 14 days after EVOS. Total PAH concentrations less than 10 µg/L were found in 2461 water samples taken at 1 and 3 m depths in the waters of Prince William Sound, Alaska after the EVOS (Neff and Stubblefield, 1995). Hydrocarbon concentrations near oil drilling platforms in the North Sea ranged from 1 to 5 µg/L (Stagg and McIntosh, 1996). Most laboratory fish cytochrome P450 system response studies have used relatively high concentrations of petroleum in water (PAH concentrations ranging from 30 to 300 µg/L) (Collodi et al., 1984; Goksøyr et al., 1991b; Payne and Fancey, 1982; Thomas et al., 1997; Vandermeulen, 1990). While few of these studies were carried out at water PAH concentrations actually found after oil spills, the lower test concentrations often failed to show responses in exposed fish. For example, the AHH activity of coho salmon exposed to 350 µg/L of Cook Inlet crude oil for 10 days was the same as the controls (Collodi et al., 1984).

Studies with different fish species and crude oils showed increases in AHH activities after exposure to oil, followed by a rapid decrease to control values after fish were transferred to clean water, i.e. depuration (Table 3). For example, salmon (*Salmo salar*) exposed to

250 µg/L of petroleum hydrocarbons from water-accommodated Bass Strait crude oil for 4 days showed high EROD activities after 2 days (100 pmol product/min/mg protein). When transferred to oil free seawater the EROD activity returned to control levels (30 pmol product/min/mg protein) within 4 days (Gagnon and Holdway, 1998). Besides studies showing increased AHH and EROD activities after oil-in-water exposure, there are also studies showing no changes in AHH or EROD activities after oil in water exposure. Flounder, *Platichthys flesus*, exposed to several concentrations of diesel oil for 120 days showed no significant difference from control fish for both EROD and AHH activities (Addison and Edwards, 1988; Stegeman et al., 1988). The low molecular weight 2–3 ring PAH found in diesel oil did not result in cytochrome P450 monooxygenase responses. There were no significant differences between the liver EROD activities of control and oil-exposed (15–130 µg/L of petroleum hydrocarbons of North Sea crude) juvenile cod, *Gadus morhua* (Goksøyr et al., 1991b). Thus, while there can be induction of AHH/EROD activities after crude oil exposure, the majority of hydrocarbons entering the water during oil spills are the 2–3 ring aromatic hydrocarbons, which do not elicit a response.

### 3.2.2. Exposure via food

Fish showed increased AHH and EROD activities when oil was incorporated into their food (Spies et al.,

Table 4  
AHH and EROD activities of fish exposed to petroleum hydrocarbons via food or sediment

Fish	Oil used	Pet. hydrocarbon conc. of sediment or food	Exposure time (days)	AHH (pmol 3-OH Bp/mg protein)	EROD (pmol/min/mg protein)	Reference
Sediment						
<i>P. americanus</i>	Venezuelan	0.78 µg PAH/g	120	0.29	–	Payne et al. (1988)
<i>P. americanus</i>	Venezuelan	14 µg PAH/g	120	0.84	–	Payne et al. (1988)
<i>P. americanus</i>	Venezuelan	240 µg PAH/g	120	1.24	–	Payne et al. (1988)
<i>P. americanus</i>	Control	0.08 µg PAH/g	–	0.16	–	Payne et al. (1988)
<i>L. limanda</i>	Gullfaks crude	1000 µg Pet. HCs/g	14	–	1000	Ritchie and O'Sullivan (1994)
<i>L. limanda</i>	Control	–	–	–	1000	Ritchie and O'Sullivan (1994)
Food						
<i>C. stigmaeus</i>	Seep oil	25 mg oil/fish/day	14	0.210 ± 0.130	–	Spies et al. (1982)
<i>C. stigmaeus</i>	Seep oil	5 mg oil/fish/day	14	0.162 ± 0.110	–	Spies et al. (1982)
<i>C. stigmaeus</i>	Control	–	–	0.209 ± 0.130	–	Spies et al. (1982)
<i>C. sordidus</i>	Seep oil	25 mg oil/fish/day	14	2.640	–	Spies et al. (1982)
<i>C. sordidus</i>	Control	–	–	1.870	–	Spies et al. (1982)
<i>B. saida</i> , males	Oseberg crude	200 µg oil/g food	72	–	132 ± 14	George et al. (1995)
<i>B. saida</i> , females	Oseberg crude	200 µg oil/g food	72	–	42 ± 6	George et al. (1995)
<i>B. saida</i> , males	Control	–	–	–	28 ± 6	George et al. (1995)
<i>B. saida</i> , females	Control	–	–	–	8 ± 2	George et al. (1995)

Fish assayed (hepatic tissues) included flounder (*Pleudopleuronectes americanus*), dab (*Limanda limanda*), Pacific sanddab (*Citharichthys sordidus*), speckled sanddab (*Citharichthys stigmaeus*) and Polar cod (*Boreogadus saida*). AHH activity was in pmol of 3-hydroxybenzo(a)pyrene formed/min/mg protein. EROD activity was in pmol of resorufin formed/min/mg protein. Abbreviations: pet. = petroleum; conc. = concentration.

1982; George et al., 1995) (Table 4). After Santa Barbara seep oil was added to the food (25 mg of oil/fish/day) of the flatfish, *Citharichthys stigmatæus*, liver AHH activities were  $209 \pm 130$  and  $945 \pm 490$  pmol 3-OH-benzo(a)pyrene/min/mg protein for fish fed oil-free food and oiled food, respectively (Spies et al., 1982). Santa Barbara seep oil has been described as having naphthalene-like compounds as the major PAH component, but quantitative data on PAH were not reported (Spies et al., 1980). AHH activities were not significantly different for fish fed a lower concentration of oil (5 mg of oil/fish/day) compared with controls. When polar cod, *Boreogadus saida*, were fed food containing Oseberg crude oil (200 µg/g food) the EROD activities after 72 days of continuous exposure for males were 28 and 132 pmol/min/mg protein for control and oil-fed fish, respectively (George et al., 1995). Unfortunately, these studies did not determine when the AHH and EROD activities returned to control levels after the fish were transferred to an oil free diet. Besides the role of the liver, fish intestinal cytochrome P450 monooxygenase systems are known to rapidly respond to dietary PAH, since PAH are rapidly absorbed by the intestinal mucosa of fish (Van Veld et al., 1988). Van Veld et al. (1988) showed that after spot, *Leiostomus xanthurus*, were fed food with PAH, AHH and EROD activities were highest in pyloric caeca and in the proximal half of the intestine. Intestinal EROD and AHH increased 36-fold and 17-fold, respectively compared with controls. Thus, petroleum PAH in the fish food should increase EROD and AHH activities in both intestinal and liver tissues.

### 3.2.3. Exposure via sediment

Different and contrasting results have been found for the relationship between sediment PAH and cytochrome P450 monooxygenase activities (Table 4). Payne and Fancey (1982) exposed flounder, *Pseudopleuronectes americanus*, for 4 months to sediment containing fresh Venezuelan crude oil and weathered crude oil (2600 µg/g sediment) and found that liver AHH activities in oil-exposed fish were 12-fold higher than controls. In contrast, sand dabs kept on sediment containing 1000 µg/g sediment of Gullfaks crude oil showed no increase in liver EROD activities compared with controls (Ritchie and O'Sullivan, 1994). Sediments containing mixtures of seep oil from Santa Barbara (Coal Oil Point) and reference sediment (0–105 µg/g TPAH) were used in 7-day exposures to hornyhead turbot (*Pleuronichthys verticalis*) to measure effects on serum/plasma estradiol, fluorescent aromatic compounds (FACs) in bile, hepatic CYP1A, and hepatic DNA damage (Roy et al., 2003). While liver DNA damage, measure by % DNA in the comet tail, increased with sediment PAH concentration such a dose response relationship was not observed for FACs, and CYP1A induction was only observed at the highest concentration. The induction of hepatic CYP1A

in these fish by PAHs from seep oil in sediments was not a good indicator of exposure, even at very high levels of both low (61.2 µg/g) and high (38.6 µg/g) molecular weight compounds. As noted in water exposure investigations, there can be cytochrome P450 responses after exposure to oil in sediment but some crude oils in the sediments do not elicit a response. The bioavailability of PAH from sediment also needs to be considered when carrying out sediment exposures and interpreting field and laboratory studies.

## 4. Responses of cytochrome P450 systems in marine birds and mammals to crude oil exposure

CYP1A response and EROD and AHH activities have been shown to be induced in marine birds and mammals after exposure to crude oil and certain PAH (Engelhardt, 1982; Lee et al., 1985; Taylor et al., 2001; Pealkall et al., 1987; Rattner et al., 1993). As in fish, the responses of cytochrome P450 systems in marine mammals and birds after exposure to inducers vary with age, stage of development, sex and other environmental factors (Gorsline and Holmes, 1982; Pealkall et al., 1987; Rattner et al., 1993; White et al., 1994). Rats fed brassica vegetables, e.g. cabbage, showed induction of cytochrome P450 systems as a result of the presence of MFO inducing compounds in the plants (McDanell et al., 1987). It seems likely that marine mammals would be exposed in their diets to a variety of natural occurring cytochrome P450 inducing compounds, e.g. retene. Hepatic AHH and EROD activities increased after high oral doses of crude oil in herring gulls followed by decreased EROD activity a few days after the dose of oil (Lee et al., 1985; Pealkall et al., 1987).

After EVOS, EROD activities were measured in sea otters, Harlequin ducks and Barrow's goldeneyes collected from oiled areas and non-oiled areas in PWS (Bodkin et al., 2002; Trust et al., 2000). Bodkin et al. (2002) found that sea otters collected in 1996–1998 from Knight Island (oiled in 1989) had higher levels of blood CYP1A mRNA than otters collected near Montague Island (unoiled). It was suggested that EVOS-contaminated sea otter prey species, i.e. clams and mussels, on Knight Island were responsible for their higher CYP1A mRNA, although no prey hydrocarbon data were reported to support this hypothesis. It is generally agreed that most sea otter feeding is done in the subtidal and low intertidal zones, and the majority of the EVOS oil was in the mid and high intertidal areas. There was no indication of effects on the otters from apparent higher CYP1A mRNA blood levels. Trust et al. (2000) found higher mean EROD activities in livers of Harlequin ducks from a spill zone location in the Crafton Island/Main Bay area of western PWS (mean = 205 pmol/min/mg protein; range = 92–377)

relative to those from the non-oiled Stockdale Harbor/Port Chalmers area of Montague Island (mean = 71 pmol/min/mg protein; range = 4–386). The range of values indicates the very high natural variability of EROD activities in these birds, likely due to such factors as diet, sexual stage of development and age of birds. In both the sea otter and Harlequin ducks studies it was concluded, from circumstantial evidence, that EVOS residues caused the observed response. However, as discussed earlier, EROD-inducing pyrogenic PAH such as chrysene and benzo(a)pyrene, derived from past and current human and industrial activities in PWS, have been found throughout the spill zone (Burns et al., 1997; Neff et al., 2003; Page et al., 1999, 2002). Another source of EROD inducing compounds could be hydrocarbons on the water surface, originating from a very active boating industry in western PWS since hydrocarbons can collect on fur or feathers and later be taken in during cleaning or preening of fur or feathers. Neither these nor other hydrocarbon sources were considered as sources of exposure for sea otters and Harlequin ducks.

### 5. Responses of marine invertebrate cytochrome P450 systems to oil spills and petroleum PAH

Crustaceans and polychaete worms are two groups of marine invertebrates that can metabolize petroleum hydrocarbons due to a well-developed cytochrome P450 system (Fries and Lee, 1984; James and Boyle, 1998; Lee, 1981, 1998; Lee et al., 1976). However, there are insufficient data to show that the cytochrome P450 system in these animals is induced as a result of exposure to an oil spill. A recent paper by Rebelo et al. (2003) concluded that there was no clear evidence of a bivalve CYP1A1, and that the Ah receptor pathway is lacking. Sequencing studies with *Crassostrea rhizophorae* indicate few similarities with CYP1A1, and a possible CYP2-like gene. While bivalves appear to exhibit low cytochrome P450 activity there may be alternate detoxification mechanisms.

Bivalve mollusks have a very limited ability to metabolize petroleum hydrocarbon and after exposure to an oil spill bioaccumulate petroleum hydrocarbons (Dyrynda et al., 1997; Lee et al., 1972a; Vandermeulen and Penrose, 1978). A cytochrome P450 system in low concentrations has been found in bivalve mollusks that produced quinones, rather than phenols and diols, as the major PAH metabolites (Lemaire and Livingstone, 1993; Michel et al., 1992; Porte et al., 1995). The antibody to fish CYP1A was used in two studies with bivalves collected from two oil spills (*Aegean* off Spain and *Sea Empress* off United Kingdom) (Solé et al., 1996; Peters et al., 1999). In these studies mussels, *Mytilus edulis*, with higher levels of CYP1A-like proteins were

found at sites having higher concentrations of PAH in their tissues. More work is necessary to determine the relationship of molluscan cytochrome P450 and CYP1A, including cloning and sequencing of the gene that expresses the bivalve cytochrome P450. At this time it is not clear what the fish CYP1A antibody is reacting to in bivalve protein extracts.

An antibody to benzo(a)pyrene diol epoxide-adducted protein (BPAP) was used to analyze bivalves (*Mya arenaria* and *Mytilus trossulus*) collected from a single oiled site and a single unoled site 11 years after EVOS (Downs et al., 2002). A positive response was found in *M. arenaria*, but not in *M. trossulus*, collected from a heavily oiled site (Bay of Isles). The authors concluded that the positive response in *M. arenaria* indicated metabolic activation of benzo(a)pyrene whose source was from EVOS. However, benzo(a)pyrene, a pyrogenic PAH, was not detected in *Exxon Valdez* cargo crude oil (Bence et al., 1996; Brown et al., 1999; Burns et al., 1997; Table 6) and benzo(a)pyrene was not detected in bivalves deployed at oiled sites in PWS (Short and Harris, 1996). Because the PAH concentrations in the bivalves from the Bay of Isles used for the BPAP assay were not reported in the studies by Downs et al. (2002), the origin of the biomarker response is not known. Lacking tissue hydrocarbon chemistry, assumptions as to the source of a measured biomarker response can be invalid.

Mussels collected from one oiled site after the *Sea Empress* oil spill had relatively high concentrations of benzo(a)pyrene, indeno[1,2,3-*cd*]pyrene and benzo[ghi]perylene, all very strong inducers of CYP1A, in addition to high concentrations of lower weight petrogenic PAH (Law et al., 1997). The source of these pyrogenic PAH was assumed to be nearby industrial and urban areas.

### 6. Fish bile fluorescent aromatic compounds (FAC) after oil exposure

Assays for PAH metabolites, which accumulate in the bile, have proven useful for assessing exposure of fish to oil. Bile samples are collected from fish and the relative amount of fluorescence intensities of the metabolites for selected excitation and emission wavelengths are measured. One method sets the emission and excitation wavelengths for the 2, 3, 4 and 5-ringed PAH represented by naphthalene (290 nm/380 nm), phenanthrene (256 nm/380 nm) pyrene (341 nm/383 nm) and benzo(a)pyrene (380 nm/430 nm). The results are presented as naphthalene (Nph) equivalents, phenanthrene (Phe) equivalents, pyrene (Pyr) equivalents and benzo(a)pyrene (BaP) equivalents all normalized to biliary protein (Aas et al., 2000; Krahn et al., 1986a). Normalizing the bile FAC per mg biliary protein reduces the variability in the FAC data from fish (Collier and Varanasi, 1991).

Since naphthalene, phenanthrene and their alkylated homologues are dominant PAH in many fresh crude oils (see Table 6), the bile from fish exposed to crude oil often shows a fluorescence profile with main peak around an excitation wavelength of 290 nm due to naphthalene-type metabolites (Aas et al., 2000) and at 256 nm due to phenanthrene-type metabolites (Huggett et al., 2003). In contrast the fluorescent spectrum of bile from fish in urban areas often have a peaks of excitation at between 360 nm and 420 nm due to the presence of high molecular weight PAH-type metabolites, e.g. benzo(a)pyrene-type metabolites (Barra et al., 2001).

A number of laboratory studies have measured FAC after fish were exposed to both pyrogenic and petrogenic PAH, followed by transfer to clean seawater (Arise et al., 1993; Britvic et al., 1993; DiGiulio et al., 1995; Lin et al., 1994). For example, Atlantic salmon bile exposed to a water-accommodated fraction of Bass Strait crude oil reached high concentrations of PAH metabolites (800 ng of naphthalene-type metabolites/mg protein) after 6 days of exposure and then decreased to background concentrations (200 ng/mg protein) 30 days after transfer to clean sea water (Gagnon and Holdway, 2000). Thus, the presence of elevated bile FAC is indicative of recent exposure to petrogenic and/or pyrogenic PAH (Arcand-Hoy and Metcalfe, 1999; Collier and Varanasi, 1991).

The ratio of Nph equivalents (fluorescence: 290 nm excitation/335 nm emission) to BaP equivalents (fluorescence: 380 nm excitation/430 nm emission) can be used as an indicator of the relative amount of metabolites derived from petrogenic and pyrogenic sources. The ratio of Nph/BaP-type metabolites in pink salmon exposed to crude oil was 1300, while the ratio for the same fish species exposed to a site containing creosote was 110 (Krahn et al., 1992). The ratio of Phe equivalents to BaP equivalents in fish collected from Buffalo River in the Niagara River ecosystem ranged from 11 to 4 (Eufemia et al., 1997). The same ratio for white sturgeon, *Acipenser transmontanus*, taken from an oil-spill site in the Columbia River was 105. Studies on bile FAC from fish collected from oil spills often only report Phe and Nph equivalents per mg of biliary protein, since the concentrations of BaP equivalents per mg biliary protein are often quite low after exposure only to petrogenic PAH. Bile FAC concentrations from turbot, *Pleuronichthys verticalis*, exposed for 7 days to natural oil seep sediment were 2500, 100 and 5 µg of Nph, Phe and BaP equivalents/ml bile, respectively, while the same values for fish exposed to reference sediment were 700, 20 and 1 µg equivalents (Roy et al., 2003). Concentrations of the total naphthalenes, total phenanthrenes and benzo(a)pyrene in these seep oil sediments were 25, 4 and 0.3 µg/g sediment, respectively, while control sediments contained 0.2 µg/g in naphthalenes and phenanthrenes and

benzo(a)pyrene was not detected. In summary, fish exposed to crude oil have a predominance of 2- and 3-ringed PAH metabolites in the bile, while fish exposed to pyrogenic PAH have a predominance of 4- and 5-ringed PAH metabolites.

Table 5 summarizes bile FAC of fish collected from a number of oil spills. The high concentrations of bile FAC in fish from oil-spill sites shows that fish readily take up and metabolize PAH to polar compounds that are later eliminated. Bile taken from white sturgeon, *Acipenser transmontanus*, 5 days after an oil spill in the Columbia River had elevated FAC concentrations, compared with fish from a reference site (Krahn et al., 1986b). Fish from the oiled station had 200 µg Nph equivalents/g tissue wet wt., 210 µg of Phe equivalents/g tissue wet wt. and 2.1 µg of BaP equivalent/g wet wt. The reference site upriver was 17 µg Nph equivalents/g, 9.6 µg Phe equivalents/g and 0.2 µg BaP equivalents/g. The duration of enhanced levels in the bile of fish from the spill site was not evaluated.

High bile FAC concentrations were found in pink salmon, Pacific halibut and Pacific cod collected from oiled sites near Chenega Bay 120 days after the *Exxon Valdez* spill (Hom et al., 1996). Between 120 and 460 days after the spill there was a 12-fold decrease in FAC concentrations (decreased from 6 to 0.5 µg Phe equivalents/mg biliary protein) in Chenega Bay pink salmon. Thus, one year after the spill there was a significant decrease of fish FAC suggesting a major decrease in the concentrations of bioavailable PAH. During the Alaska study fish were collected from Kodiak Harbor, an industrial site, that was not oiled by EVOS. High concentrations of FAC (up to 7.5 µg Phe equivalents/mg biliary protein in halibut) were found during both years of the two year study. The PAH sources for Kodiak Harbor were likely fishing boats, fish processing plants and fueling facilities in the harbor. A study conducted in 1999–2000, ten years after the EVOS showed there were no significant differences between bile FAC in fish from oiled and non-oiled sites in Prince William Sound, Alaska (Huggett et al., 2003). This study also measured concentrations of bile FAC and EROD activities in Pacific cod and Pacific halibut caught off an area of eroding Tertiary sediments and active natural oil seeps east of Prince William Sound that were not significantly different from the levels for Prince William Sound fish. From these results it was concluded that PAH from the natural petrogenic background in the seafloor sediments were bioavailable at low concentrations to bottom fish (Huggett et al., 2003; Page et al., 2004). In summary, FAC levels in fish bile measured in laboratory studies may return to background in 1 month. FAC levels in fish exposed to a spill generally return to baseline/reference levels within one year of a spill, and in some areas background FAC can be high because of other PAH inputs, both natural and anthropogenic.

Table 5  
Bile fluorescent aromatic compounds (FAC) in fish collected after oil spills and from oil seeps

Spill and reference sites	Oil type and amount spilled ( $\times 10^6$ L)	Fish species	Time after spill (days)	Concentrations of PAH metabolites in bile			Reference
				$\mu\text{g}$ Naph equiv/mg bile ( $\times 10^3$ )	$\mu\text{g}$ Phen equiv/mg bile ( $\times 10^3$ )	$\mu\text{g}$ BaP equiv/mg bile ( $\times 10^3$ )	
Columbia R. spill	Industrial oil (0.7)	<i>A. transmontanus</i>	20	210		2.1	Krahn et al. (1986b)
Ref. site	–	<i>A. transmontanus</i>		17	9.5	0.2	Krahn et al. (1986b)
Gulf War	Kuwait crude (960–1300)	<i>L. khallopterus</i>	$\approx 300$	–	$20 \pm 21$	–	Krahn et al. (1993)
Ref. site (Rennie S.)	–	<i>L. khallopterus</i>	$\approx 300$	–	$10 \pm 2.2$	–	Krahn et al. (1993)
Ref. site (Qatar)	–	<i>L. khallopterus</i>	$\approx 300$	–	$17 \pm 13$	–	Krahn et al. (1993)
Oil seep (Calif. Coast)	Seep crude (8000 L/day)	<i>R. toxodes</i>	Continuous	$110 \pm 90$	$140 \pm 150$	–	Spies et al. (1996)
Ref. site	–	<i>R. toxodes</i>	–	$22 \pm 11$	$30 \pm 20$	–	Spies et al. (1996)
Alaska, Snug Harbor	North Slope crude (75)	<i>P. bilineatus</i>	$\approx 120$	$42 \pm 25$	$11 \pm 7$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Snug Harbor	North Slope crude (75)		$\approx 460$	$42 \pm 3$	$9 \pm 6$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Snug Harbor	North Slope crude (75)		$\approx 760$	$24 \pm 14$	$5 \pm 3$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Snug Harbor	North Slope crude (75)	<i>S. malma</i>	$\approx 460$	–	$20 \pm 2$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Snug Harbor	North Slope crude (75)	<i>H. elassodon</i>	$\approx 120$	–	$8 \pm 3$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Snug Harbor	North Slope crude (75)	<i>H. elassodon</i>	$\approx 460$	–	$4 \pm 1$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Snug Harbor	North Slope crude (75)	<i>H. elassodon</i>	$\approx 760$	–	$3 \pm 1$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Tonsina Bay	North Slope crude (75)	<i>P. bilineatus</i>	$\approx 120$	–	$11 \pm 1$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Tonsina Bay	North Slope crude (75)	<i>P. bilineatus</i>	$\approx 460$	–	$6 \pm 1$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Kukak Bay	–	<i>P. bilineatus</i>	$\approx 120$	–	$2 \pm 1$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
(Ref. site)	–	<i>S. malma</i>	$\approx 120$	–	$1 \pm 0.5$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
(Ref. site)	–	<i>H. elassodon</i>	$\approx 120$	–	$2 \pm 1$	–	Collier et al. (1995, 1996), Varanasi et al. (1995)
Alaska, Bay of Isles	–	<i>H. octogrammus</i>	$\approx 3600$	–	$5 \pm 1$	–	Jewett et al. (2002)
Alaska, Barnes Cove	–	<i>H. octogrammus</i>	$\approx 3600$	–	$5 \pm 2$	–	Jewett et al. (2002)
(Ref. site)	–						
Alaska, Oiled areas	–	<i>S. caurinus/S. maliger</i>	$\approx 3600$	–	1.7	–	Huggett et al. (2003)
Alaska, Non-oiled area	–	<i>S. caurinus/S. maliger</i>	$\approx 3600$	–	1.6	–	Huggett et al. (2003)

Fish assayed included sheiry (*Lethrinus khallopterus*), white sturgeon (*Acipenser transmontanus*), rock sole (*Pleuronectes bilineatus*), Dolly Varden (*Salvelinus malma*), flathead sole (*Hippoglossoides elassodon*), surf perch (*Rachochilus toxodes*), masked greenling (*Hexagrammos octogrammus*) and copper/quillback rockfish (*Sebastes caurinus*, *Sebastes maliger*). Concentration of PAH metabolites in bile are given as  $\mu\text{g}$  of naphthalene equivalents/mg bile protein,  $\mu\text{g}$  of phenanthrene equivalents/mg bile protein and  $\mu\text{g}$  of benzopyrene equivalents/mg bile protein. Abbreviations: Naph = naphthalene, phen = phenanthrene, BaP = benzo(a)pyrene, ref = reference.

## 7. Marine mammal bile fluorescent aromatic compounds (FAC) after oil exposure

Work on bile FAC in harbor seals and Stellar sea lions after EVOS is summarized by Hom et al. (1999). In 1989, approximately 120 days after the spill, harbor seals from the heavily oiled Bay of Isles had FAC concentrations of  $72 \pm 93 \mu\text{g}$  of Phe equivalents/g biliary protein compared with FAC concentrations of 0.017 to  $8 \mu\text{g}$  of Phe equivalents/g biliary protein from harbor seals collected from non-oiled sites. By 1990 the FAC in harbor seals in the Bay of Isles had decreased to  $12 \mu\text{g}$  of Phe equivalents/g biliary protein (one sample). Similar decreases in bile FAC were found in harbor seals sampled in 1989 and 1990 from oiled Herring Bay. Stellar sea lions were not sampled from the same sites as harbor seals and those that were sampled were not from oiled areas. Sea lions had bile FAC ranging from 0.039 to  $8.5 \mu\text{g}$  Phe equivalents/g biliary protein. The assumption was made that EVOS was the primary contributor to the bile FAC found in harbor seal sampled in oiled areas in 1989. It should be noted that both seals and sea lions do not necessarily remain in one bay but move about for food and during breeding (King, 1983). While EVOS likely contributed to the seal FAC in heavily oiled bays, it cannot be assumed, without detailed tissue hydrocarbon chemistry that all phenanthrene-type metabolites found in the harbor seal bile were derived from EVOS. Hom et al. (1999) reported the results of a limited study of aromatic compounds (AC), including the PAH, in blubber, liver and muscle of a small number of harbor

seals and sea lions from Prince William Sound and the Gulf of Alaska after EVOS. The highest AC concentrations were found in seals from heavily oiled Herring Bay which were  $280 \pm 350$  and  $26 \pm 29 \text{ ng/g}$  wet wt. in 1989 and 1990, respectively. PAH in harbor seals from other sites, both oiled and non-oiled, were much lower than these Herring Bay seals. As with fish, it appears there is a decrease in the FAC levels of marine mammals within a year after oil spill exposure. Most marine mammal studies after oil spills have sampled a relatively small number of animals and the FAC data show a very large variation making interpretation of the data difficult.

## 8. PAH, cytochrome P450 system responses, FAC increases and higher order biological effects

Fish exposed to oil spills often show increases in cytochrome P450 monooxygenase activities, i.e. AHH and EROD activities, CYP1A levels and bile FAC concentrations. For most investigated spills the increases in these parameters were transitory, on the order of a few months, followed by a decrease to baseline levels within a year after the spill. The very strong cytochrome P450 inducers, i.e. dibenzo(*a,h*)anthracene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, are either absent or at very low concentrations in most crude oils (Table 6). Chrysene and its alkylated homologues, weak cytochrome P450 inducers, are present at concentrations ranging from 2 to 400 mg/L in most crude oils (Bence et al., 1996; Bence, private communication). As noted earlier, these compounds

Table 6  
PAH in various crude oils and oiled sediments

PAH	Kuwait crude (ng/g)	North Sea crude ( $\mu\text{g/g}$ )	EVC ( $\mu\text{g/g}$ )	Heavily weathered EVC (ng/g)	Sediments with EVC (ng/g)	Sediments Gulf War (ng/g)
Naphthalene	200	1200	620	1	n.d.	5
Alkyl naphthalenes	2700	5500	5300	71	8000	150
Fluorene	20	270	90	1	n.d.	5
Alkyl fluorenes	630	n.a.	1100	350	15,000	75
Phenanthrene	50	240	260	6	n.d.	7
Alkyl phenanthrenes	900	n.a.	2300	1100	2100	150
Anthracene	n.d.	2	n.d.	n.d.	n.d.	n.d.
Fluoranthene	n.d.	10	2	7	n.d.	n.d.
Pyrene	5	20	10	n.d.	160	2
Alkyl pyrenes	145	n.a.	80	140	3300	5
Chrysene	5	20	50	190	840	1
Alkyl chrysenes	120	80	390	880	1500	18
Benz( <i>a</i> )anthracene	n.d.	11	2	n.d.	n.d.	1
Benzo( <i>b</i> )fluoranthene	n.d.	4	6	30	130	1
Benzo( <i>k</i> )fluoranthene	n.d.	n.d.	n.d.	n.d.	n.a.	1
Benzo( <i>e</i> )pyrene	n.d.	n.a.	n.a.	n.a.	130	1
Benzo( <i>a</i> )pyrene	n.d.	1	n.d.	2	n.d.	n.d.
Dibenz( <i>a,h</i> )anthracene	n.d.	n.d.	1	5	n.d.	n.d.
Indeno [1,2,3- <i>cd</i> ]pyrene	n.d.	n.d.	1	n.d.	n.a.	n.d.

Data was taken from Burns et al. (1997) for EVC (*Exxon Valdez* crude) and EVC heavily weathered, Sauer et al. (1993) for Kuwait crude and Gulf Water sediment one year after spills, Shigenaka and Henry (1995) for oiled sediments from Smith Island, Prince William Sound, two years after *Exxon Valdez* spill and Aas et al. (2000) for North Sea crude oil. Abbreviations: n.d. = not detected; n.a. = not available.

may be important contributors to observed induction of cytochrome P450 systems in fish collected from oil-spill sites. The primary contributors to FAC observed in fish after oil spills are naphthalene- and phenanthrene-like metabolites. Thus, a combination of increased bile FAC and cytochrome P450 system responses are good indicators of PAH exposure after an oil spill. Collier et al. (1993) and Hellou and Upshall (1995) found that sediment PAH concentrations correlated with fish bile FAC, but not with fish EROD activities. Similarly, Lin et al. (1994) found that concentrations of Bass Strait crude oil in the water correlated with Atlantic salmon bile FAC, but not with fish EROD activity. The lack of correlation between crude oil hydrocarbon concentrations in water or sediment and fish EROD activity may be due to the low amounts of strong cytochrome P450 inducers in most crude oils. In contrast, bile FAC are more reflective of the major petroleum PAH components, particularly naphthalenes and phenanthrenes.

There is no proven relationship between petroleum TPAH concentrations, FAC, cytochrome P450 system responses and higher order biological effects in fish. Woodin et al. (1997) reported higher CYP1A in intertidal fish in oiled areas of Prince William Sound in Alaska compared to unoiled reference areas, but noted that a relationship between the health of these intertidal fish and induction of CYP1A could not be established. Collier et al. (1995) noted that mechanistic linkages between the induction of CYP1A and such biological effects as liver lesions, immunosuppression or reduced growth or survival have not been established.

The relationship between high sediment PAH concentrations and higher order biological effects has been investigated at a number of coastal sites containing primarily pyrogenic hydrocarbons. Hepatic neoplasm and related lesions have been reported in fish from Puget Sound (WA) and Elizabeth River (VA) at sites with PAH concentrations up to 2200 µg/g sediment (Vogelbein et al., 1990). Moore and Myers (1994) noted significant correlations between bile FAC, induction of CYP1A and liver neoplasia in English sole, *Parophrys vetulus*, collected from sites in Puget Sound with high sediment PAH. While histopathological lesions were found in plaice collected from some heavily oiled embayments in the *Amoco Cadiz* spill zone on the Brittany coast, France (Haensley et al., 1982), other studies on fish from oil-spill sites did not find histopathological lesions (Ritchie and O'Sullivan, 1994; Wiedmer et al., 1996). While not related to oil spills, EROD has been correlated with histopathological changes (volume density of lipofuscin/ceroid in hepatocytes) in immature demersal fish inhabiting a contaminated site (Au and Wu, 2001). Increases in the abundance of lipofuscin granules have been correlated with EROD activities when benzo(a)pyrene has either been injected or added to the diet of fish, but depuration demonstrated that these responses were reversible (Au et al., 1999, 2004). High concentrations of crude oil hydrocarbons in sediments, while linked in some cases to elevated EROD in exposed fish, are not linked to the effects, e.g. lipofuscin granules, liver neoplasia, often seen in fish exposed to high concentrations of pyrogenic PAH in sediments.

Table 7

Sediments with mixture of pyrogenic and petrogenic PAH (all values are in ng/g sediment)

PAH	Buffalo River, New York	Black Creek, New York	Penobscot Bay, Maine	Elizabeth River, Virginia	Drier Bay, Alaska	Sleepy Bay, Alaska
Naphthalene	81	n.d.	n.a.	n.a.	n.a.	n.a.
Alkyl naphthalenes	66	n.d.	n.a.	n.a.	635	65
Fluorene	68	n.d.	n.d.	n.a.	270	8
Alkyl fluorenes	n.a.	n.a.	n.a.	n.a.	406	32
Phenanthrene	440	12	250	2500	2500	84
Alkyl phenanthrenes	82	n.d.	n.a.	n.a.	1400	98
Anthracene	180	n.d.	49	n.a.	722	14
Fluoranthene	890	39	3700	42,000	4200	93
Pyrene	740	30	502	28,000	4500	127
Alkyl pyrenes	n.a.	n.a.	n.a.	n.a.	3700	121
Chrysene	540	22	276	19,000	2000	54
Alkyl chrysenes	n.a.	n.a.	n.a.	n.a.	3500	87
Benz(a)anthracene	450	12	512	11,000	2000	44
Benzo(b)fluoranthene	430	35	601	17,000	2600	47
Benzo(k)fluoranthene	160	17	400	n.a.	970	14
Benzo(e)pyrene	190	17	376	6000	1500	26
Benzo(a)pyrene	350	13	376	9000	2400	39
Dibenz(a,h)anthracene	64	n.d.	37	n.a.	300	5
Indeno [1,2,3-cd]	270	14	198	n.a.	1500	20

Data taken from Eufemia et al. (1997) for Buffalo River and Black Creek, from Johnson and Larsen (1985) for Penobscot Bay, from Bieri et al. (1986) for Elizabeth River and from Page et al. (2002) for Drier Bay and Sleepy Bay. Abbreviations: n.d. = not detected; n.a. = not available.

The distribution of individual PAH in sediments with primarily pyrogenic PAH are quite different from the PAH profiles of crude oil or oiled sediments (Neff, 2002; Krahn et al., 1992; Page et al., 1996, 1999) (Tables 6 and 7). The PAH profile of crude oil is dominated by high concentrations of 2- and 3-ringed compounds, particularly naphthalenes and phenanthrenes (Table 6) with alkyl homologues being more abundant than unsubstituted PAH in each series. The pyrogenic PAH found in some contaminated coastal sites, include high concentrations of a number of 4 to 6-ringed unsubstituted PAH, such as pyrene, benzo(a)pyrene, benzo(k)fluoranthene, dibenzo[a,h]anthracene, and indeno[1,2,3,-cd]pyrene, which are very strong inducers of cytochrome P450 systems (Tables 1 and 7). Benzo(a)pyrene has been shown to induce neoplasia in fish (Hendricks et al., 1985) and it is assumed that this compound as well as other strong cytochrome P450 inducers are responsible for the high incidence of hepatic neoplasms found at sites with high concentrations of pyrogenic PAH. In the Puget Sound area, the percent of neoplasms in English sole from Eagle Harbour, Duwamish Waterway and President Point (reference site) were 18%, 21% and 0%, respectively, while the concentrations of benzo(a)pyrene in sediments at these sites were 2300, 73 and 41 ng/g sediment, respectively (Baumann, 1989). Fish with liver lesions in Puget Sound were found to have significantly higher concentrations of benzo(a)pyrene-like metabolites in bile than did fish without lesions (Krahn et al., 1984). No correlations were found between concentrations of naphthalene- or phenanthrene-like PAH metabolites and liver lesions.

As noted earlier, fish collected from oil spills have predominantly naphthalene- or phenanthrene-like metabolites and very low concentrations of benzo(a)pyrene like metabolites in their bile. Wiedmer et al. (1996), citing the results of benzo(a)pyrene studies, suggest that fish exposed to oil have an increased potential for mutagenesis and carcinogenesis and diminished reproductive potential. However, as noted earlier, benzo(a)pyrene is generally not detected in crude oils and is not a good surrogate for oil spill studies. A better surrogate for crude oil would be phenanthrenes, fluorenes or chrysenes (Table 6).

After an oil spill, it is important to determine the contribution of all sources of PAH, both petrogenic and pyrogenic, to observed cytochrome P450 systems responses, bile FAC and higher order biological effects on fish populations. A good example of the importance of non-spill PAH sources is shown by the analysis of sediments at various bays in Prince William Sound sampled after the *Exxon Valdez* spill (Page et al., 1996, 1999, 2002). Many of these bays, including some oiled by the *Exxon Valdez* spill, had relatively high sediment concentrations of pyrogenic hydrocarbons. The PAH profile of Sleepy Bay sediments in 1999 and 2000, which was

heavily oiled by the *Exxon Valdez* spill, showed inputs of both petrogenic and pyrogenic PAH (Page et al., 2002; Table 7). Analysis of a 1999 subtidal sediment sample from this bay showed that 63% of the PAH were pyrogenic while 8% of the PAH were similar to weathered *Exxon Valdez* oil. Benthic sediment samples from Drier Bay, which was not oiled by the *Exxon Valdez* spill, had concentrations of pyrogenic PAH of up to 39,000 ng TPAH/g sediment dry wt. There were high concentrations of carcinogenic 4 to 6-ringed pyrogenic PAH, including benzo(a)pyrene, benzofluoranthenes and in-deno[1,2,3-cd]pyrene, whose source was likely copper mines and canneries which formerly operated in this bay (Table 7).

In a study of biomarkers in nearshore fish in the *Exxon Valdez* spill zone, Jewett et al. (2002) found the highest bile FAC concentrations ( $5300 \pm 972$  Phe equivalents/mg bile protein) in masked greenling (*Hexagrammus octagrammus*) caught in unoiled Barnes Cove, Drier Bay, indicative of exposure to non-spill related PAH. Pyrogenic PAH are likely to be the main influence on bile FAC and cytochrome P450 systems in fish from bays where there has been a history of human and industrial activity.

## 9. Conclusions

There is a body of literature showing that exposure of fish to PAH in water, sediment or food can result in increased cytochrome P450 responses and bile FAC concentrations. Some fish collected from an oil spill zone often show increases in the levels of these biomarkers during the first few months after the spill followed by decreasing levels during the remainder of the year. Long-term, multi-year biomarker work at oiled and un-oiled sites has not always taken into account the influence of non-spill PAH from pyrogenic, petrogenic and biogenic sources on these biomarkers. We suggest that a much greater effort than is often observed is needed after an oil spill to comprehensively identify all PAH sources in the spill zone before applying biomarker analyses to document exposure to petroleum hydrocarbons from the spill. The studies carried out to date have not demonstrated that these biomarkers can be used to assess oil spill PAH exposure in marine invertebrates. There is some evidence of increased bile FAC and cytochrome P450 responses in marine mammals and birds from oil-spill sites, but more work needs to be carried out on this topic since these animals migrate over large distances and thus are exposed to PAH from a variety of sources. Fish from areas with high pyrogenic PAH concentrations in the sediment, e.g. portions of Puget Sound, have high bile FAC concentrations, induction of CYP1A activities, and increased incidence of liver neoplasia. When lesions are observed in fish species in

harbor areas, the predominant PAH present in the sediments are pyrogenic. Fish from areas with high petrogenic PAH in the sediment have increased bile FAC, possible induction of CYP1A activities but no increase in the incidence of liver neoplasia. An important caution in using CYP1A activity as a measure of exposure to PAH is that results are species/tissue specific and age, sex, season, and temperature effects must be taken into account. There is no clear linkage between CYP1A induction and long-term effects on individuals or populations of fish, birds or mammals.

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