Exxon Valdez Oil Spill
Restoration Project Final Report

Exposure to Hydrocarbons Ten Years after the *Exxon Valdez* Oil Spill: Evidence from Cytochrome P4501A Expression and Biliary FACs in Nearshore Demersal Fishes

Restoration Project 99379
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October 2000

TO BE SUBMITTED TO MARINE ENVIRONMENTAL RESEARCH

Exposure to hydrocarbons ten years after the *Exxon Valdez* oil spill: evidence from cytochrome P4501A expression and biliary FACs in nearshore demersal fishes

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Abstract

Three biomarkers of hydrocarbon exposure, CYP1A in liver vascular endothelium, liver ethoxyresorufin *O*-deethylase (EROD), and biliary fluorescent aromatic compounds (FACs), were examined in the nearshore fishes masked greenling (*Hexagrammos octogrammus*) and crescent gunnel (*Pholis laeta*) collected in Prince William Sound, Alaska, 7 to 10 years after the *Exxon Valdez* oil spill (EVOS). All biomarkers were elevated in fish collected within the original oil spill trajectory compared to reference sites. In 1998, endothelial CYP1A in masked greenling from sites that were heavily oiled in 1989 was significantly higher than in fish collected outside the spill. In 1999, fishes collected from sites adjacent to intertidal mussel beds containing lingering *Exxon Valdez* oil had elevated EROD and endothelial CYP1A, and high concentrations of biliary FACs. Fish from sites near unoiled mussel beds, but within the original spill trajectory, also showed evidence of hydrocarbon exposure, although there were no correlations between sediment petroleum hydrocarbon and any of the biomarkers. Our data show that 10 years later, nearshore fishes within the original spill were still exposed to residual EVOS hydrocarbons.

Keywords

Exxon Valdez; oil spill; Prince William Sound; biomarkers; fishes; cytochrome P4501A; CYP1A; EROD; FACs

Abbreviations (probably not appropriate for MER)

CYP1A = cytochrome P450 1A, EROD = ethoxyresorufin *O*-deethylase, EVOS = *Exxon Valdez* oil spill, FACs = fluorescent aromatic compounds, IHC = immunohistochemical, NPH = naphthalene, PAH = polycyclic aromatic hydrocarbons, PHN = phenanthrene, THC = total petroleum hydrocarbon concentrations.

1. Introduction

When the super tanker *Exxon Valdez* ran aground in March 1989 in northern Prince William Sound (PWS), Alaska and spilled nearly 42 million liters of crude oil, it became the largest spill in US history (Spies, Rice, Wolfe, & Wright, 1996). Nearshore environments were severely impacted by the spill and associated cleanup efforts, especially in the first three years subsequent to the spill (Spies et al., 1996; Peterson, 2000). Affected resources in the intertidal and shallow subtidal regions included dominant plants, a suite of infauna and epifauna, and many species of fish , birds, and marine and terrestrial mammals. More than 20 fish species examined after the spill showed evidence of exposure to and effects of petroleum, including cytochrome P450 1A (CYP1A) induction, genetic damage, physical deformities, reduced abundance and growth, and/or compromised survival of some life stages (Table 1).

Concentrations of *Exxon Valdez* oil in sediments and in animal tissues diminished greatly within the first several years following the spill (Armstrong, Dinnel, Orensanz, Armstrong, McDonald, & Cusimano et al., 1995; Boehm, Page, Gilfillan, Stubblefield, & Harper, 1995; Jewett, Dean, Smith, & Blanchard, 1999; Murphy, Heintz, Short, Larsen, & Rice, 1999). However, recent evidence indicates that relatively high concentrations of oil have persisted at some sites within PWS. In 1995, six years after the spill, mean total petroleum hydrocarbon concentrations (THC) in sediments and mussels from selected mussel beds were twice background concentrations (Carls, Babcock, Harris, Irvine, Cusick, & Rice, 2000). Beneath some low-energy cobble beaches, THC concentrations in 1997 were up to 32,000 μg/g dry weight (Hayes & Michel, 1999). There is also evidence that nearshore vertebrate species, including pigeon guillemots (*Cepphus columba*) (Seiser, Duffy, McGuire, Roby, Golet, & Litzow, 2000), sea ducks (Esler, Schmutz, Jarvis, & Mulcahy, 2000; Trust, Esler, Woodin, & Stegeman, 2000), and sea otters (*Enhydra lutris*) (Ballachey, Stegeman, Snyder, Blundell, Bodkin, & Dean et al., 1999; Monson, Doak, Ballachey, Johnson, & Bodkin, 2000), were still being exposed to hydrocarbons up to nine years after the spill. Thus, there is concern about the potential for oil retained within PWS (Carls et al., 2000) to have adverse impacts on nearshore resources.

We examined two intertidal fishes, masked greenling (*Hexagrammos octogrammus*) and crescent gunnel (*Pholis laeta*) to determine whether these representative nearshore fishes were exposed to hydrocarbons, and if so, to determine the spatial extent and potential sources of contamination 7 to 10 years after the *Exxon Valdez* spill (EVOS). Masked greenling and crescent gunnel are numerically dominant fishes in nearshore PWS and are important members of the food web. Both species are common in nearshore kelp and eel grass beds, where they attain mean densities of 3 to 4 individuals/100 m^{-2} (Dean, Haldorson, Laur, Jewett, & Blanchard, 2000). Both are solitary, and are territorial when guarding their eggs in the fall (Hughes, 1986; Pers. Observ.). Both species feed on a variety of benthic organisms but mainly consume crustaceans, including gammarid amphipods, isopods, tanaids, hermit crabs and helmet crabs

(McConnaughey, 1978; Blackburn, Anderson, Hamilton, & Starr, 1983; Hughes, 1986; Rogers, Wangerin, & Rogers, 1983; Rosenthal, 1983, S. Jewett unpublished data). River otters (*Lontra canadensis*) consume both fish species, while crescent gunnels are eaten also by mink (*Mustela vison*), pigeon guillemots, and a number of fishes (Blackburn et al., 1983; Kuletz, 1983; Rogers et al., 1983; Johnson, 1985; Bowyer, Testa, Faro, Schwartz, & Browning, 1994). Masked greenling and crescent gunnel are relatively abundant, widely distributed and easily captured, and can serve as surrogate indicators of exposure for other vertebrates in nearshore benthic habitats.

Cytochrome P450 1A is a protein that is induced by and plays a critical role in the oxidation of numerous exogenous organic compounds, including polycyclic aromatic hydrocarbons (PAH) and planar halogenated aromatic hydrocarbons. The metabolism of some of these chemicals by CYP1A yields oxidized products that may be inactive, or in some cases, more toxic than the parent compounds. Thus, metabolism of PAH by CYP1A can potentially result in deleterious physiological effects. CYP1A induced in vertebrates by PAH, including those found in oil, can serve as a sensitive biomarker of exposure to these environmental contaminants. In this study, the extent of potential hydrocarbon exposure was determined by examining the expression of CYP1A the expression in liver of masked greenling and crescent gunnel collected from sites that varied with respect to distance from shorelines that were heavily oiled following the EVOS.

Many fluorescent metabolites of polycyclic aromatic hydrocarbons are excreted via the hepatobiliary system in fish and their presence in bile reflects recent exposure to these contaminants (Krahn, Burrows, Ylitalo, Brown, Wigren, & Collier et al., 1992). In addition to measuring CYP1A, we also measured biliary fluorescent aromatic compounds (FACs) in fishes collected from sites near oiled mussel beds, to further determine if the EV oil that remains at these sites is a source of hydrocarbon exposure for resident fish. The levels of CYP1A and biliary FACs in fish within the mussel beds were compared with the degree of oiling in sediments at the same sites.

Determining potential sources of contamination and their persistence and distribution is important in evaluating persistence of effects of the EVOS on nearshore species and making better decisions with respect to management and habitat restoration of resources areas within Prince William Sound.

2. Materials and methods

In 1996 and 1998, we determined exposure of fishes to hydrocarbons by measuring the induction of CYP1A in masked greenling and crescent gunnel collected from sites that varied with respect to the extent of oiling on adjacent shorelines and proximity to the spill zone. A preliminary study in 1996 examined greenling collected from a site that was heavily oiled (Herring Bay) compared to an unoiled site just outside the trajectory of the spill (Jackpot Bay). In

1998, sampling was expanded to include 5 heavily oiled sites, 2 sites that were adjacent to unoiled beaches but within the trajectory of the spill and within 1 km from heavily oiled shorelines, and at 3 sites approximately 5 km or more outside of the spill's trajectory. All sites were within relatively sheltered low-energy embayments with gravel, cobble, or boulder substrate along the shoreline and with mixed cobble, sand, and mud in the nearshore subtidal zone and with predominately kelp (*Agarum clathratum* and *Laminaria saccharina*) and eelgrass (*Zostera marina*) vegetation. The locations of the collection sites relative to the EVOS are shown in Figure 1, and the nature of the sites, species collected and year of sampling are given in Table 2.

In July of 1996, 14 masked greenling were collected at Herring Bay and 6 were taken from Jackpot Bay. Based on the analysis of CYP1A by immunohistochemical assay, we estimated that a sample size of eight fish per site would be sufficient to detect a 50% difference in CYP1A levels between sites with an 80% power ($\alpha = 0.05$). In July of 1998, 8 masked greenlings per site were collected from 10 sites and 8 crescent gunnels per site were collected from two oiled sites (Herring Bay and Bay of Isles), from one unoiled site within the spill zone (Mummy Bay), and from one site outside of the spill zone (Port Chalmers).

Masked greenling were collected along shallow (< 5 m) shoreline segments by spearing in 1996, and by hook and line or baited pots in 1998. Crescent gunnel were also collected by pots as well as by hand in the intertidal zone. All fish were killed by a blow to the head, measured (fork length), and sexed. The peritoneal cavity of fishes was opened to ensure complete preservation of internal organs in 10% neutral buffered formalin solution. The fish were shipped to Woods Hole Oceanographic Institution (WHOI), MA, for embedding, sectioning and immunohistochemical analysis of CYP1A as described previously (Smolowitz, Hahn, & Stegeman, 1991).

In the summer of 1999, sample sites were selected to examine the possible relationship between high residual concentrations of *Exxon Valdez* oil in mussel beds and exposure to hydrocarbons (as indicated by elevated levels of CYP1A) in nearshore fishes. Masked greenling were collected from 8 sites within 200 m of intertidal mussel beds where oil was found in relatively high concentrations in prior years (Babcock, Irvine, Harris, Cusick, & Rice, 1996) and from near mussel beds at two sites that were unoiled but within the oil spill trajectory (Table 2, Fig. 1). Crescent gunnel were collected from 7 oiled mussel beds and one unoiled site. Six to 8 greenling and/or crescent gunnels were collected from each site. All sample sites were locations where sediments were simultaneously collected for hydrocarbon analysis by personnel from the National Oceanic and Atmospheric Administration/Auke Bay Laboratory (NOAA/ABL). CYP1A induction (IHC staining and EROD activity) and concentrations of bile fluorescent aromatic compounds (FACs) were determined in these fish as indicators of hydrocarbon exposure.

Sampling procedures in 1999 were as described for 1998, except that one half of each liver was preserved in 10% formalin solution (for IHC analysis) and the other half was frozen in liquid nitrogen (for EROD measurement). Bile was also collected and stored cryogenically according to the protocol of Stehr, Myers, & Willis (1993). Bile could not be collected from some fish with empty gall bladders. All bile samples were archived frozen at UAF until selected samples were sent to NOAA, National Marine Fisheries Service (NMFS), Seattle, WA for FAC analysis.

Sediments were collected from mussel beds for determination of hydrocarbon concentrations. Mussel beds ranged in size from approximately 20 to 500 m^2 and most were situated on mixed sand and gravel substrates, with mussels relatively evenly dispersed throughout the sampling area. A transect, generally 30 m long and parallel to the water line, was established through the middle of the mussel bed. Three pooled subsamples of surficial sediments (0–2 cm) under the mussels were collected with a HC-free stainless steel spoon into HC-free glass jars. Surficial sediment samples were also taken in each bed from spots where a hydrocarbon sheen or odor was observed.

2.1. Analyses of CYP1A , biliary FACs in bile and total hydrocarbons.

CYP1A levels were evaluated by immunohistochemical detection of protein (IHC) and ethoxyresorufin O-deethylase (EROD), using methods described before (Smolowitz et al., 1991; Hahn, Woodward, Stegeman, & Kennedy, 1996). For IHC analysis, CYP1A was immunodetected in 5 μ sections of paraffin-embedded liver using monoclonal antibody 1-12-3 to scup (a marine teleost) CYP1A, which cross reacts with greenling and crescent gunnel CYP1A. As described previously (Woodin, Smolowitz, & Stegeman, 1997), a scalar score of occurrence X intensity was determined for CYP1A staining in specific cell types. Scores for immunohistochemical staining of CYP1A in hepatocytes have been shown to correlate very well with the levels of CYP1A determined by protein immunoblotting of microsomes prepared from the same livers (Woodin et al., 1997).

The analysis of EROD activity was carried out with post mitochondrial supernatant preparations (PMS). Average gunnel liver size was <40mg, and for this reason PMS were prepared and analyzed instead of microsomes. Livers that had been at - 80 ^{o} were thawed to 2^{o}C, homogenised in ice cold buffer and centrifuged at 600 X g for 10 min followed by centrifugation at 12,000 X g for 10 min as described previously (Stegeman, Binder, & Orren, 1979). EROD activities were determined for the resulting PMS by using a Cytoflour fluorescence plate reader (Hahn et al., 1996), and the data were expressed as pmol resorufin/min/g liver.

Fluorescent metabolites of PAHs (FACs) are primarily excreted via the hepatobiliary system in fish. FACs in bile of masked greenling were measured at NOAA, Seattle, WA, according to the protocol of Varanasi, Collier, Krone, Krahn, Johnson, & Myers et al. (1995). PAHs of differing ring structure are distinguished and quantified by this method

(Krahn et al., 1992). FACs were analysed in fish collected in 1999 which exhibited a wide range of EROD activities. Fluorescence wavelengths appropriate for phenanthrene (PHN) and naphthalene (NPH) were monitored, since these wavelengths have been shown to be most useful for assessing petroleum exposure in fish (Krahn et al., 1992). Concentrations of FACs are presented as ng PHN equivalents/mg total bile.

Sediment samples were analyzed for hydrocarbons at the NOAA/ABL using ultraviolet fluorescence as adapted from Krahn, Ylitalo, Joss, & Chan (1991) and Krahn, Ylitalo, Buzitis, Chan, Varanasi, & Wade et al. (1993). Hydrocarbons were extracted twice from sediments with methylene chloride, separated by high-performance liquid chromatography (HPLC), and quantified using a fluorescence detector at the optimal wavelengths for phenanthrene (260 nm excitation, 380 nm emission) by comparison to known amounts of *Exxon Valdez* oil. Total petroleum hydrocarbon (THC) concentrations were reported as μg/g sediment wet weight. The method detection limit for THC was 50 μg/g.

2.2. Statistical analysis

We tested the hypothesis that hydrocarbon exposure did not differ among sites with respect to proximity to oiled shorelines using a one-way, nested analysis of variance. For fishes collected in 1998, liver endothelial cell CYP1A staining scores were tested for differences among oiling categories (oiled, unoiled within the spill zone and unoiled outside of the spill zone) with sites nested within oiling category. In cases in which there was a significant difference among oiling categories, contrasts were performed to determine differences between individual categories. Bonferroni's multiple comparisons (Milliken & Johnson, 1984) were used to contrast individual sites.

Differences among sites for CYP1A (IHC and EROD) measured in fish collected in 1999 were tested using a one-way ANOVA and site means were contrasted using the Bonferonni procedure described above. Also, for sites in which IHC levels were determined in both 1998 and 1999, we tested for differences between years, between sites, and for a significant interaction between site and year using a two-way ANOVA. In cases where there were significant differences between years, we also performed contrasts to determine differences between years within each site. In all the analyses, IHC scores and EROD activities were log-transformed (x+1) prior to statistical treatment to reduce heteroscedasticity, and data were combined for all sexes and sizes of fish as preliminary analyses indicated no differences attributable to either sex or size.

Relationships between concentrations of hydrocarbons from oiled mussel bed sediments and various indicators of hydrocarbon exposure in fishes (CYP1A, EROD and FACs) were explored using Pearson Product Moment correlation. Mean sediment THC concentrations at each site sampled in 1999 were correlated with means for each of the

fish exposure indices. We also performed correlation analyses to explore possible relationships between IHC CYP1A scores, EROD activity, and FAC concentrations within individual fish.

3. Results

The average lengths of masked greenling and gunnel were 213 mm (SE = 4.2) and 134 mm (SE = 2.8), respectively. Analysis of otoliths from greenling ranging in size from 198 mm to 316 mm revealed that these fish ranged from 1 to 3 years of age (S. Jewett, unpublished data). No age data were obtained for gunnel.

Probing of liver with monocloanl antibody 1-12-3 showed that CYP1A was more strongly expressed in vascular endothelium, with slight levels in hepatic parenchyma (Fig. 2). The liver vascular endothelium staining scores of 14 masked greenling collected from the oiled area at Herring Bay in 1996 averaged 1.7 (± 0.66 SE); no staining was observed in 6 greenling from the reference site at Jackpot Bay.

CYPIA levels in liver vascular endothelium measured by IHC differed significantly between sites in 1998, for both masked greenling ($p = 0.005$) and crescent gunnel ($p = 0.01$; Table 3 and Fig. 3). CYPIA was detected at 7 of 10 sites for masked greenling and 1 of 4 sites for crescent gunnel. For masked greenling, endothelial CYP1A staining was significantly higher in fishes collected from sites that were adjacent to shorelines that were heavily oiled following the EVOS compared to unoiled sites outside of the oil spill's trajectory (Table 3). Endothelial CYP1A in fishes from unoiled sites within the spill zone also tended to be higher than for fishes from sites outside the zone, but differences were not significant ($p = 0.069$, Table 3). CYP1A staining scores for greenling were highest at two heavily oiled sites, Bay of Isles and Herring Bay (Fig. 3). Mean IHC scores for gunnel liver endothelium did not differ significantly between oiling categories ($p = 0.186$, Table 3), but were significantly higher at Herring Bay than at Bay of Isles or the two sites adjacent to unoiled shorelines (Fig. 3).

In 1999, fish were collected from sites near intertidal mussel beds where high concentrations of PAHs were present in 1995, and at several nearby mussel beds that were unoiled but within the spill trajectory along Knight Island. IHC scores for *H. octogrammus* did not differ significantly ($p = 0.06$) between sites, although mean site scores were highest at Bay of Isles and Herring Bay, two heavily oiled sites (Fig. 4). Mean scores for *P. laeta* endothelial CYP1A were significantly different ($p = 0.001$) for oiled vs. unoiled sites, with the highest values occurring at the oiled sites of Sleepy Bay and Herring Bay (Fig. 4).

In a two-way analysis of *H. octogrammus* liver endothelial CYP1A at sites sampled in multiple years, there was a difference between sites ($p = 0.01$), but no reduction over time ($p = 0.55$), and no interaction between site and time ($p = 0.99$; Fig. 5). Similar analysis for *Pholis laeta* revealed no site differences ($p = 0.06$) between Herring Bay and Bay of

Isles, and no interaction between them ($p = 0.76$), but a significant increase in activity ($p = 0.02$) at both sites between 1998 and 1999 (Fig. 5).

Only IHC analyses were conducted on tissues collected in 1996 and 1998. Declining CYP1A IHC signal, perhaps due to the reduction of residual EV oil, led to the use of EROD, a sensitive CYP1A-dependent catalytic assay, on 1999 samples. EROD activity was measurable in all fish of both species at all sites sampled in 1999 (Fig. 6). As seen for endothelial CYP1A IHC scores in 1998 and 1999, EROD activities were generally higher at heavily oiled sites, but there was broad overlap between sites. For greenling, EROD activity at Herring Island (within Herring Bay) was significantly higher than at two sites on the far western Sound (Foul Bay and Chenega Island) and was higher at Bay of Isles than at Foul Bay (Fig. 6). Greenling from Herring Island had the highest mean EROD of 344 ± 83 pmol/min/g liver. The highest single EROD value, 705 pmol/min/g liver, was observed in an animal from Sleepy Bay. Contrasts did not discriminate differences between sites for crescent gunnels (*P. laeta),* which exhibited lower mean EROD activities than the greenling collected at the same sites.

FACs analysis was conducted on 30 bile samples from *H. octogrammus* collected in 1999 that had a range of EROD values from 7 to 705 pmol/min/g liver. The concentrations of biliary FACs determined at phenanthrene (PHN) and naphthalene (NPH) wavelengths were highly correlated ($n = 30$, $r = 0.97$, $p < 0.001$), therefore, only the concentrations measured at the PHN wavelength pair are reported. Nearly all processed bile had FAC concentrations indicative of exposure to organic chemicals (Fig. 7). We did not test for differences in FAC values between sites because samples selected for analysis were not randomly chosen from the fish collected. However, there were no obvious trends that would suggest differences among sites. The average FACs of all 30 samples was 4,135 ± 356 (SE) ng PHN equivalents/mg total biliary protein. The average site FAC concentrations ranged from 2,100 ± 613 ng PHN/mg protein at oiled Disk Island to nearly 5,300 ± 972 at Barnes Cove.

Although IHC scores, EROD activity, and FAC concentrations together showed that fishes within the spill trajectory were effected by hydrocarbon exposure in 1999, there were no significant positive correlations between these measures within individual fish (Table 4).

Relatively high concentrations of petroleum hydrocarbons were found in sediments from oiled mussel beds adjacent to fish collection sites in 1999 (Table 5). Six of the 10 sites (Chenega Island, Disk Island, Foul Bay, Upper Passage, Herring Island and Bay of Isles) had THC concentrations from composited or spot surface samples that exceeded nearly 5,000 µg/g wet weight. THC was often higher in subsurface depths. For example, in Bay of Isles the mean THC value of composited samples was 1,796 µg/g at 0-2 cm depths and 9,102 µg/g at 4-6 cm. Sediment hydrocarbon levels were compared with CYP1A and FAC values in resident fish. However, there were no significant

positive correlations between sediment hydrocarbon concentrations and IHC scores, EROD activities, or FACs for masked greenling or crescent gunnel collected from these sites (Table 4). In fact, a significant inverse correlation was observed between sediment TPH concentrations and IHC scores for gunnel.

4. Discussion

Soon after the EV spill elevated levels of CYP1A were reported in 11 species and elevated biliary FACs in 9 species of pelagic and demersal fishes at various life stages (Table 1). When a small number of masked greenling and crescent gunnel were examined seven years after the spill (1996), there was little expectation of finding evidence of EVO exposure. Declining CYP1A levels (measured as hepatic aryl hydrocarbon hydroxylase) and biliary FACs were reported in nearshore demersal fishes collected from 1989 through 1991 (Collier, Krone, Krahn, Stein, Chan, & Varanasi, 1996). Although measurable levels of CYP1A and/or FACs suggested continued exposure to oil in yellowfin sole (*Pleuronectes asper*), rock sole (*P. bilineatus*), and flathead sole (*Hippoglossoides elassodon*) collected in 1991 from shallow (< 30 m) oiled sites, the values were near those of reference sites. CYP1A immunohistochemical staining was elevated in masked greenling from oiled Herring Bay in 1996 relative to reference sites, suggesting exposure to EVO in nearshore fishes as many as 7 years after the spill. Subsequent sampling of this species and crescent gunnel in 1998 and 1999 revealed that CYP1A levels were elevated in liver vascular endothelia at most sites 9 to 10 years after the spill. Teal, Farrington, Burns, Stegeman, Tripp, & Woodin et al. (1992) observed CYP1A induction in the marsh fish *Fundulus heteroclitus* 20 years after the West Falmouth oil spill.

Laboratory experiments in the intertidal prickleback fish *Anoplarchus purpurescens* have shown that the levels of hepatic CYP1A induced by exposure to EV oil returned to control levels (near zero detection) within 4 weeks after placing the fish over clean sediment (Woodin et al., 1997). There could be species differences, but generally CYP1A levels decline after cessation of exposure to rapidly metabolized inducers such as PAH. Thus, even the low vascular IHC staining scaled scores (< 2) seen in 1996-99 suggest persistent exposure to hydrocarbons. The presence of CYP1A staining in hepatic vessels is not a surprising result, as vascular endothelium is the first organ interacting with blood-borne hydrocarbon xenobiotics. Depending on the levels and route of exposure, the removal of these compounds by hepatic blood vessel CYP1A could reduce the dose and hence the response in hepatocytes. Hepatocyte CYP1A staining is known to correlate well with liver microsomal measures of CYP1A (Woodin et al., 1997). The lack of CYP1A IHC signal in hepatocytes seen here, although low levels of EROD were detectable in liver microsomes, may be attributable to the lower sensitivity of IHC.

Although EROD values in 1999 suggest a slight induction, these activities were relatively low compared to levels seen historically in fish from highly contaminated sites. EROD activities in livers of demersal fishes exposed to contaminants vary greatly. For example, in several species of flatfish exposed to a suite of organic contaminants, EROD ranged from 175 to 5,000 pmol resorufin/min/mg of liver microsomal protein (e.g., Stegeman, Teng, & Snowberger, 1987; Collier, Anulacion, Stein, GoksØyr, & Varanasi, 1995; Kirby, Neall, & Tylor, 1999). Assuming a typical 5-10% microsomal protein yield, the highest mean EROD activity determined in this study for masked greenling collected from Herring Island can be calcualted at just 3 to 6 pmol resorufin/min/mg microsomal protein. Thus, although the EROD values in greenling and gunnel in 1999 exhibit significant site differences, they suggest a minimal residual hydrocarbon impact when compared to other polluted sites. However, even low EROD activity might be attributed to contaminant exposure.

Bile fluorescent aromatic compounds (FACs) may be one of the best indicator of recent exposure to polynuclear aromatic hydrocarbons (Arcand-Hoy & Metcalfe, 1999). EVOS derived biliary FACs in demersal fish declined from 1989 through 1991, with little difference existing between fish collected from oiled and reference sites by 1991 (Collier et al., 1996). Nevertheless, a hydrocarbon signature was still present in biliary FACs, even at sites thought not to have been impacted by oil, whether within and outside the spill trajectory. The mean values of FACs in masked greenling in this study ranged from 2,100 to 5,300 ng PHN/mg total biliary protein, with an average for all sites of 4,135 ± 356 ng PHN equivalents/mg total biliary protein. These values are similar to those reported for a variety of demersal fishes three years after the spill. In 1991, FACs measured in flathead sole were significantly higher at oiled PWS sites than at reference sites, with means of approximately 8,000 and 3,600 ng PHN/mg protein, respectively (Armstrong et al., 1995; calculated by converting NPH to PHN equivalents using the linear relationship PHN = 0.2806 x NPH determined here, $n = 30$, $r^2 = 0.94$). In the bile of fish collected in 1991, mean FACs for yellowfin sole, rock sole, and flathead sole ranged from 1,760 to 2,380, 2,000 to 18,990, and 1,110 to 3,030 ng PHN/mg protein, respectively, with no apparent trend with respect to oiled or reference sites in any of these flounders (Collier et al., 1996; Varanasi et al., 1995). The lack of correlation of FAC levels with the nature of the collection sites in those studies is not a surprising result given the mobility of these species. Our FACs data for 1999 indicate that exposure to hydrocarbons is still occurring, even at sites considered unoiled but within the broad trajectory of the spill.

We cannot be certain whether the contamination we observed was EVO or from some other hydrocarbon source. Pyrogenic as well as petrogenic PAHs from sources other than the EVOS occur within the Sound (Page, Boehm, Douglas, & Bence, 1995; Boehm, Page, Gilfillan, Bence, Burns, & Mankiewicz, 1998; Short, Kvenvolden, Carlson, Hostettler, Rosenbauer, & Wright, 1999). However, the significantly higher levels of CYP1A IHC staining observed in

fishes collected from sites adjacent to heavily oiled shorelines compared to unoiled sites outside of the spill trajectory suggests that residual EVO is a major contributor to the elevated CYP1A levels we observed. Furthermore, similar geographic patterns of CYP1A expression were observed in harlequin and goldeneye ducks (Trust et al., 2000) and sea otters (Ballachey et al., 1999). Those studies found higher levels of CYP1A present in animals from heavily oiled bays (Bay of Isles and, in the case of sea otters, Herring Bay) compared to unoiled sites on Montague Island that were outside of the spill trajectory. Trust et al. (2000) presented supporting evidence that the differences between sites might not be due to exposure to polychlorinated biphenyls, which were present at low levels throughout the Sound. Also, analyses presented by Short et al. (1999) indicated that much of the total PAH fraction that was attributable to non-EVO may be from coal deposits, which may be less biologically available and therefore less likely to induce CYP1A expression. On the other hand, if background hydrocarbons in PWS are mainly from oil seeps (and source rock erosion), as suggested by Boehm et al. (1998), then some of the CYP1A induction could result from those sources.

While it seems likely that EVO was a source of contamination effecting CYP1A expression and biliary FACs in these fishes, we do not know the route of exposure or the location of environmental reservoirs of oil that may be persisting sources of contamination. In spite of the fact that we observed relatively high concentrations of hydrocarbons in several oiled mussel beds throughout the Sound, it is not clear that these were sources of contamination in nearshore fishes. There were no positive correlations between TPH concentrations in sediments from mussels beds and any of the measures of hydrocarbon exposure in fishes collected from nearby sites. Other investigators also have failed to show a relationship between sediment hydrocarbons and biomarkers of exposure in fish (e.g., Armstrong et al., 1995; Stagg, McIntosh, & Mackie, 1995; Kirby et al., 1999). The lack of concordance may be due to the patchy spatial distribution of shoreline oil, frequency and intensity of storms that can mobilize subsurface oil, avoidance by fish of oiled patches, and possibly the patchy distribution of contaminated prey. Alternatively, it may be that oil in mussels beds is not a primary source of contamination in nearshore fishes. The route of hydrocarbon exposure is also uncertain, but water, substrate, and diet are all possibile sources (Smolowitz et al., 1991; Van Veld, Wolfgang, Cochran, GoksØyr, & Stegeman, 1997; Woodin et al., 1997).

The three biomarkers we used to assess exposure in 1999 all indicated that greenling and gunnel from sites within the EVO spill trajectory were exposed to hydrocarbons. However, there was no correlation between liver vascular CYP1A IHC scores, hepatic EROD activity, or biliary FAC concentrations within individual fish. The lack of correlation between hepatic vascular CYP1A and hepatic EROD in these fish is not surprising, as endothelium can express high levels of CYP1A without concomitant levels of catalytic function (EROD), possibly due to limiting amounts of NADPH: P450 reductase in this tissue (Stegeman, Woodin, Klotz, Wolke, & Orme-Johnson, 1982).

Penetrance of inducing hydrocarbons into surrounding cells may be less at lower levels of exposure (e.g., Schlezinger & Stegeman, 2000). The levels of CYP1A in hepatocytes of these fish were slight and often below the limits of detection with the IHC procedure.

FAC concentrations in bile are relatively short term (hours to days) indicators of recent hydrocarbon exposure, while CYP1A protein expression generally occurs within a day and can persist for several weeks after hydrocarbon exposure, based on dose-response relationships in other fish species. The specific time course and dose-response of CYP1A expression following hydrocarbon exposure in greenling and gunnel have not been determined. FACs were determined at the optimum wavelength for two ring (NPH) and three ring (PHN) components of EVO. These compounds are not known to be inducers of CYP1A in fish. Larger 4 and 5 ring components which are inducers of CYP1A were not measured, and the proportion of residual oil represented by these less volatile compounds could change relative to PHN and NPH with time. Thus, the levels of CYP1A would not correlate with the FACs signal.This study, with others, suggests that EVO has persisted at some locations for 10 years after the spill (Hayes & Michelle, 1998, Carls et al., 2000), and that organisms within the spill zone are still exposed to hydrocarbons (Ballachey et al., 1999; Seiser et al., 2000; Trust et al., 2000). It is predicted that THC concentration in sediments beneath mussel beds will not decline to background concentrations for nearly three decades after the spill (Carls et al., 2000). There was no evidence of adverse effects of the spill on population densities of either greenling or gunnel in 1990 (Laur & Haldorson, 1996), but other fishes found in the intertidal and shallow subtidal were found to have reduced abundances at oiled sites up to four years after the spill (Barber, McDonald, Erickson, & Vallarino, 1995; Wertheimer & Celewyez, 1996; Bue, Sharr, & Seeb, 1997; Murphy et al., 1999). Also, there is evidence that 6 to 9 years after the spill, hydrocarbon exposure was correlated with reduced survival of some nearshore vertebrate species including sea otters (Monson et al., 2000) and sea ducks (Esler et al., 2000; Trust et al., 2000). While lower survival has not been linked to exposure to EVO, the collective evidence supports a conclusion that EVOS still is available to biota in nearshore systems 10 years after the spill.

Acknowledgements

Financial support was provided by the *Exxon Valdez* Oil Spill Trustee Council. We thank the following personnel who contributed to various aspects of this study: Dennis Jung of Coastal Resources Associates, Inc. for assistance in all sampling; Judy Berger of Alaska Department of Fish and Game (ADF&G) for assistance with the cryogenic preservation of tissues; Kristen Munk of ADF&G for age determination of masked greenling; John Wekell of NOAA/NMFS Seattle for conducting additional FAC analyses in 1999, and Pat Harris and Mark Carls of NOAA/ABL for assistance with 1999 sampling. The findings and conclusions presented, however, are ours, and do not necessarily reflect the views or positions of the Trustee Council.

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FIGURE CAPTIONS

Figure 1. Study sites in Prince William Sound where masked greenling (*Hexagrammos octogrammus*) and crescent gunnel (*Pholis laeta*) were sampled for determining exposure to *Exxon Valdez* oil. Shaded area indicates the oil spill trajectory, originating at Bligh Reef.

Figure 2. Liver of *Hexagrammos octogrammus* collected in 1999 from Herring Bay, Prince William Sound, immunostained with mouse monoclonal antiscup CYP1A (A) or with UPC10 nonspecific mouse monoclonal antibody (B). Arrows indicate sites of vascular endothelial staining.

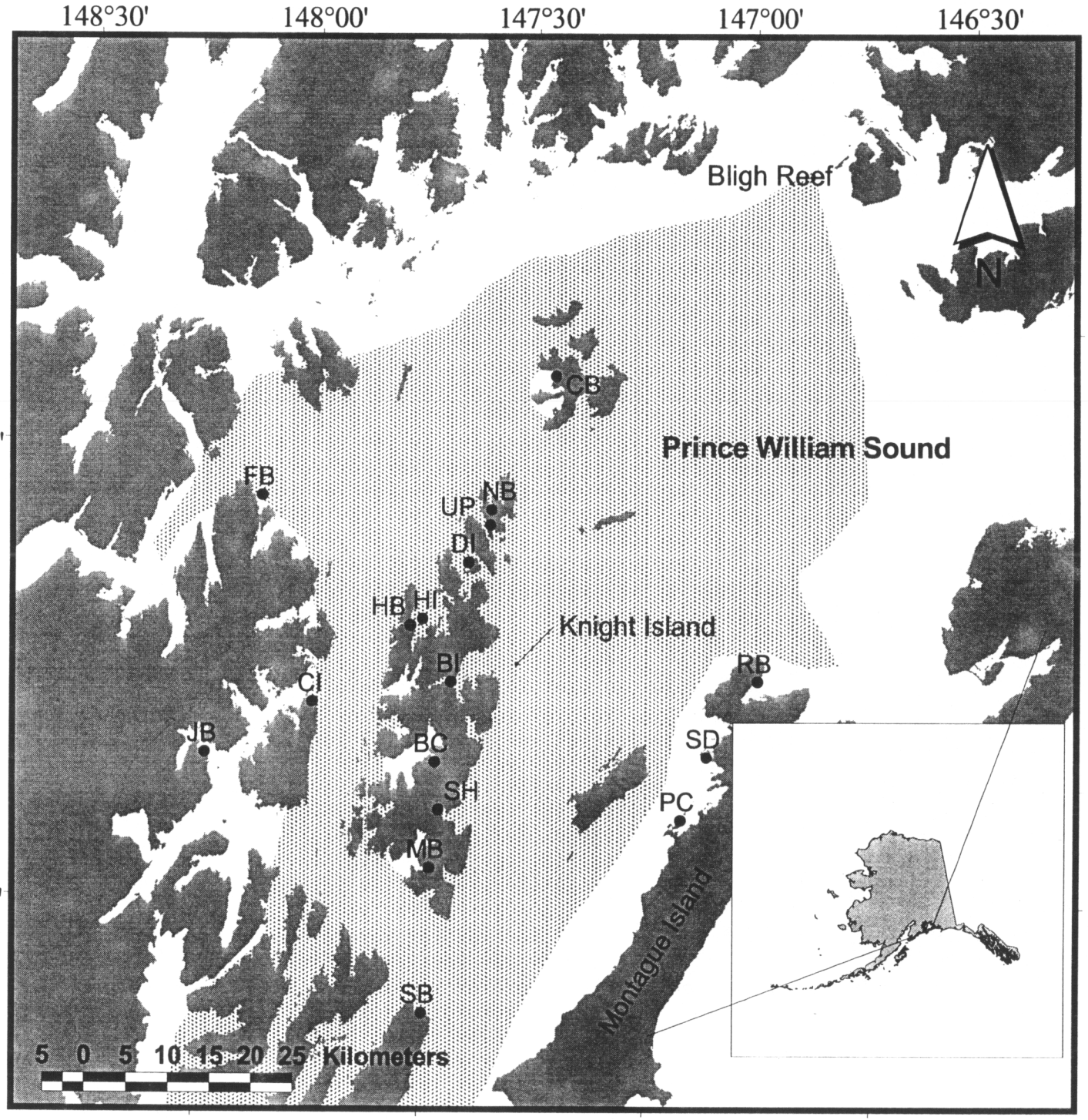
Figure 3. Mean (± SE) CYP1A IHC scores in liver vascular endothelia of (A) masked greenling *(Hexagrammos octogrammus*) and (B) crescent gunnel (*Pholis laeta*) from sites oiled (●), unoiled within the EVOS trajectory (Ø), and unoiled outside the spill trajectory (O) in Prince William Sound, 1998. Sites statistically similar are denoted by a common letter over the histograms.

Figure 4. Mean (± SE) CYP1A IHC scores in liver vascular endothelia of (A) masked greenling (*Hexagrammos octogrammus*) and (B) crescent gunnel (*Pholis laeta*) from sites oiled (●)and unoiled within the EVOS trajectory (Ø) in Prince William Sound, 1999. Sites statistically similar are denoted by a common letter over the histograms.

Figure 5. Temporal variation of CYP1A IHC scores in liver vascular endothelia of (A) masked greenling (*Hexagrammos octogrammus*) from 4 sites and (B) crescent gunnel (*Pholis laeta*) from 2 sites in western Prince William Sound, Alaska. Years statistically different are denoted by different letters.

Figure 6. Mean (± SE) CYP1A EROD values in (A) masked greenling (*Hexagrammos octogrammus*) and (B) crescent gunnel (*Pholis laeta*) from sites oiled (●) and unoiled within the EVOS trajectory (Ø) in Prince William Sound, 1999. Sites statistically similar are denoted by a common letter over the histograms.

Figure 7. Concentrations (mean ± SE) of biliary fluorescent aromatic compounds (FACs) in masked greenling (*Hexagrammos octogrammus*) collected in 1999 at sites oiled (●) and unoiled within the EVOS trajectory (Ø). The value at the end of each site is the number of bile samples analyzed.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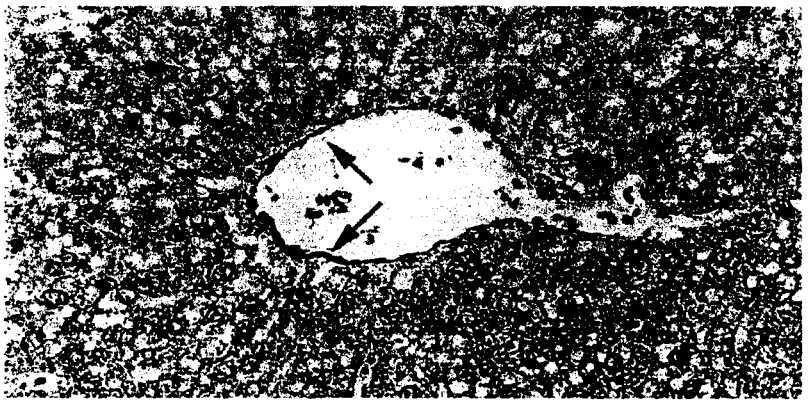

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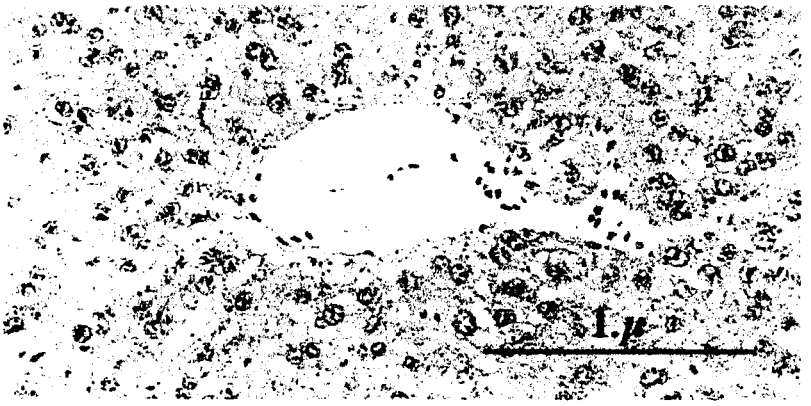
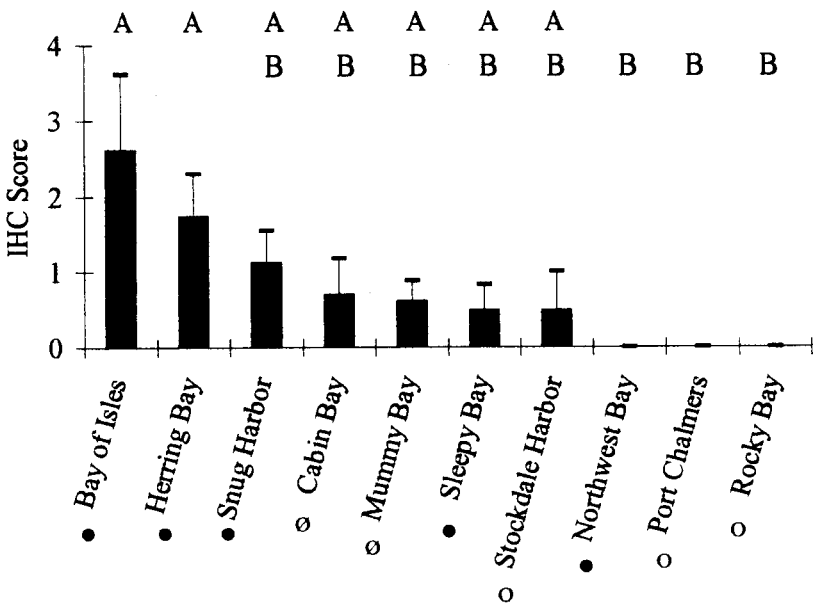
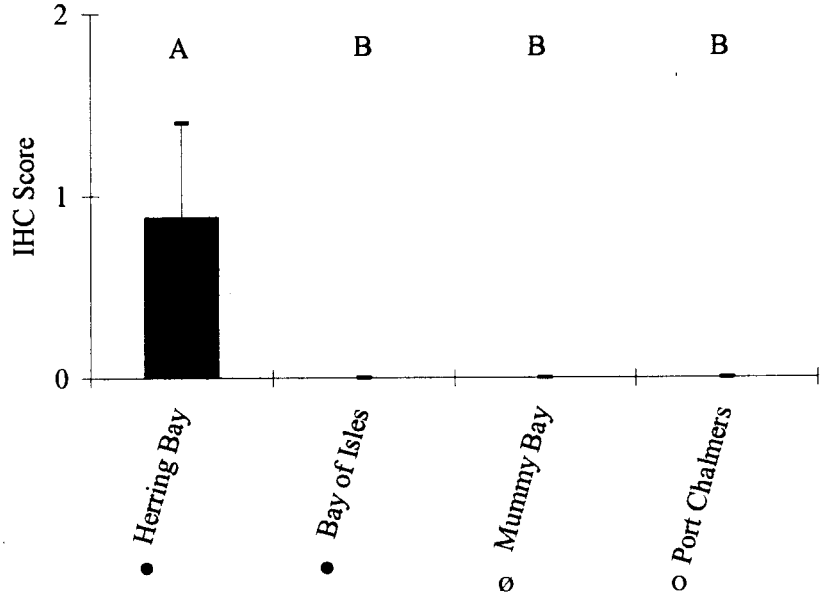


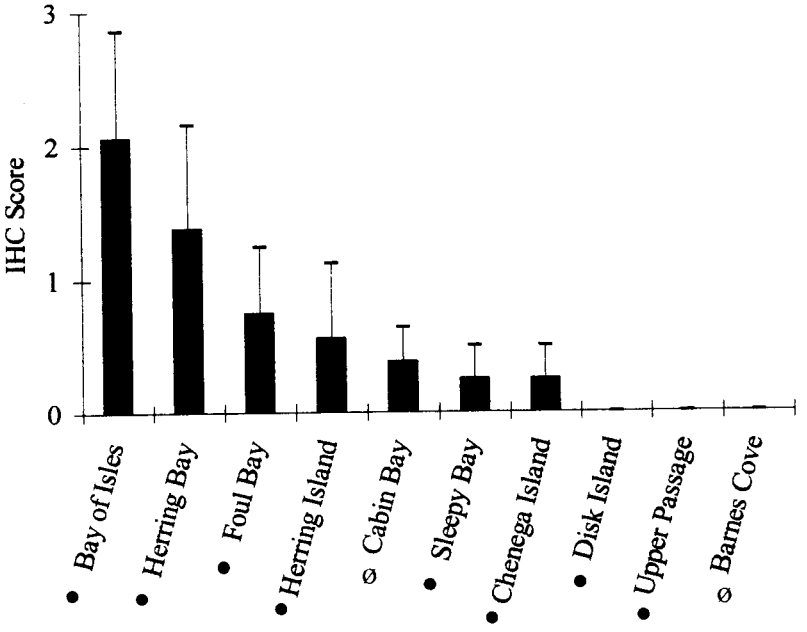
A *Hexagrammos octogrammus*



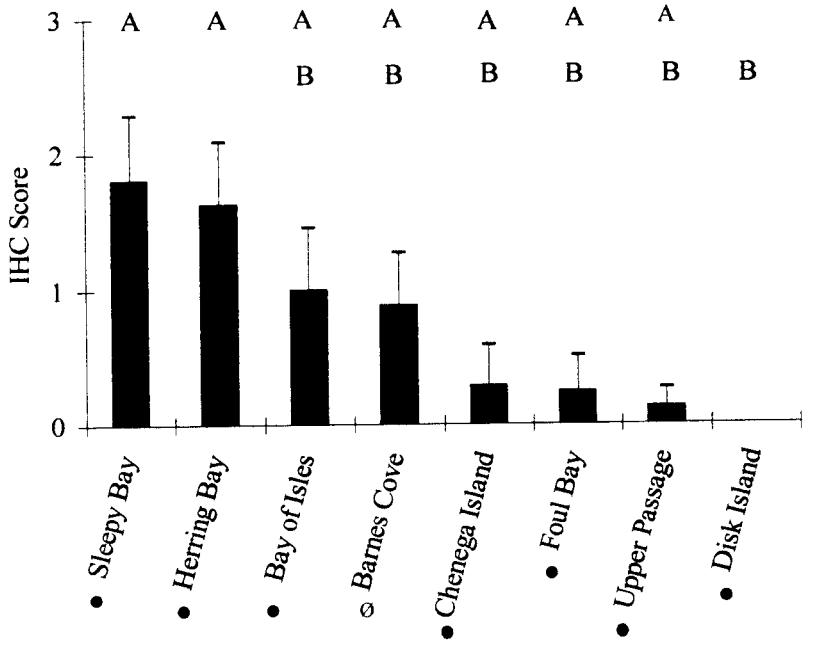
B *Pholis laeta*



A *Hexagrammos octogrammus* ANOVA $p = 0.06$

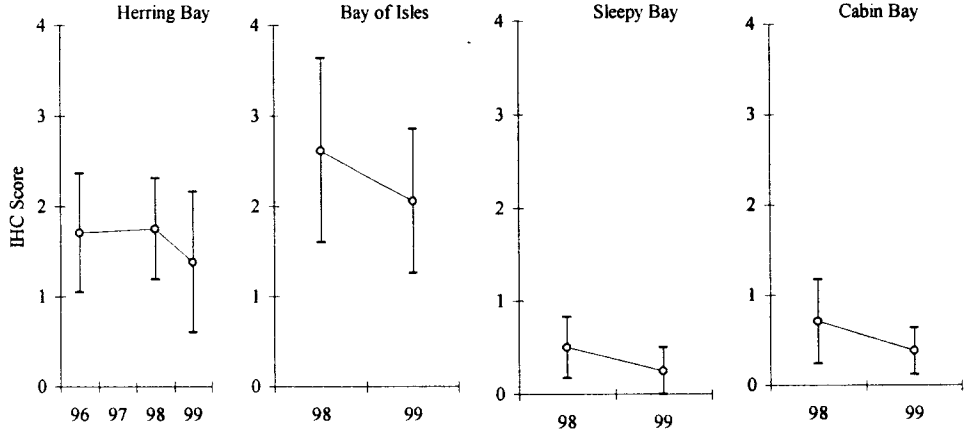


B *Pholis laeta* ANOVA $P = 0.001$



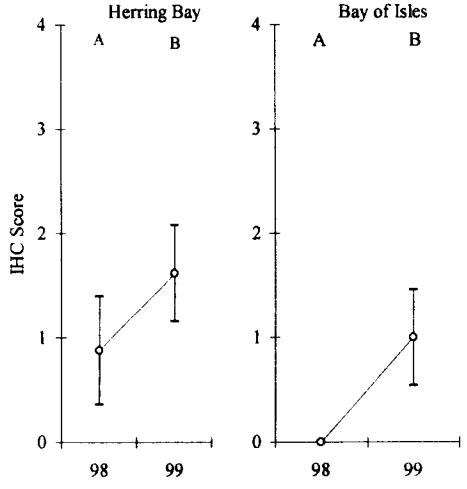
A *Hexagrammos octogrammus*

ANOVA: Site - p = 0.01, Year - p = 0.55, Interaction - p = 0.99

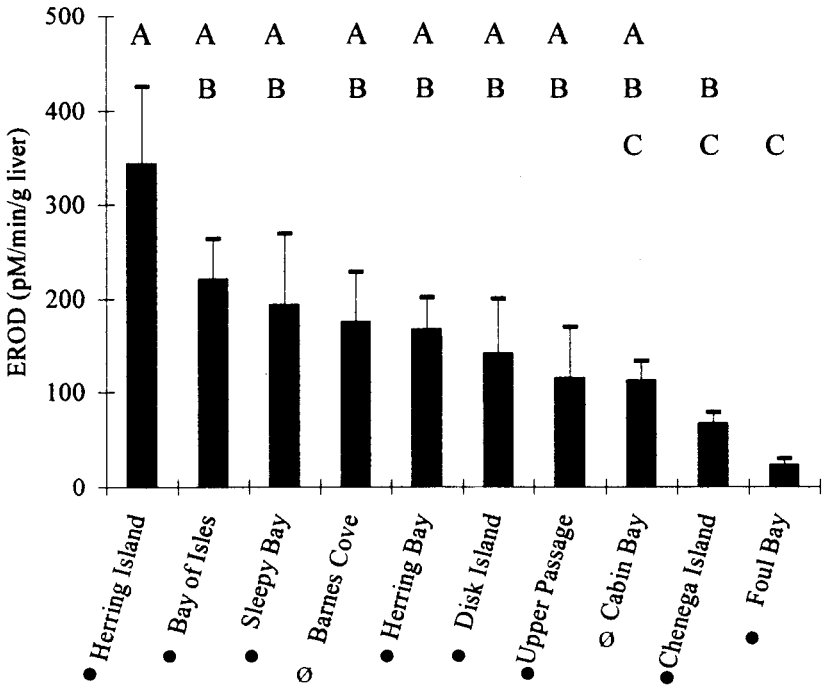


B *Pholis laeta*

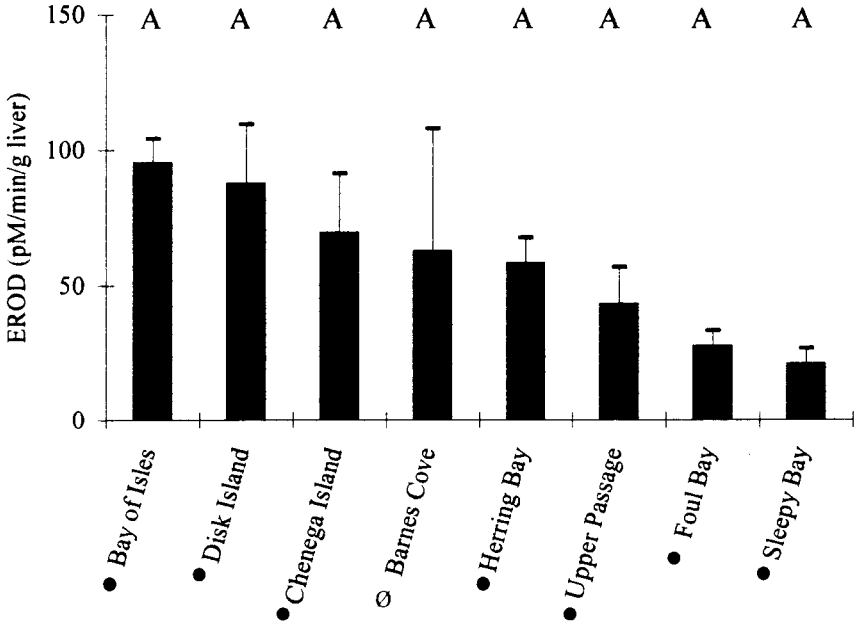
ANOVA: Site - p = 0.06, Year - p = 0.02, Interaction - P = 0.76



A *Hexagrammos octogrammus* ANOVA $p < 0.001$



B *Pholis laeta* ANOVA $p = 0.019$



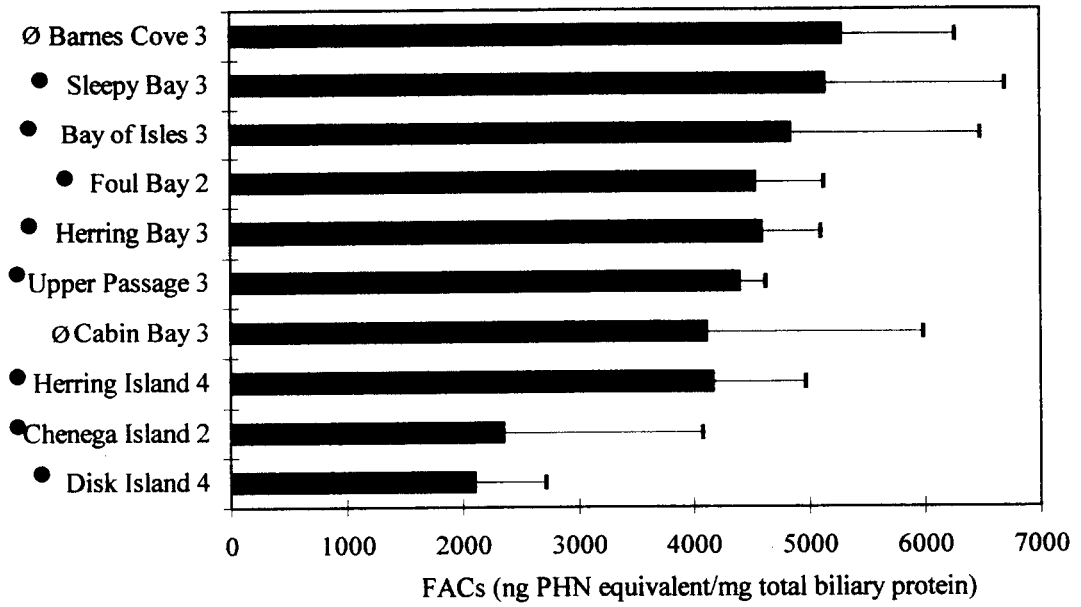

Ø Barnes Cove 3
Sleepy Bay 3
Bay of Isles 3
Foul Bay 2
Herring Bay 3
Upper Passage 3
Ø Cabin Bay 3
Herring Island 4
Chenega Island 2
Disk Island 4
0
1000
2000
3000
4000
5000
6000
7000
FACs (ng PHN equivalent/mg total biliary protein)

Table 1
Summary of effects of the *Exxon Valdez* oil spill on fishes, 1989-92.

SPECIES	LIFE STAGE	YEAR	CONCLUSION	SOURCE
Pacific herring - *Clupea pallasi*	Eggs	1989-90	Lower proportion of developed eggs at 1 site in 1989	Pearson et al. 1995
	Eggs, larvae	1989-90	No effects on eggs; reduced larval production	Brown et al. 1996
	Eggs, larvae	1989	Egg, larval mortality; reduced larval growth	McGurk & Brown 1996
	Larvae	1989	Reduced size, injested food, growth, more cytogenetic damage	Marty et al. 1997
	Larvae	1989	Genetic damage	Hose & Brown, 1998
	Larvae	1989	Morphological deformaties, genetic damage, lower growth	Norcross et al. 1996
	Larvae	1989	Morphological deformaties, genetic damage	Hose et al. 1996
	Adults	1989-91	Hepatic necrosis	Marty et al. 1999
	Adults, larvae	1992	Lower reproduvtive success	Kocan et al. 1996
Pink salmon - *Oncorhynchus gorbuscha*	Eggs, fry, juveniles	1989-91	No observed spill effects	Brannon et al. 1995
	Eggs, alevins	1989, 1991	Cytochrome P4501A induction only in alevins	Wiedmer et al. 1996
	Embryos, preemergernt fry	1989-92	Embryo mortality	Bue et al. 1996
	Juveniles	1989-90	Oil in diet, but no conclusive spill effects on diet	Sturdevant et al. 1996
	Juveniles	1989-90	Cytochrome P4501A induction	Carls et al. 1996
	Juveniles	1989-90	Lower growth, abundance, survival	Wertheimer & Celewycz 1996
	Juveniles	1989-91	Lower growth, cytochrome P4501A induction	Willette 1996
	Adults	1989	Aromatic compound metabolites in bile - MS/GS	Krahn et al. 1992
	Adults	1989-90	Aromatic compound metabolites in bile - FACs	Hom et al. 1996
	Adults	1989-91	No observed spill effects	Maki et al. 1995
Chum salmon - *O. keta*	Juveniles	1989-90	Cytochrome P4501A induction	Carls et al. 1996
	Juveniles	1989-90	Oil in diet, but no conclusive spill effects on diet	Sturdevant et al. 1996
Coho salmon – *O. kisutch*	Adults	1989-90	Aromatic compound metabolites in bile - FACs	Hom et al. 1996
Cutthroat trout – *O. clarki*	Subadults, adults	1989-90	Lower growth, survival	Hepler et al. 1996
Dolly Varden - *Salvelinus malma*	Subadults, adults	1989-90	Lower growth, survival	Hepler et al. 1996
	Adults	1989-90	Cytochrome P4501A induction, bile FACs	Collier et al. 1996
Walleye pollock - *Theragra chalcogramma*	Adults	1989	Aromatic compound metabolites in bile - MS/GS	Krahn et al. 1992
	Adults	1990-91	Cytochrome P4501A induction; bile FACs	Collier et al. 1996
Pacific cod - *Gadus macrocephalus*	Juveniles	1990	No observed spill effects	Laur & Haldorson 1996
	Adults	1989-90	Aromatic compound metabolites in bile - FACs	Hom et al. 1996
Quillback rockfish - *Sebastes maliger*	Adults	1990	Hemosiderosis	Khan & Nag 1993
Kelp greenling - *Hexagrammos decagrammus*	Adults	1990	Hemosiderosis	Khan & Nag 1993
Sculpins - Cottidae	Juveniles	1990	No observed spill effects	Laur & Haldorson 1996
High cockscomb - *Anoplarchus purpurescens*	Adults?	1990	Cytochrome P4501A induction	Woodin & Stegeman 1993
Arctic shanny - *Stichaeus punctatus*	Juveniles	1990	No observed spill effects	Laur & Haldorson 1996
Ribbon prickleback - *Phytichthus chirus*	Adults?	1990	Cytochrome P4501A induction	Woodin & Stegeman 1993
Black prickleback - *Xiphister atropurpureus*	Adults?	1990	Cytochrome P4501A induction	Woodin & Stegeman 1993
Crescent gunnel – *Pholis laeta*	Adults?	1990	Cytochrome P4501A induction	Woodin & Stegeman 1993
Pacific halibut - *Hippoglossus stenolepis*	Adults	1989-90	Aromatic compound metabolites in bile - FACs	Hom et al. 1996
Flathead sole - *Hippoglossoides elassodon*	Adults	1989-91	Cytochrome P4501A induction; bile FACs	Collier et al. 1996
Yellowfin sole - *Pleuronectes asper*	Adults	1989-91	Cytochrome P4501A induction; bile FACs	Collier et al. 1996
	Adults	1990	Hemosiderosis	Khan & Nag 1993
Rock sole - *P. bilineatus*	Adults	1989-91	Cytochrome P4501A induction; bile FACs	Collier et al. 1996
Intertidal fishes - 21 species	Juveniles, adults	1990-91	Lower density and biomass in 1990	Barber et al. 1995

Table 2
Study sites in Prince William Sound where masked greenling (mg) (*Hexagrammos octogrammus*) and crescent gunnel (cg) (*Pholis laeta*) were sampled for determining exposure *to Exxon Valdez* oil. See Figure 1.

			Site Oiling Category		
Site	Site code	Year sampled	Moderately to heavily oiled	Unoiled within spill zone	Unoiled outside spill zone
Herring Bay	HB	96, 98, 99	mg & cg		
Bay of Isles	BI	98, 99	mg & cg		
Sleepy Bay	SB	98, 99	mg		
Upper Passage	UP	99	mg & cg		
Disk Island	DI	99	mg & cg		
Chenega Island	CI	99	mg & cg		
Foul Bay	FB	99	mg & cg		
Herring Island	HI	99	mg		
Northwest Bay	NB	98	mg		
Snug Harbor	SH	98	mg		
Cabin Bay	CB	98, 99		mg	
Barnes Cove	BC	99		mg & cg	
Mummy Bay	MB	98		mg & cg	
Jackpot Bay	JB	96			mg
Rocky Bay	RB	98			mg
Stockdale Harbor	SD	98			mg
Port Chalmers	PC	98			mg & cg

Table 3
Results from nested ANOVAs comparing log-transformed IHC scores of liver vascular endothelia of masked greenling (*Hexagrammos octogrammus*) and crescent gunnel (*Pholis laeta*) collected in 1998 adjacent to shorelines that were moderately to heavily oiled, unoiled but within the spill zone, and unoiled outside the spill zone. Contrasts between the three oiling categories are given for masked greenling.

		Hexagrammos octogrammus		
Source	DF	SS	F	*p*
Oiling category	2	3.43	6.68	0.002
Sites within oiling category	7	5.72	3.18	0.005
Contrasts				
Oiled vs unoiled within spill zone	1	0.311	1.21	0.270
Oiled vs unoiled outside spill zone	1	3.431	13.35	0.001
Unoiled within vs outside spill zone	1	0.879	3.42	0.069

		Pholis laeta		
Source	DF	SS	F	*p*
Oiling category	2	0.362	1.79	0.186
Sites within oiling category	1	0.723	7.15	0.012

Table 4
Pearson Product Moment correlations (r) between (A.) mean total petroleum hydrocarbons (THC) in surface sediments and liver vascular endothalia IHC scores and EROD activity in masked greenling (*Hexagrammus octogrammus*) and crescent gunnel (*Pholis laeta*) collected near study sites in 1999, and (B.) biliary FACs (phenanthrene), IHC scores, and EROD activity in individual fish.

A.		*Hexagrammus octogrammus*	
Comparison	N	r	*p*
THC vs IHC	10	-0.23	0.517
THC vs EROD	10	-0.39	0.271
		Pholis laeta	
THC vs IHC	8	-0.75	0.032
THC vs EROD	8	0.13	0.759
B.		*Hexagrammus octogrammus*	
FAC vs IHC	30	0.066	0.728
FAC vs EROD	30	0.015	0.939
EROD vs IHC	73	-0.04	0.734
		Pholis laeta	
EROD vs IHC	39	0.149	0.364

Table 5
Mean and maximum concentrations of total petroleum hydrocarbons (THC) in surface sediment collected in 1999 from mussel beds at study sites oiled (O) and unoiled (U) within the EVOS trajectory.

Sites	Number of Pooled Samples	Mean THC (µg/g wet wt.)	Number Pooled & Spot Samples	Maximum THC (µg/g wet wt.)
Chenega Island (O)	1	9,621	2	25,168
Disk Island (O)	1	5,116	4	9,942
Foul Bay (O)	1	4,596	4	27,375
Upper Passage (O)	2	3,710	6	28,231
Herring Island (O)	3	3,241	6	4,966
Bay of Isles (O)	5	1,796	11	18,933
Herring Bay (O)	1	17	1	17
Sleepy Bay (O)	1	15	1	15
Barnes Cove (U)	1	14	2	14
Cabin Bay (U)	1	3	1	3