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Biomarkers of PAH Exposure in an Intertidal Fish Species from Prince William Sound, Alaska: 2004−2005

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Polycyclic aromatic hydrocarbon (PAH) exposure biomarkers were measured in high cockscomb prickleback (*Anoplarchus purpurescens*) fish collected from both previously oiled and unoiled shore in Prince William Sound (PWS), Alaska, to test the hypothesis that fish living in the nearshore environment of the sound were no longer being exposed to PAH from the *Exxon Valdez* oil spill. Pricklebacks spend their entire lives in the intertidal zone of rocky shores with short-term movements during feeding and breeding restricted to an area of about 15 meters in diameter. Fish were assayed for the PAH exposure biomarkers, bile fluorescent aromatic compounds (FAC), and liver ethoxyresorufin O-deethylase (EROD) activity (a measure of cytochrome P450 1A (CYP1A) monooxygenase activity). Bile FAC concentrations and EROD activities were low and not significantly different in fish from previously oiled and unoiled sites. The similar low EROD activity and bile FAC concentrations in fish from oiled and unoiled shores, supports the hypothesis that these low-level biomarker responses were not caused by exposure of the fish to residues of the spilled oil.

Introduction

Approximately 37 000 metric tons of Alaskan North Slope crude oil were released into Prince William Sound (PWS), Alaska, following the grounding of the oil tanker, *Exxon Valdez,* in March 1989. Much of the oil was swept through PWS into the Gulf of Alaska (GOA) by a winter storm, but approximately 40% came ashore along 783 km (486 miles) (16%) of the PWS shoreline (*1*, *2*). Cleanup activities in 1989, aided by harsh winter storms, removed much of the shoreline oil in the year after the spill (*3*) and, by 1992, approximately 10 km of shoreline remained visibly oiled (*2*). The National Marine Fisheries Service estimated that approximately 11.3 ha (27.9 acres) of the PWS shore remained oiled in 2001, with most oil present as light residues in the subsurface of the middle and upper intertidal zone under boulder/cobble overburden (*4*).

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During the summer after the spill, scientists from the National Oceanographic and Atmospheric Administration (NOAA) collected several species of finfish from spill path and unoiled areas of PWS and the northern GOA (*5*, *6*, *7*). Bile was analyzed for metabolites of polycyclic aromatic hydrocarbons (PAH), as fluorescent aromatic compounds (FAC). As expected, fish collected in the path of the spilled oil showed evidence of exposure to the spilled oil. FAC levels had dropped significantly in fish collected in the spring and summer of 1990 from the spill path and were at or near the levels in fish from outside the path. Fish from all areas showed similar low levels of FAC.

Fish were collected in PWS and the Gulf of Alaska (GOA) in 1999 and 2000 to test the hypothesis that fish inhabiting waters in the path of the spill were still being exposed to remnants of the spilled oil (*8*). Fish, including some of the species that NOAA sampled, were collected from the path of the spill as well as from outside the spill path in PWS and in non-spill path areas of the eastern GOA. Biomarkers (FAC concentrations, ethoxyresorufin-O-deethylase, or EROD activity, and immunohistochemically detected CYP1A) were measured in bile and tissue of kelp greenling (*Hexagrammus decagrammus*), Pacific cod (*Gadus macrocephalus*), Pacific halibut (*Hippoglossus stenolepis*), rockfish (*Sebastes caurinus* and *Sebastes maliger*), and rock sole (*Pleuronectes bilineatus*) to determine exposure to bioavailable PAH. There were no significant differences in biomarker levels in fish from oiled and un-oiled areas, so the hypothesis was rejected (*8*). However, low levels of CYP1A induction were found in fish from both the oiled and un-oiled areas, including the GOA well east and upcurrent of the spill path.

That study examined fish that were collected >50 meters from shore. Consequently, the data could not test the hypothesis that near-shore and intertidal communities were being exposed to small amounts of oil seeping into the sound from intertidal surface and buried oil deposits. This is an important concern, because near-shore and intertidal areas of PWS are habitat for numerous species of intertidal and shallow subtidal animals, including the early life stages of the two most important commercial fisheries species in the sound, pink salmon (*Onhorhynchus gorbuscha*) and Pacific herring (*Clupea pallasi*). The intertidal zone is important habitat for several species, such as high cockscomb pricklebacks (*Anoplarchus purpurescens*), clams (*Protothaca staminea* and *Saxidomus giganteus*), and mussels (*Mytilus trossulus*) that are food for sea otters (*Enhydra lutris*), and harlequin ducks (*Histrionicus histrionicus*) (*9*).

Neff et al. (*9*) recently reported that mean PAH concentrations were low, ranging from 2.2 to 246 ng/g dry wt (parts per billion), in clams, mussels, and high cockscomb pricklebacks collected in 2002 from the intertidal zone of 17 previously oiled PWS shores. The following hypothesis was formulated to assess if intertidal communities were being exposed to bioavailable fractions of oil from remnant buried intertidal deposits: Fish inhabiting the intertidal zone of beaches previously oiled by the *Exxon Valdez* spill have the same level of biomarkers of oil exposure as do fish from non-oiled beaches, indicating no or negligible continuing input of PAH from surface or buried intertidal oil deposits to the marine environment. To test this hypothesis, complementary biomarkers (EROD activity and FAC concentrations) were measured in high cockscomb pricklebacks collected in 2004 and 2005 from the intertidal zones of oiled and unoiled PWS shores. Most of the previously oiled sites surveyed in this investigation contained subsurface oil residues in 2001 (*4*) and 2002 (*9*). In 2004, samples also were collected from

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shores adjacent to sites of past human commercial activities. Fish tissues collected in 2004 were analyzed for EROD activity and those collected in 2005 were analyzed for both EROD activity and FAC concentrations. The study reported here substantiates the low-level exposure to bioavailable PAH as indicated by the PAH biomarkers, FAC and EROD, in intertidal fish tissues.

Experimental Section

Species Selection. High cockscomb pricklebacks were chosen to compare PAH-exposure biomarkers on oiled and un-oiled shores because they are the most abundant intertidal fish on the rocky shores of PWS (*10*). They live in crevices among the cobbles and boulders of the lower shore at low tide and forage in the intertidal zone when the tide is in. Short-term movements during feeding and breeding are restricted to an area of about 15 meters in diameter. Females deposit eggs under intertidal rocks in February and March; the eggs hatch as planktonic larvae that remain in the plankton for only a few days before settling on the lower shore. Thus, pricklebacks spend their entire life in the intertidal zone. Females grow faster and to larger size than males (*11*). They feed on a mixed diet of green algae, amphipods, crabs, and polychaete worms (*11*). In turn, they are fed upon by river otters, mink, some sea ducks, pigeon guillemots, and subtidal fish (*10*).

The high abundance and limited migrations of high cockscomb pricklebacks in the lower intertidal zone makes them ideal candidates for sentinels of exposure to bioavailable PAH from buried intertidal oil deposits. The density and biomass of intertidal fish, including high cockscomb pricklebacks, decreased on oiled shorelines for 2 years after the spill, but populations recovered by 1991 (*10*). Experimental exposure of pricklebacks to β-naphthoflavone or Prudhoe Bay crude oil in sediment or food resulted in a strong induction of CYP1A (averaging 100-fold and >50 fold respectively) (*12*). Pricklebacks collected from oiled shores in June, 1990 (1 year after the spill) had levels of CYP1A in several tissues that were elevated relative to levels in fish from un-oiled shores (*12*), indicating the utility of these fish as sentinels of oil exposure.

Site Selection. Sites were selected to represent a range of oiling conditions: those that had been oiled by the 1989 spill, sites that had never been oiled, and sites that were locations of past industrial activity (former herring processing plants), now abandoned (Figure 1, Table 1). The unoiled reference sites are located at the head of northwestward- or westward-facing bays (West Twin Bay on Perry Island and Lower Herring Bay, and Drier Bay on Knight Island, Figure 1). There is no evidence that oil slicks from the spill entered any of these bays. Fourteen shore sites were sampled in a 7-day period in June 2004 and seven sites were sampled during a 9-day period in June 2005. The sites initially oiled by EVOS are in Herring Bay, Lower Passage, and Bay of Isles in the northern part of the Knight Island group (Figure 1) and represent a range of current oiling conditions based upon studies conducted in 2001 (*4*), 2002 (*9*, *13*), 2004 (*14*), and as noted at the time of fish collection. Sites where intertidal subsurface oil (SSO) residues were documented in 2001, in subsequent years, or at the time of collection are noted in Table 1. Three of these sites also are locations where peat is exposed in the intertidal zone. Because peat is a known inducer of CYP1A (*15*), its presence must be considered when interpreting biomarker results.

Field Collections. High cockscomb pricklebacks were collected by carefully turning over exposed rocks at low tide in the −1 to +1 m mean low water intertidal zone of exposed bedrock/rubble, boulder/cobble, and cobble/pebble shores. Fish were placed immediately in a clean bucket of aerated site water for transport back to the shipboard laboratory where they were kept alive prior to processing.

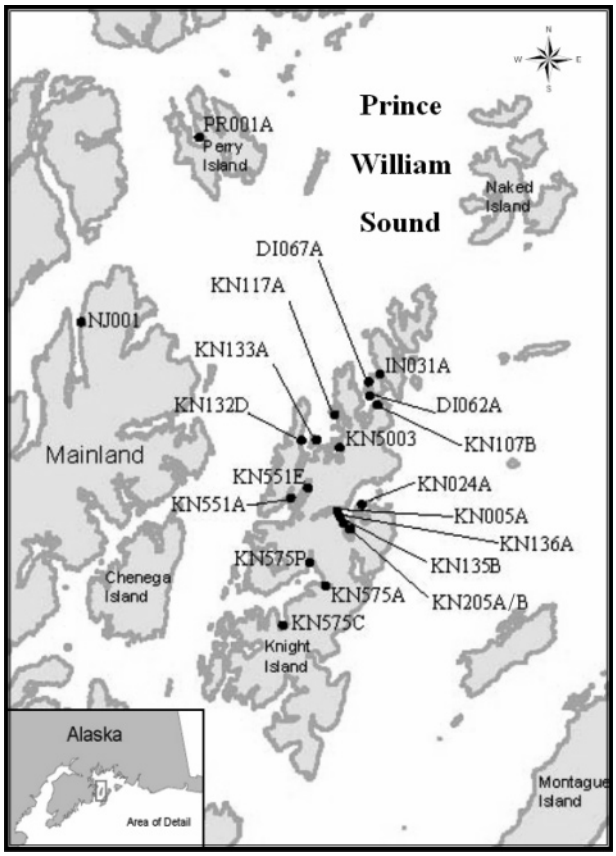

FIGURE 1. Map of western Prince William Sound, Alaska showing shoreline SCAT segments where high cockscomb prickleback fish were collected in 2004 and 2005 for PAH exposure biomarker analysis.

TABLE 1. Oiled, Unoiled, and Human Activity Shoreline Segments (each Approximately 100 m Long) in Prince William Sound, Alaska[a]

oiled in 1989	unoiled	human activity
2004 sites		
DI-067A (SSO, peat)	KN-551A	KN-575P
IN-031A (SSO, peat)	KN-575A	NJ-001A
KN-024A		
KN-107B (SSO)		
KN-117A (SSO)		
KN-132D (SSO)		
KN-133A (SSO)		
KN-135B (SSO)		
KN-136A (SSO, peat)		
KN-205A		
2005 sites		
DI-062A	KN-551E	
KN-005A	KN-575C	
KN-205B	PR-001A	
KN-5003		

[a] Based on designations by the Shoreline Cleanup Assessment Team (SCAT), of sites where high cockscomb prickleback fish (*Anoplarchus purpurescens*) were collected for PAH biomarker analysis in 2004 and 2005. Locations of SCAT segments are shown in Figure 1.

Sample Preparation. The fish were dissected within 2 h of collection, and the length of each fish was recorded. Previous studies have reported the rate at which induced CYP1A decline during depuration of pricklebacks experimentally exposed to oil (*12*). Also, a half-life of about 40 h has been determined for CYP1A protein in another species (*15*). These studies indicate that there would not be a measurable change in the amounts of CYP1A in pricklebacks

during the 2 h after collection. Clean nitrile gloves were worn during the handling and dissection of all fish. All sampling utensils were cleaned with a clean lab wipe, and sequentially rinsed with methylene chloride, acetone, and distilled water. The syringes used for bile sampling were rinsed with distilled water, methylene chloride, acetone, and then distilled water again (three times each) prior to sampling each fish.

The fish were killed by cervical scission just prior to dissection. The peritoneal cavity was opened with micro-scissors, taking care not to puncture the gall bladder. A bile sample was collected from each fish by carefully piercing the gall bladder with a syringe and withdrawing up to 25 μL of bile, or by excising the complete gall bladder with micro forceps. The bile/gall bladder was immediately transferred into a pre-cleaned 2-mL glass vial with a Teflon seal and stored frozen in a liquid nitrogen dry shipper. Care was taken not to expose any of the liver tissue to the bile, as bile components can disrupt membranes and can bind to many CYPs, with a possible inhibition or inactivation of EROD enzyme activity in the sample. If the liver tissue was contaminated with bile, the sample was not collected and the entire fish was discarded. Duplicate bile samples were collected at random to evaluate the precision of the FAC measurements. Several bile "field blanks" consisting of 25 μL of distilled water drawn into a syringe, dispensed into a sample vial, and stored in the same manner as the bile samples, were collected during the sampling.

A sample of liver for measurement of EROD activity was collected from each fish by carefully excising the liver with micro scissors and transferring it into a 2-mL cryo vial, which was immediately frozen by immersion in liquid nitrogen. These samples were stored in a liquid nitrogen dry shipper until analysis. Due to the small size of this species, the whole liver (minus the gall bladder and bile duct) usually was collected. Liver weights ranged from 10 to 47 mg. In the 2004 sampling, equivalent portions of liver were pooled from 2 to 6 fish and the pools frozen; the remainder of the liver was devoted to other analyses. In the 2005 sampling, individual livers were frozen from all fish.

Analytical Methods. Bile samples were analyzed for FAC by the Geochemical and Environmental Research Group of Texas A & M University. The method for FAC analysis was the same as that used for fish samples reported by Huggett et al. (*8*) and described by Krahn et al. (*17*, *18*). Metabolites with fluorescence maxima similar to those of naphthalene, phenanthrene and benzo(a)pyrene were quantified as naphthalene equivalents, phenanthrene equivalents, and benzo-(a)pyrene equivalents, respectively, and normalized to bile protein.

The EROD analyses were performed at the Woods Hole Oceanographic Institution. Liver tissue was thawed on ice and microsomal fractions were prepared from individual livers or pooled liver samples by methods reported by Stegeman et al. (*19*). A few samples yielded amounts of microsomal material insufficient for replicate assay of both protein and EROD. Those samples are not included in the results. Microsomal EROD was measured fluorimetrically in liver microsomal fractions by a modification of a plate-based assay with a Cytofluor fluorescent plate reader as described by Hahn et al. (*20*). Kinetic activities were determined as picomoles (pmol) of product (resorufin) per volume of enzyme in the assay by comparison with the fluorescent signal generated by known amounts of resorufin measured under equivalent volume, buffer, and instrument sensitivity conditions. Resulting activities were normalized to the amount of microsomal protein present in each sample as determined by the bicinchoninic acid method of Smith et al. (*21*).

Statistical Methods. Our objective was to test the null hypothesis of equal mean biomarker expression (EROD activity and bile FAC concentration) at oiled and reference sites, against the alternative hypothesis of higher mean levels at the oiled sites.

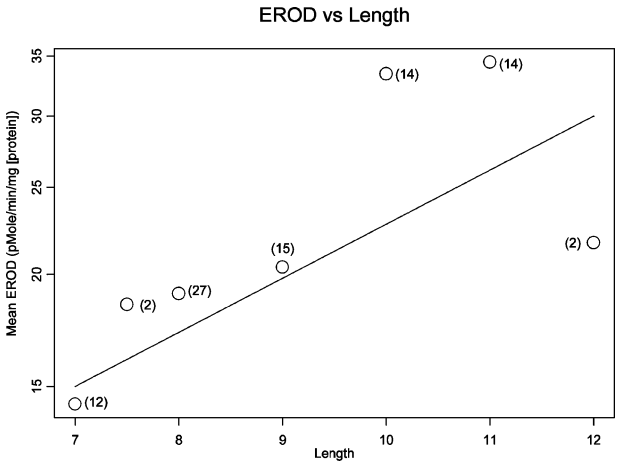

FIGURE 2. Relationship between mean hepatic EROD activity (pmol/min/mg protein) and length (cm) for high cockscomb pricklebacks collected in the intertidal zone of Prince William Sound, Alaska, in 2005. Sample size in parentheses.

There was a positive relationship between length of fish and EROD activity (Figure 2). Adult females are larger than adult males (*11*), so most of the larger fish probably were females. It was not possible to determine the sex of most fish because they were past the reproductive season, and a sex difference would not be expected, and the reason for the association with length is not known. Regardless, in statistical analyses, fish length may serve as a surrogate for fish sex. The data also showed significant intra-site variability. Consequently, site-to-site as well as fish-to-fish (within sites) variability are incorporated into a nested ANOVA: the mean level of a biomarker ($\bar{Y}$) is a function of *treatment* (reference or previously oiled) and *site* within *treatment*. Where μ is the overall grand mean,

$$\bar{Y} = \mu + \text{length} + \text{treatment} + \text{site(treatment)} + \text{error}$$

In addition to ANOVA, we used regression analysis to test for a significant positive correlation among biomarkers, where the null hypothesis of no slope was tested against the alternative hypothesis of a positive slope.

All tests were one-tailed at $\alpha = 0.05$. A one-tailed test is more sensitive for finding an effect at previously oiled sites than a two-tailed test. Data were log-transformed to achieve additivity (*22*, *23*), and residuals were assessed for normality with qqnorm plots (*24*). For EROD, the relationship between oiled and unoiled means did not differ for 2004 and 2005 (no significant year-by-treatment interaction), therefore, we combined data over years to increase statistical power over tests on years separately. For the benefit of the reader, we plot untransformed data on log-scaled axes.

Results and Discussion

There is no statistical evidence that mean levels of biomarkers in the high cockscomb prickleback were higher at oiled than at either reference or human activity sites. Table 2 shows mean EROD activities and bile FAC concentrations, standard errors, and *p*-values. Mean values of all parameters for fish from reference sites nominally exceeded those at oiled sites. The high magnitude of all *p*-values (all greater than 0.665) supports the null hypothesis that there is no difference in mean parameter values among fish from oiled sites, reference sites, and human activity sites.

Figures 3 and 4 show both the means and distributions of individual values for EROD activity and FAC concentra-

TABLE 2. Summary of Mean Hepatic EROD Activity and Concentrations of Bile FAC in the High Cockscomb Prickleback from Oiled, Reference, and Human Activity Shores in Prince William Sound in 2004 and 2005[a]

biomarker	oil	reference	human activity	*p*-value [d]
EROD[b]	20.4 (4)	28.2 (5)	63.2(14)	>0.960[e]
FAC[c]: naphthalene	4.5 (0.8)	4.9 (1.9)		0.710
FAC: phenanthrene	0.48 (0.1)	0.78 (0.3)		0.667
FAC: benzo(a)pyrene	0.010 (0.002)	0.019 (0.004)		0.665

[a] Mean ± 1 SE. [b] pmol/min/mg [protein]. [c] mg Ph equiv/g biliary protein. [d] The one-tailed alternative hypothesis was for higher mean EROD and FAC levels at oiled sites. [e] Linear contrasts for oil vs reference, and oil vs human activity, *p*-values = 0.966 and 0.978, respectively.

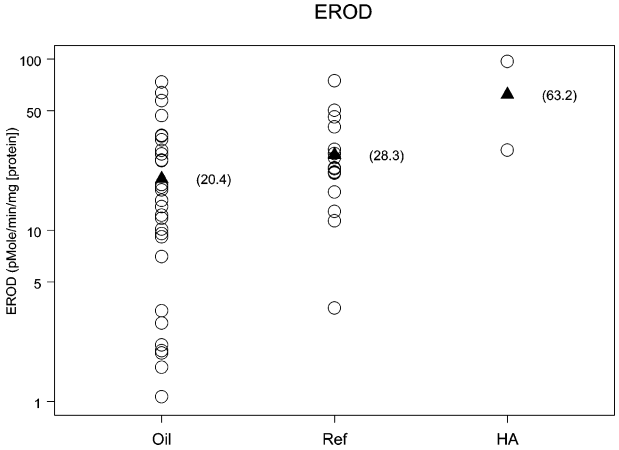

FIGURE 3. Hepatic EROD activity in high cockscomb pricklebacks collected in 2004 and 2005 from previously oiled (oil), reference (ref), and human activity (HA) sites in Prince William Sound, Alaska. Sample sizes: oil (*31*), ref (*16*), HA (*2*).

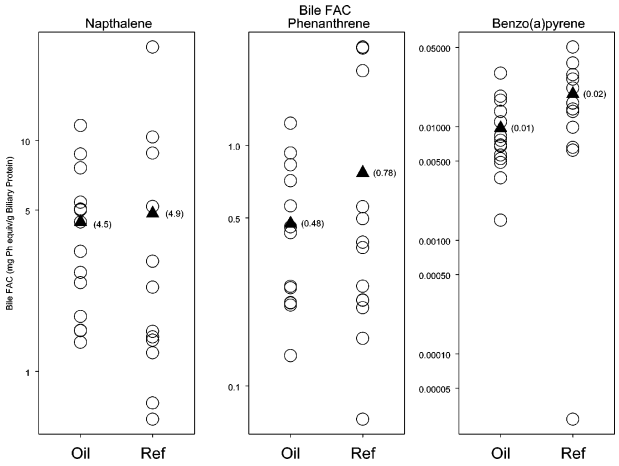

FIGURE 4. Bile FAC concentrations, measured as naphthalene equivalents, phenanthrene equivalents, and benzo(a)pyrene equivalents in high cockscomb pricklebacks collected at oiled (Oil) and unoiled reference (ref) sites in Prince William Sound, Alaska in 2005. Sample sizes: oil (*14*), ref (*13*).

tions, respectively. The highest values for both EROD and FAC biomarkers occurred at human activity and reference sites, respectively. The individual EROD activities at the different sites ranged from 0.38 to 73.5 pmol/min/mg protein in livers of fish from oiled sites, 3.52 to 74.8 pmol/min/mg protein at reference sites, and 29.4 and 97.1 pmol/min/mg protein at the two HA sites. Overall mean bile FAC concentrations in fish from oiled and reference sites were 4.5 and 4.9 mg naphthalene equivalents/g biliary protein, 0.48 and 0.78 mg phenanthrene equivalents/g biliary protein, and 0.01 and 0.02 mg benzo(a)pyrene/g biliary protein, respectively. A significant correlation (p-value < 0.001) between naphthalene equivalents and phenanthrene equivalents, validates

TABLE 3. Coefficient of Determination (R^2) among Biomarkers for the High Cockscomb Prickleback

	FAC:naph	FAC: phen	FAC: benz
EROD	0.10	<0.001	0.001
FAC: naph		0.75[a]	0.02
FAC: phen			0.04

[a] *p*-value <0.001

laboratory calibration and quantification of bile FAC and indicates that the intertidal fish from oiled and unoiled shores were exposed to low concentrations of low molecular weight, possibly petrogenic, PAH.

Liver microsomes of high cockscomb pricklebacks collected from oiled shores in PWS just over 1 year after the spill contained (on average) up to sevenfold more CYP1A protein, measured by immunoblotting, than did microsomes of fish collected at the same time from unoiled shores (*12*). Moreover, CYP1A is strongly induced in pricklebacks exposed to known AHR agonists, or by experimental exposure to PBCO (*12*). Thus, if bioavailable fractions of spilled oil PAH were still leaching from intertidal sediments in 2004 and 2005, one would expect elevated hepatic EROD activity. The levels of EROD activity and bile FAC concentrations were similarly low in fish collected from oiled and unoiled shores in 2004 and 2005. This indicates that in 2004/2005, fish from previously oiled sites were not being exposed to levels of PAH that were any greater than the exposures at previously unoiled sites, suggesting that this exposure was not due to residues of the spilled North Slope crude oil.

There was not a significant correlation between EROD activity and FAC concentration in 2005 (Table 3). We reported earlier a poor correlation between EROD activity and bile FAC concentrations in fish caught offshore in PWS and GOA (*8*). Most of the FAC in intertidal fish in this study and in offshore fish reported earlier (*8*) were naphthalene and phenanthrene equivalents. These 2- and 3-ring PAH are poor inducers of EROD activity (*24*). Concentrations of benzo(a)pyrene equivalents were low, indicating that exposures to the most powerful PAH inducers also were low consistent with the low level expression of CYP1A. The portion of CYP1A induction due to petroleum PAH exposure may be small compared to that caused by other inducers.

There were intertidal peat deposits on three shores where oil formally had been observed. EROD activities in high cockscomb pricklebacks from these shores were slightly higher, but not significantly so, than activities in fish from previously oiled shores where no intertidal peat was observed. Thus, natural CYP1A inducers in peat (*15*) may have made a small contribution to the level of induction observed in fish on some oiled shores.

The results reported here show that high cockscomb pricklebacks inhabiting the intertidal zones of beaches previously oiled by the *Exxon Valdez* oil spill were not being exposed to higher concentrations of bioavailable PAH than fish living at un-oiled shores in 2004 and 2005. This is important because it indicates that bioavailable petroleum

PAH from buried, weathered oil either are not being released by wave action and tidal pumping into intertidal water as the tide falls or, if so, they are being released at concentrations, that do not produce detectable changes in the indices measured in fish here. Heintz et al. (*26*, *27*) and Carls et al. (*28*) inferred from laboratory studies with eggs and larvae of pink salmon and Pacific herring that dissolved PAH leaching from buried intertidal weathered oil deposits could harm fish inhabiting intertidal and near-shore areas. Because high cockscomb pricklebacks spend their entire lives in the intertidal zone, within an area of about 15 meters in diameter (*10*), if dissolved PAH from buried oil were harming them, one might expect lower numbers of these fish on shores with buried oil deposits. Barber et al. (*9*) reported lower than normal abundance of intertidal fish on the most heavily oiled shores during the 2 years after the spill. After 1991, intertidal populations were normal and abundant. This observation and the fact that there were no significant differences in EROD activities or FAC levels in fish collected on oiled and non-oiled shores in 2004 and 2005, indicates that these intertidal fish are no longer exposed to harmful concentrations of dissolved or dispersed, bioavailable PAH from the oil spilled in 1989.

Acknowledgments

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