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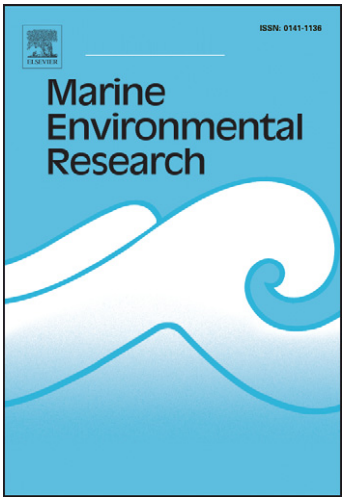


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# SEMIPERMEABLE MEMBRANE DEVICES LINK SITE-SPECIFIC CONTAMINANTS TO EFFECTS: PART 1 - INDUCTION OF CYP1A IN RAINBOW TROUT FROM CONTAMINANTS IN PRINCE WILLIAM SOUND, ALASKA

KATHRINE R. SPRINGMAN*, JEFFREY W. SHORT[1], MANDY R. LINDEBERG[1], JACEK M. MASELKO[1], COLIN KHAN[2], PETER V. HODSON[2], STANLEY D. RICE[1]

[1]Auke Bay Laboratory, Alaska Fisheries Science Center, National Marine Fisheries Service, NOAA, 11305 Glacier Highway, Juneau, Alaska 99801-8626 USA

[2]Queen's University, Kingston, Ontario, K7L 3N6 Canada

*Corresponding author contact information:

Telephone: 530-400-4141; fax: 707-937-6212; e-mail: krspringman@gmail.com

Current address: P.O. Box 315, Littleriver, CA 95456

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**Abstract**

Extracts from semi-permeable membrane devices (SPMDs) deployed on beaches in Prince William Sound (PWS), Alaska, were used to evaluate if complex contaminant mixtures from different sources can be distinguished by the resulting cytochrome P450 1A (CYP1A) activity in exposed test animals. Deployment sites included canneries, salmon hatcheries, and beaches where lingering oil remains from discharges during the 1964 earthquake or the 1989 *Exxon Valdez* oil spill. Other sites were selected at random to evaluate region-wide contaminant inputs or were located in salmon streams to evaluate contaminants carried and released by migrating salmon carcasses following reproduction. Following standard deployments of approximately 28 d, an aliquot of the accumulated contaminants was intraperitoneally injected without cleanup into juvenile rainbow trout (*Oncorhynchus mykiss*). After 2d and 7d, the activity of CYP1A was measured by the ethoxyresorufin-*o*-deethylase (EROD) assay. Exposure to extracts from the oiled sites and one hatchery site with numerous creosote pilings elicited strong EROD responses, whereas fish exposed to salmon stream extracts elicited weak but significant responses during late summer compared to late spring. Responses from the other sites were not significant, indicating contaminants from these sources are unlikely to cause CYP1A induction in resident biota. Rather than simply assessing extant contaminants, this method evaluates the potency of the different sites for bringing about aryl hydrocarbon receptor responses in resident biota.

## 1. Introduction

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Biochemical responses can be sensitive indicators of pollution exposure. Such responses, termed biomarkers, are indicators in biological fluids, cells or tissues signaling modifications in the biological system due to contaminants (NRC 1987). Biomarker responses have been extensively investigated and provide a convenient and toxicologically relevant means of detecting exposure to low concentrations of contaminants (Dickerson et al., 1994). While biomarkers may indicate exposure to specific classes of contaminants, these responses may provide little information regarding the contaminant source, beyond that inferred from the geographic and temporal patterns of biomarker expression (Short and Springman 2006). Even the location of contaminant sources may be ambiguous when biomarkers are assessed in mobile biota. Moreover, biomarkers usually do not account for chemical speciation or availability (Bucheli and Fent 1995).

Biomarker expression can be linked more directly to contaminant sources via an intermediary contaminant sampler such as semipermeable membrane devices (SPMDs). These passive samplers may be deployed to concentrate lipophilic organic contaminants from acknowledged or probable sources. At equilibrium with the exposure medium, SPMDs accumulate contaminants according to their octanol-water partition coefficient ($K_{ow}$) values (Huckins et al., 2006). The contaminants retained in the SPMD are capable of membrane passage, and as such are potentially a greater risk to biota. Once retrieved by dialysis, these contaminants may be injected into test organisms such as fish, which can then be examined for resulting changes. This relates the response expression to bioavailable contaminants present at the deployment site. Previous studies have assessed the potency of cytochrome P450 1A (CYP1A) in fish liver cells (PLHC-1) to inducers absorbed by environmentally exposed SPMDs (Parrott et al., 1999; Villeneuve et al, 1997). Sundberg et al., (2005) injected trout eggs with

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72 fractionated SPMD extracts to evaluate the induction potency of different contaminant classes
73 found in a region where a single point source was dominant. In all these studies, the
74 contaminants recovered from the SPMDs were purified by silica gel chromatography or other
75 means to facilitate chemical analysis prior to their use for the biological assays. Because
76 purification may remove contaminants that affect the selected biomarker, none of these methods
77 assess the induction potential of the environment *per se*, but only the target contaminants of the
78 study. Our aim in this work is to extend these earlier methods to evaluate the CYP1A-induction
79 potential of *all* nonpolar, lipophilic contaminants absorbed by SPMDs deployed in the
80 environment under study by injecting recovered contaminants into juvenile rainbow trout
81 without clean-up.

82 In situations where multiple contaminant sources are plausible, the biomarker response of
83 injected contaminants extracted from SPMDs provides a method of evaluating the relative
84 potency of different sources. The biomarker response of animals injected with the SPMD extract
85 provides an indication of the induction potency of contaminants from the source(s) sampled by
86 the deployed SPMD. This approach quantifies the biochemical effect triggered by the mixture
87 of nonpolar compounds as a group, and does not require identification of the particular
88 contaminants causing induction. This may be a considerable advantage in cases where a positive
89 biomarker response is evident in resident biota, multiple potential sources are present, and the
90 identity of the contaminants that are driving the biomarker response is not apparent.

91 Prince William Sound (PWS), Alaska provided an excellent setting to test the utility of
92 this method. It is a sparsely populated region where relatively few candidate sources are
93 present, one of which is oil from the 1989 *Exxon Valdez* oil spill that is still found on beaches in
94 western PWS. This oil is a CYP1A inducer (Woodin et al., 1997). Induction of CYP1A has

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been reported in resident sea otters (*Enhydra lutris*) and harlequin ducks (*Histrionicus histrionicus*) that frequent beaches where oil persists (Short et al., 2006, Bodkin et al., 2002, Trust et al., 2000). However, confounding potential sources of CYP1A inducers are possible. These include: (1) asphalt and heavy bunker fuels released from storage tanks damaged by the 1964 Alaska earthquake (Kvenvolden et al 1995); (2) fuel spills from commercial fishing, marinas, salmon hatchery docks and other marine traffic (Page et al., 1999); (3) broad-scale background pollutants from atmospheric deposition of persistent organic pollutants (Stern et al., 1997); (4) polycyclic aromatic hydrocarbons (PAH) associated with oil seeps and erosion of hydrocarbon-rich source rocks and coal outcrops (Short et al., 2004; Short et al., 1999; Page et al., 1995); (5) PAH from forest fires (Page et al., 1999); (6) contaminants from marine vessels (Page et al., 1995); and (7) persistent organic pollutants associated with spawning migrations of adult salmon returning to their natal streams (Krümmel et al 2003; Ewald et al., 1998). Concerns regarding these potential sources of CYP1A inducers in the region continue to be raised (Boehm et al., 2007, Huggett et al., 2006, Neff et al., 2006, Lee and Anderson, 2005, Page et al., 2004, Huggett et al., 2003, Jewett et al., 2002). Injecting fish with extracts from SPMDs and measuring their CYP1A activity provides a way of identifying which sources are capable of stimulating a response. Those that do not, can be eliminated as causes of the CYP1A induction observed in wildlife in that region.

Here, we extended the approach used by Sundberg et al. (2005) to evaluate which pollution sources in PWS were capable of stimulating CYP1A induction. In fish, the activity of this enzyme, as determined with the ethoxyresorufin-*o*-deethylase (EROD) assay, is an established biomarker of exposure to PAH and structurally related compounds. The activity of this enzyme is particularly suited to monitor discharges of petroleum or other PAH sources, co-

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118 planar polychlorinated biphenyls (PCB), and dioxins (Whyte et al., 2000; Stegeman and Hahn
119 1994). Our overall objectives were to identify which of these sources contained compounds that
120 induced CYP1A in exposed biota, and to determine which of the bioavailable hydrocarbons were
121 responsible. We report our results in two parts. Here (Part I), our objectives are to describe and
122 validate the method, which includes an assessment of the precision, sensitivity, and dynamic
123 range. We present the results of our field deployments of SPMDs in PWS to evaluate the
124 CYP1A induction potential of prospective contaminant sources. In Part II, (Short et al., 2008),
125 we relate the CYP1A induction results to the chemical composition of the contaminants
126 accumulated by the SPMDs.
127
128 **2. Methods**
129
130 *2.1 Sampling Design*
131
132 Prince William Sound is a complex, fjord-type ecosystem with a sea surface area of about
133 8,800 $km^2$ (Schmidt 1977). Most of its 7,000 inhabitants reside in Cordova, Valdez or Whittier
134 (Fig. 1), while fewer than 200 live in the vicinity of Chenega or the regional salmon hatcheries.
135 The latter group is near the path followed by oil released by the T/V Exxon Valdez during the oil
136 spill in 1989. Past industrial activities within the spill path were sporadic. These include 2 large
137 mine sites at Latouche, 6 fish processing plants, and a number of smallholdings devoted to
138 mineral prospecting, artisanal sawmills and fur-farming. All of these were abandoned during the
139 mid-20$^{th}$ century (Wooley 2000). Current industrial activities include the Alyeska oil terminal in

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Port Valdez, commercial fishing and oyster farming, 5 large salmon hatcheries, and seasonal tourism.

We deployed SPMDs to evaluate the CYP1A induction potential of 6 classes of contaminant sources: (1) lingering, subsurface, Alaska North Slope (ANS) oil from the *Exxon Valdez* oil spill ($N$ = 5 sites, 3 of which were replicated); (2) human activity ($N$ = 6) sites, including an abandoned industrial site with Monterey Formation bunker oil from storage tanks damaged by the 1964 Alaska earthquake; (3) salmon hatcheries ($N$ = 5); (4) salmon streams ($N$ = 5), because salmon carcasses can release accumulated CYP1A-inducing pollutants as they decompose in their natal streams following reproduction; (5) random sites ($N$ = 9) to evaluate area-wide pollution sources including atmospherically transported contaminants; and (6) a site at Constantine Harbor where PAH associated with organic-rich rocks eroded from geologic sources east of PWS are incorporated into the intertidal sediments (Short et al., 1996). At the ANS sites, we deployed additional SPMDs at adjacent paired control sites to account for local inducing agents other than ANS. We also deployed SPMDs at a remote site at Graves Harbor in southeast Alaska ~ 300 km east of PWS and upcurrent of the geological hydrocarbon sources to serve as a negative regional field control site, and at Cordova Harbor to serve as a positive field control.

The SPMDs were deployed at most sites in spring from mid-May to mid-June 2004, prior to the annual spawning migration of salmon. At the salmon stream sites, they were again deployed in summer from mid-August to mid-September 2004, after adult salmon had returned to spawn. Five of the random site deployments were during spring, and the other 4 during summer to assess region-wide seasonal differences. The random selections for these sites were made within each of 5 sectors partitioning PWS (see Fig. 1 in Short et al., 2008). Each sector

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was allocated 1 or 2 sites to ensure dispersion, and each site was located on a randomly-selected shore segment within a sector as described in Part II (Short et al., 2008).

*2.2 Semipermeable membrane devices*

The SPMDs were obtained from Environmental Sampling Technologies (St. Joseph, MO), and deployed in 30 cm long cylindrical cages, each loaded with 5 SPMDs woven on stainless steel carriers stacked within the cage. Each SPMD had dimensions of 91.4 × 2.5 cm and contained 1 ml of ultra-high purity (UHP) triolein. The cages were half buried in pits excavated at the mid-tide level of deployment site shorelines. After placement in a pit, the excavated material was replaced to the level of the beach surface, surrounding, but not covering, the cage. This arrangement allowed the SPMDs to sample the atmosphere at low tide (Lohmann et al., 2001, Ockenden et al., 1998), surface seawater at high tide, and interstitial water flow within the upper 15 cm of beach while minimizing biofouling. Cages were deployed for the standard deployment period of 27 – 28 days, then retrieved for dialysis. At the ANS and human activity sites where contamination was greater, the cages were placed in patches of subsurface oil to ensure uptake of organic contaminants. A field blank cage with 5 UHP SPMDs was briefly exposed to the atmosphere during two of the deployments and then stored for dialysis.

*2.3 SPMD processing*

Contaminants were recovered by dialysis into hexane at the laboratory of Environmental Sampling Technologies, following physical removal of external sediment and biofouling as per

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185 Huckins et al. (2000). The dialysates from all 5 SPMDs within a cage were pooled. Following
186 concentration of the combined dialysates to ~1 ml, samples were sent to Auke Bay Laboratory in
187 sealed ampules. The contents were partitioned into subsamples for chemical analysis (3 aliquots
188 each containing 5% of the dialysate) and for injection into juvenile trout for CYP1A induction (2
189 aliquots, each with 42.5% of the dialysate). One each of the aliquots for injection and for
190 chemical analysis were archived in the event of accidental losses. Results of the chemical
191 analysis of the dialysates are presented in Part II (Short et al., 2008). The aliquot used for
192 injection was exchanged into peanut oil (Spectrum Chemical Mfg., Gardena, CA) under nitrogen
193 at ~ 35 ºC. Samples for induction tests were coded with un-informative sample identification
194 numbers and sent to California for blind bioassay.
195 Quality assurance samples included field and dialysis blanks, a solvent control, and a
196 positive laboratory control (β-naphthoflavone or BNF, a model CYP1A inducer). The β-
197 naphthoflavone was suspended in peanut oil and doses of 2.5 mg $kg^{-1}$ were prepared for three
198 fish weight classes, ≤ 7.5 g, 7.5-10.0 g, ≥ 10.0g, to maintain a uniform injection volume of 50 µl
199 $fish^{-1}$.
200
201 *2.4 Animal Exposure*
202
203 Juvenile rainbow trout (*Oncorhynchus mykiss*), Mount Shasta strain, average weight 9.2
204 ± 2.6 g, were obtained from California Department of Fish and Game Mount Shasta Hatchery.
205 They were maintained at 13 ± 1°C in aerated, flow-through water systems for at least a week
206 prior to testing and fed a commercial diet (Silver Cup, #3, Nelson & Sons Inc., Murray, UT) until
207 the day prior to dosing.

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208 The dialysate aliquot from each sample was partitioned further into 10 sub-aliquots, each
209 injected into a single juvenile trout, so each trout received 4.2% of the dialysate from an SPMD
210 deployment in a 50 µl injection. For each sample, 10 fish were anesthetized in a solution of ~50
211 mg $l^{-1}$ tricaine methane sulfonate (MS222) and injected intraperitoneally (i.p.) with the extract.
212 Many compounds can induce CYP1A, including planar hydrocarbons that meet the
213 requisite structural criteria, but these compounds may reach peak induction at different times.
214 The positive control, β-naphthoflavone (BNF), is at its peak induction ~ 48h after i.p. injection in
215 rainbow trout, which is similar to the response from PAHs in this species (Lemaire et al., 1996).
216 Of 209 polychlorinated biphenyls (PCBs), those that can induce CYP1A strongly in rainbow
217 trout show peak induction at 6 d following i.p. injection (Huuskonen et al., 1996). We sacrificed
218 5 fish of each group in an overdose of MS222 at 2 d to examine the CYP1A responses of those
219 compounds that are metabolized more readily, such as PAH, while allowing the remaining 5 fish
220 7 d until sacrifice to evaluate effects from compounds which reach peak induction later, such as
221 PCBs. The weight and standard length of each individual were recorded, and the liver excised
222 and frozen in liquid nitrogen within two minutes of death. This was repeated with the remaining
223 5 fish from each group at 7 d post-injection. The livers were shipped frozen to Queen's
224 University, Kingston, Ontario, Canada for evaluation of CYP1A induction.
225
226 *2.5 EROD bioassay*
227
228 CYP1A induction was estimated by measuring the activity of ethoxyresorufin-*o*-
229 deethylase (EROD), based on an original method by Pohl and Fouts (1980) modified for a
230 microplate spectrofluorometer (Hodson et al., 1996). Liver samples stored at -84°C were

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231 thawed on ice and a sub-sample of 15-60 mg was homogenized in 500 – 750 µL of ice-cold 20
232 mM Na-HEPES buffer in 150 mM KCl (pH7.5). Homogenates were centrifuged for 20 minutes
233 at 2°C and 9000 x G, and the supernatant and microsome layer (S-9 fraction) were recovered and
234 stored at -84°C. Each S-9 fraction was thawed on ice and triplicate aliquots of 50 µL were
235 incubated with 50 µL of 2.2 µM 7-ethoxyresorufin in 100 mM HEPES buffer (pH 7.8) in a 96-
236 well microplate held in the dark for 10 minutes at room temperature. The reaction was initiated
237 with 10 µL of 1.1 µM of NADPH. Fluorescence of resorufin, the reaction product, was
238 measured every minute for 12 minutes at excitation and emission wavelengths of 530 and 586
239 nm with a SpectraMax Gemini spectrofluorometer (Molecular Devices, Sunnyvale CA, USA).
240 Crude activity was estimated as the rate of increase in fluorescence and converted to a molar
241 basis by reference to a resorufin standard curve (0-20 pM) included with each plate. Molar
242 specific activity (picomole resorufin per mg protein per minute; pmol mg protein$^{-1}$ min$^{-1}$) was
243 calculated by normalizing crude activity to S-9 protein concentrations. Protein was determined
244 against a standard curve of bovine serum albumin (0-2.5 mg l$^{-1}$) by mixing 10 µL of each S-9
245 fraction with 200 µL of Biorad Reagent (Biorad, Hercules, CA, USA) and measuring absorption
246 at 600 nm with a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale CA,
247 USA). Data were acquired and analyzed with Molecular Devices SOFTMAX PRO software.
248 Samples were analyzed blind, and the analytical batches were composed of samples selected at
249 random.
250
251 *2.6 Data analysis*
252

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The CYP1A induction caused by contaminants recovered from the 5 SPMDs in a deployment device is presented as the antilog of the logarithmic mean EROD value of the 5 fish injected for each time group (2 d and 7 d). The logarithmic data transformation is used as the EROD activities are log-normally distributed (Hodson et al. 1996), justifying parametric statistical comparisons. Consequently, following logarithmic transformation, we used analysis of variance (ANOVA) to assess the significance of differences between EROD values. Groups of SPMD results within contaminant source classes are presented as the mean for individual SPMD deployments. Variation is based on the logarithmic standard error, presented as the antilog, which gives an asymmetric interval.

To clarify notation in these statistical tests, we use "$N$" to represent the number of sites in a class, as described in Section 2.1. Because the number of individuals compared is the number of fish injected, we use "$n$" to denote the number of fish within a group, and "$m$" to denote the number of SPMDs that were used to prepare the extract for that group. Although 5 fish were usually injected with the equivalent of an extract aliquot from one SPMD, the number of corresponding EROD analyses was occasionally fewer because of processing errors, and greater in some cases because all the extract was used to evaluate the response at 2 d.

## 3. Results and Discussion

### *3.1 Analytical Performance*

The ability of the method to detect and distinguish contaminant burdens injected into the juvenile trout depends on the sensitivity, precision, and dynamic range of the EROD response,

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which are demonstrated by the laboratory and field control treatments. The basal EROD activity of unhandled fish, 0.932 pmol mg protein$^{-1}$ min$^{-1}$ (Table 1), defines the minimum activity expected in the absence of stress. Injection of the peanut oil solvent caused a significant doubling of activity after 2 d ($P = 0.002$), subsiding by 7 d to a level not significantly different than the basal activity ($P = 0.56$; Table 1). The lower responses at 7 d compared with 2 d were the result of recovery from the stress of the initial injection and handling (Lemaire et al., 1996, Blom and Förlin, 1997). Consequently, increases that more than double the basal activity following injection were probably the result of factors other than handling stress.

Injection of some of the field- and dialysis-blank SPMD extracts caused greater EROD activity than expected from handling stress, suggesting low-level contamination introduced during manufacture. The mean EROD activity of these blanks was twice that of the corresponding activities caused by injection of peanut oil at 2 d and at 7d (Table 1), both significant increases ($P < 0.05$). Analysis of variance showed marginally significant differences among the field blanks (FB; $m = 4$) at 2 d ($P = 0.063$) and clearly significant differences at 7 d ($P < 0.001$). The 7 d activities of one of the field blanks and one of the dialysis blanks were not significantly different than their corresponding 2 d activities ($P > 0.20$), the other field blank and dialysis blank are significantly lower ($P < 0.01$) at 7 d. These results suggest that all the field and dialysis blanks contained traces of contaminants capable of stimulating small increases in EROD activity 2 d following extract injection, extending in some cases to 7 d. Such low-level traces of contaminants in un-deployed SPMDs have been noted previously by Boehm et al., (2005). However, the alkylated PAH recovered from SPMDs by Boehm et al. (2005) may have been introduced as laboratory artifacts during their non-standard dialysis of the samplers (Schwartz, 2006).

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Oleic acid, an impurity in the triolein used for SPMDs, may contribute to some of the observed responses. The fraction containing these compounds has produced positive responses in the Microtox assay (Sabaliûnas et al., 1999). However, Huckins et al. (2006) noted that while deployed, any remaining oleic acid or methyl oleate impurities diffuse to the exterior surface of the membrane where they are removed. This environmental release does not occur in quality control SPMDs (field blanks and dialysis blanks), creating the potential for differential responses. Purified (UHP) triolein substantially decreases these impurities (Lebo et al, 2003; also, summary of SPMD screening in Supplementary Data), thus decreasing but not precluding this risk, which could be magnified without sample cleanup. These results indicate that additional purification of SPMDs may be necessary to achieve maximum sensitivity when the contaminants are extracted, injected into fish and the EROD response measured. In our study, this additional purification was provided by environmental exposure when SPMDs were deployed at sites distant from obvious contaminant sources within PWS.

The low EROD responses produced by SPMDs deployed at random sites, Constantine Harbor, regional field control and the local ANS control sites provide a robust estimate of the precision of the method in the absence of contaminants. The mean EROD response for these sites was not significantly different from the responses from the peanut oil injections at either 2 d or 7 d ($P > 0.47$, $m = 16$; Table 1), but were significantly below the corresponding field and dialysis blank responses ($P < 0.001$). This indicates that impurities initially present in the SPMDs had diffused into the environment during deployment, an effect that is also evident in PAH data presented for reference sites in PWS presented by Boehm et al. (2005). As with the peanut oil injections, the 2 d responses were significantly greater than the 7 d responses by a factor of 2 ($P < 0.001$), confirming the effect of handling stress. Analysis of variance did not

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shows significant differences among the individual SPMDs at 2 d following injection ($P$ = 0.318), but did at 7 d ($P < 0.001$), which we attribute to slight variation in fish responses to handling stress.

Comparison of the positive and negative control samples illustrates the analytical range of the method. The positive laboratory control dose of 2.5 2.5 mg $kg^{-1}$ BNF is near the threshold for immune system impairment in juvenile trout similar to those we used (Springman et al., 2005), providing a biologically meaningful laboratory benchmark. The corresponding 2 d and 7 d EROD responses near 70 pmol mg $protein^{-1}$ $min^{-1}$ (Table 1) were about 40-fold greater than the response from the peanut oil injection. The contaminants accumulated by the SPMDs deployed at the Cordova Harbor field control site elicited about half the response of the BNF dose (Table 1), confirming the ability of the method to detect CYP1A-inducing contaminants when present. Cordova Harbor, a confined harbor with limited seawater exchange, is one of the most polluted in the United States based on petroleum-derived contaminant concentrations in mussels within the harbor (Miles et al., 2001).

The EROD responses of the rainbow trout we injected are comparable with responses expected for feral fish and other biota occupying the environment of the deployed SPMD. A valid comparison of induction from bioavailable CYP1A inducers depends on a comparable absorption capacity in both fish and SPMD. Direct experimental comparison of uptake rates and accumulated burdens for SPMDs vs. fish shows that SPMDs accumulate polychlorinated biphenyls (PCBs) about twice as fast per gram SPMD as per gram wet fish tissue, resulting in body burdens about twice as great (Echols et al., 2000; Meadows et al., 1998). With our method, 42.5% of the contaminants accumulated by 22.5 g of SPMDs was injected into 10 rainbow trout of 9.2 g mean wet weight, so the effective ratio of SPMD to wet fish tissue is 1:10, which is

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comparable to that in Meadows et al. (1998). If lipophilic contaminants are accumulated twice as fast by SPMDs than fish, then our fish received about one-fifth the body burden they would have accumulated, had they been exposed to the deployment conditions of the SPMD that produced the extract we injected into them. However, the entire complement of contaminants accumulated by the SPMD during the 28 d SPMD deployment period was directly administered to our naïve test fish. Assuming fish absorb, biotransform and eliminate xenobiotics according to first-order kinetics, the time required to attain 20% of the equilibrium body burden when exposed to a constant aqueous contaminant concentration is ($0.22 * t_{1/2}/0.693$), where $t_{1/2}$ is the half-life of the contaminant in the fish (Meador et al., 1995). Half-lives of 3- and 4-ring PAH in rainbow trout are 2 to 9 days (Niimi and Dookhran 1989; Niimi and Palazzo 1986), implying 20% accumulation within 0.6 – 2.9 days. Even longer times would be required for more persistent contaminants such as PCBs due to their longer half-lives. Our intraperitoneal injection lead to complete absorption of contaminants within a few hours, and would therefore be about as effective for stimulating CYP1A induction as placing the fish in the environment sampled by the deployed SPMD. Consequently, it is unlikely that biota exposed to the same environment as our deployed SPMDs would have CYP1A activities above their basal level if an injection of contaminants from the SPMD fails to stimulate induction.

Having described the analytical performance and characteristics of our SPMD-extract injection method, we now assess results from SPMD deployments at sites within or near obvious local contaminant sources within PWS.

*3.2 ANS Sites*

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368 The SPMDs deployed at the ANS sites consistently accumulated inducers responsible for
369 the strongest CYP1A responses of our study (Fig. 2). The overall mean response was 39 pmol
370 mg protein$^{-1}$ min$^{-1}$ for 2 d, significantly exceeding the ANS control site responses ($P < 0.001$).
371 Mean responses for these sites ranged from 23 – 69 pmol mg protein$^{-1}$ min$^{-1}$. At one site where
372 3 SPMDs were deployed within 2 m of each other, the mean responses ranged from 24 – 36
373 pmol mg protein$^{-1}$ min$^{-1}$, providing an indication of field reproducibility of the method. The
374 consistency of the responses from the ANS sites shows that the lingering ANS oil remains a
375 potent source of CYP1A-inducing compounds, and that those compounds are in a form that can
376 be absorbed by SPMDs and presumably by biota. The subsurface oil patches where the SPMDs
377 were deployed consisted of semi-liquid oil that readily produced surface sheens on contact with
378 water, so the CYP1A inductive potential of the extracts derived from these deployments is not
379 surprising, given the demonstrated CYP1A induction potency of ANS oil (Woodin et al., 1997).
380 Induction of EROD activity persisted for 7 d following injection of SPMD extracts from
381 most of the ANS sites (Fig. 2). The 7 d response was greater than the 2 d response at one of the
382 sites on Disk Island, but otherwise were lower by factors of 2 – 3 at 3 other sites, and by factors
383 of 5 -10 at the remaining 2 sites (KN114A and KN109A). The results from the ANS sites
384 suggest more complex interactions which incorporate site-specific characteristics, such as those
385 found at Disk Island and areas of Knight Island (KN136A). Exposure to some forms of peat
386 may induce CYP1A (Kopponen et al., 1993), and co-exposure to humic acids can modulate
387 induction (Matsuo et al. 2006). These sites are located in areas where there is peat beneath the
388 oil, and one of the Disk Island deployments was within a stream. The 2 d responses from all
389 three sites (Fig. 2) are similar to each other and to other oiled sites. However, the 7 d responses
390 corroborate the elevated CYP1A activity that results from co-exposure to humic substances and

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petroleum (Matsuo et al., 2006), in that the 7d response is more persistent at the Disk Island location where the peat is not flushed by the surface stream. ANS sites received the same oil in 1989, yet the response elicited by bioavailable hydrocarbons in 2004 varies in persistence and intensity from site to site. Such location-specific factors may sometimes affect the potential toxicity of bioavailable PAH.

*3.3 Human Activity and Salmon Hatchery Sites*

The EROD responses from the human activity and salmon hatchery sites were usually similar or only slightly greater than responses at the random and other uncontaminated sites (Fig. 3), with two exceptions: McClure Bay and Armin F. Koernig (AFK) hatchery. Excluding these two sites, the mean 2 d and 7 d EROD responses of and salmon hatchery sources ranged from 1.9 – 5.7 pmol mg protein$^{-1}$ min$^{-1}$ ($m$ = 9, $n$ = 45) and from 1.3 – 2.9 pmol mg protein$^{-1}$ min$^{-1}$ ($n$ = 44), respectively. The responses that are significantly greater than those of the uncontaminated sites are indicated in Fig. 3. These sites include surface patches of Monterey Formation asphalt at Latouche, Port Ashton, Port Audrey and Thumb Bay, and unidentified contaminants near the hatchery at Main Bay. At Latouche, the EROD response remained significantly elevated through 7 d.

These small but significant EROD responses indicate that the CYP1A induction potencies of the contaminants at most human activity and salmon hatchery sites are minor when compared with induction potential from oiled ANS sites. This may be because either these human activity and salmon hatchery contaminants are less bioavailable, or are not strong inducers, or both. Page et al. (2006) reported substantial concentrations of PAH associated with

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414 asphaltic remnants of Monterey Formation bunker oil contaminating a total of ~ 2.1 ha of
415 surficial sediments at Latouche, Port Ashton, Port Audrey and Thumb Bay. Our results indicate
416 the CYP1A induction potency of these sediments is weak in contrast to lingering ANS oil
417 (compare Figs. 2 and 3). Latouche, Port Ashton, Port Audrey and Thumb Bay account for 95%
418 of the total area contaminated by Monterey Formation bunker oil that also lies within the path of
419 the *Exxon Valdez* oil spill (Page et al., 2006). This combined area amounts to ~ 20 % of the area
420 contaminated by lingering *Exxon Valdez* oil (Short et al., 2004, 2008). The combination of
421 weaker induction and the smaller size of the combined Monterey Formation bunker oil spills
422 demonstrates these sources of EROD-inducing contaminants are minor in comparison with
423 lingering *Exxon Valdez* oil.

424 In contrast, SPMD extracts from the AFK hatchery and McClure Bay sites both produced
425 substantial EROD responses (Fig. 3), indicating the presence of stronger and more bioavailable
426 CYP1A inducing contaminants. The 2 d mean EROD responses are greater than 31 pmol mg
427 protein$^{-1}$ min$^{-1}$ and remained elevated after 7 d, especially at McClure Bay, where the 7 d
428 response was nearly half the 2 d response. The deployment site at AFK is near a large pier
429 supported by several hundred creosote pilings, and creosote pilings support numerous other piers
430 or their remnants within ~ 1- 2 km of the deployment site, unlike the other hatcheries where steel
431 or concrete pilings are in use. These creosote pilings are the likely source of the CYP1A-
432 inducing contaminants at AFK. Creosote contains numerous PAH that are potent CYP1A
433 inducers (Hyötyläinen and Oikari 1999) and the large surface area of the pilings near the AFK
434 deployment site would promote dissolution of PAH into surrounding waters. Similarly, the
435 SPMD deployment at McClure Bay was within a patch of semi-liquid, subsurface Monterey
436 Formation bunker fuel oil that was released from a nearby storage tank ruptured during the 1964

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Alaska earthquake. In contrast with surficial deposits of Monterey Formation asphalt at the other human activity sites, the EROD response from the McClure Bay deployment shows that burial can impede weathering such that the associated contaminants remain readily bioavailable after 40 years. This corroborates the continuing bioavailability of contaminants associated with the ANS oil, as well as the extraordinary persistence of remaining ANS oil (Short et al., 2004, 2008). Also, the elevated 7 d EROD responses confirms our results from the oiled ANS sites, in that prolonged EROD responses are associated with this hydrocarbon source as well.

The prolonged, increased EROD responses from SPMD extracts deployed at some sites suggests possible interactive effects, since elevated 7 d EROD activity occurs only at those sites with a history of hydrocarbon exposure. For example, PAH oxidation may contribute to CYP1A induction at favorable sites for microbial growth. This prolonged EROD response may increase the duration that significant EROD responses are detectable in monitored, mobile biota exposed to lingering oil in PWS, such as sea otters (Bodkin et al., 2002), and harlequin ducks (Trust et al. 2000).

*3.4 Salmon Streams*

The EROD responses from the summer SPMD deployments in the 5 salmon streams consistently exceeded those of the spring deployments, implying slight seasonal variability at these sites. The 2 d and 7 d responses from salmon stream spring deployments are not significantly different ($P > 0.105$) than the respective responses from the random sites sampled in spring (Fig. 4). In contrast, the 2 d and 7 d responses from summer deployments, averaged over the 5 salmon stream sites, are 3.6 and 2.0 pmol mg protein$^{-1}$ min$^{-1}$ ($n = 25$), which are 41%

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and 44% greater than respective responses from the random sites sampled during summer ($P <$ 0.036). The increases of the 2 d responses are consistent for all 5 salmon streams, and for 4 of the streams for the 7 d responses. The increase during late summer but not during late spring indicates a seasonally transient input that subsides from late summer to the following spring. This summer increase is localized to the salmon streams. Differences between random site deployments during spring compared with summer are not significant ($P > 0.15$; Fig. 4) and are within the range of responses from peanut oil injections. Thus the seasonal increase observed at the salmon stream sites is probably not the result of a region-wide contaminant input. The timing of this increase is in agreement with contaminants released from decomposing salmon carcasses, following their migration to their natal stream to reproduce.

*3.5 Constraints on EROD Induction by Background Contaminants*

The consistent absence of CYP1A induction following injection of contaminants extracted from our random and control site SPMDs into juvenile trout shows that the contaminant background is a negligible source of dissolved, lipophilic CYP1A inducers in PWS. Background contaminants are by definition broadly distributed, so they should routinely cause induction if they include CYP1A inducers. But our results show that none of the $m = 16$ SPMD deployments at random and control sites elicited CYP1A induction above the stress caused by peanut oil injection, and that deployment at these sites actually removed CYP1A inducing compounds that were initially present in the SPMDs at deployment. We therefore conclude that, apart from small, localized pollution sources that are usually readily identifiable (e.g. creosote pilings, remnant oil deposits from the 1964 earthquake or the 1989 *Exxon Valdez* oil spill, etc.),

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483 the marine waters of PWS are among the least contaminated coastal waters with respect to
484 dissolved, lipophilic CYP1A inducers.

485 The low levels of dissolved CYP1A-inducing contaminants in the marine waters of PWS
486 implied by our random and control site results are consistent with most other studies of
487 contaminants in the region. As reviewed recently by Boehm et al. (2007), measured background
488 concentrations of total PAH in the marine waters of PWS are usually less than 20 ng $L^{-1}$ (often
489 near or below the detection limits of the methods used), nearly all of which are 2- and 3-ring
490 PAH that are not potent CYP1A inducers (Barron et al., 2004). The SPMDs deployed by Boehm
491 et al. (2005) on the beach surface at their reference site contained a mean total PAH amount of
492 39 ng $SPMD^{-1}$, implying aqueous concentrations in the neighborhood of 1 ng $L^{-1}$, again with
493 compounds capable of inducing CYP1A likely to be a very small proportion. In a survey of
494 PAH bioavailability at PWS sites oiled by the 1989 *Exxon Valdez* oil spill, Neff et al. (2006)
495 measured PAH concentrations of sea lettuce (*Ulva fenestrate*), reasoning that the mucilaginous
496 surface coating of this macroalgae would efficiently collect dissolved and particle-bound PAH
497 from seawater at higher tides and from the atmosphere at lower tides, much as our SPMDs
498 absorbed dissolved PAH from these media. They found mean concentrations of 11.3 ng $g^{-1}$ dry
499 wt., again implying ambient total PAH concentrations below 1 ng $L^{-1}$ (assuming a wet:dry wt
500 ratio of 10:1, and an effective $K_{ow}$ of $10^4$). In a study of PAH in lipid-rich copepods in central
501 PWS, Carls et al. (2006) found total PAH of 409 ng $g^{-1}$, entirely of 2- and 3-ring PAH, and
502 equivalent to aqueous total PAH concentrations below about 20 ng $l^{-1}$. Finally, the PAH
503 concentrations detected in the SPMDs deployed at the random and control sites of this study also
504 imply ambient dissolved total PAH concentrations in the low ng $L^{-1}$ range, with the more potent
505 CYP1A inducers such as PCBs and chrysenes usually below their quantitation limits in the

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506 SPMDs (Short et al., 2008). These studies all indicate ambient concentrations of the more potent
507 CYP1A inducers such as chrysene that are well below 1 ng $L^{-1}$ in the marine waters of PWS, and
508 hence well below concentrations that might plausibly stimulate a background elevation of
509 CYP1A in PWS biota.

510 Neff et al. (2003) concluded that background PAH in deep-water sediments of PWS are
511 bioavailable based on differences in concentration patterns detected in polychaete worms (*Nereis*
512 *virens*) following prolonged exposure, but the supporting evidence presented is equivocal. The
513 PAH burden of the polychaete worms declined during exposure to the PWS sediments, were near
514 typical detection limits of the method used, and no evidence of statistical significance was
515 reported, suggesting the PAH pattern differences may simply reflect analytical variability.

516 Huggett et al. (2003) and Page et al. (2004) have argued that marine fish in the northern
517 Gulf of Alaska are chronically exposed to CYP1A-inducing hydrocarbons derived from oil seeps
518 and eroding hydrocarbon source rocks, but this conclusion seems premature. Their argument is
519 based on detecting measurable liver EROD responses in 4 species of fish, and fluorescent
520 aromatic compounds (FAC) in their bile. However, these authors failed to consider the
521 possibility that the low values they reported are merely the basal responses for the fish species
522 they tested. A thorough familiarity of this endogenous activity is necessary for accurate
523 interpretation of EROD induction. Of several biomarkers examined in 15 species of fish, EROD
524 induction showed the most variable basal activity between species (Förlin et al., 1995). An
525 accurate determination of the factors that can modulate it is a central concern when using this
526 biomarker to gauge contaminant impacts (Flammarion and Garric, 1997). Basal activity levels
527 were not reported for any of the fish species tested by Huggett et al. (2003), so there is no basis
528 for evaluating the significance of the EROD activities they presented. Similarly, the bile FAC

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529 responses for a single fish species considered by Huggett et al. (2003) show a marginally
530 significant increase above three samples from the lower of two reference sites that were collected
531 a decade earlier by different researchers and analyzed by a different laboratory. Moreover, the
532 identity of the compounds that caused the bile FAC response, presumably peaks within the same
533 HPLC retention time interval as 3-ring PAH conjugates, is not clear. Unfortunately, Huggett et
534 al. (2003) failed to verify the fluorescent compounds they detected in their bile samples as
535 conjugates of PAH by gas chromatography-mass spectrometry (Krahn et al.1992). These peaks
536 may have been interferences from naturally occurring compounds (Krahn et al. 1984), including
537 naturally occurring PAH such as retene (Lipiatou and Saliot 1992, 1991), or the metabolites of
538 organic pigments ingested during feeding. Also, the fish sampled were not controlled for sex,
539 age, reproductive status or differences in recently ingested prey, which may affect bile FAC
540 results (Collier and Varanasi 1992). Indeed, it appears that the basal response for bile
541 fluorescent compounds has not been established unequivocally for any species, making
542 interpretation of the evidence presented by Huggett et al. (2003) and Page et al. (2004)
543 problematic.

544 Woodin et al. (1997) did detect a slight but apparently significant increase in liver EROD
545 responses in an intertidal fish (gunnel, *Anoplarchus purpurescens*) collected from two reference
546 areas in PWS compared with basal responses, but it is not clear whether this CYP1A-inducing
547 agent was from a widespread background source or from undetected localized sources.

548 Overall, it seems unlikely that marine sediments contaminated by PAH from natural
549 petrogenic sources are capable of causing significant CYP1A induction in biota, when the extract
550 from the SPMD deployed at the Constantine Harbor failed to do so following injection into
551 juvenile trout. This deployment site is a mudflat containing ~560 ng PAH $g^{-1}$ sediment derived

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entirely from the eroded geologic sources (Short et al., 1996), and this concentration is within a factor of 3 of the greatest background concentration found anywhere within PWS (Page et al., 1996). The failure to accumulate sufficient PAH to stimulate a detectable EROD response when injected, after deployment of the SPMD in intimate contact with the Constantine Harbor sediments for nearly a month with the Constantine Harbor sediments, is strong evidence that the PAH associated with these sediments is not sufficiently bioavailable to induce CYP1A in exposed biota. Also, the general absence of EROD responses from SPMDs deployed at random and control sites is consistent with the low intensity of human impacts (apart from the *Exxon Valdez* oil spill) in PWS. Although human activities have affected scores of sites within PWS (Page et al., 2006; Wooley, 2000), most of these sites were either concentrated around Sawmill Bay or else were smallholdings that usually impacted less than 100 m of shoreline each. These impacts were distributed over more than a century and their cumulative total affects less than 0.2% of the present day shoreline in PWS (Boehm et al., 2004). These small scale impacts, coupled with a low population density, results in fewer and more localized contaminant sources. The absence of CYP1A-inducing contaminants in our SPMDs from random and control sites is therefore not surprising.

## 4. Conclusions

The results of this study demonstrate the value of SPMD dialysate injection for elucidating contaminant sources and exposure effects. Method sensitivity is illustrated by the small but significant differences among EROD responses, such as those between spring and summer salmon stream EROD activities. Significant EROD induction occurs following injection

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575 of dialysates from SPMDs exposed to part per trillion aqueous concentrations of potent inducers,
576 especially chrysenes (Short et al., 2008). This shows that the SPMD/EROD assay as described
577 in this study to be a cost-effective method for detecting these contaminants in comparison with
578 direct chemical analysis of the dialysate. Another advantage over direct chemical analysis is that
579 the identity of the EROD-inducing agents need not be known. Instead, this method assesses the
580 inductive potential of the bioavailable complex mixture, with all its inherent interactions, at a
581 given site. The dynamic range is sufficiently large to indicate contamination gradients that may
582 suggest sources, and to quantify the effects of the bioavailable contaminant fraction following
583 direct exposure. Method reproducibility is demonstrated by the biochemical responses from the
584 oiled sites, particularly EL56C where the replicates were 2 meters apart, and the apparent
585 absence of false negatives. Thus, the method provides a reliable means of evaluating exposure
586 to the bioavailable fraction of hydrophobic contaminants, as the response elicited is based on the
587 absorption of contaminants into the SPMD, which serves as a standardized surrogate for local
588 biota.

589 This method overcomes potential drawbacks associated with biomarkers when compared
590 to chemical analysis, as presented by Forbes et al. (2006), including cost, relevance, sensitivity,
591 reproducibility, and consistency. Technological advances, such as enzyme-linked
592 immunosorbent assay (ELISA) columns and assay kits, have led to substantial increases in ease
593 of use for EROD and other biomarkers. The EROD assay is relatively simple in both method
594 and required equipment; by comparison, determination of chemical residues in tissue or
595 environmental samples is much more complex and equipment-intensive, leading to higher per
596 sample costs than those spent on biomarkers (Dickerson, 1994; EPA, 1986). For each site in this
597 study, the cost of EROD assays was about 16% of that for chemical analyses. Finally, this

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method allows an assessment of the total contribution of the complex mixture that can be taken up by the targets, whether these compounds are detected analytically or not.

The SPMD extract injection method complements field testing of biota from contaminated areas. This method measures the response that the bioavailable contaminant fraction can produce with few confounding variables such as gender, age, reproductive status or condition. CYP1A induction was selected here as it is a well-established biomarker of PAH exposure. For breadth of applicability, the test organism we used is an appropriate choice as rainbow trout P450 systems may be as complex as their mammalian counterparts (Buhler and Wang-Buhler 1998). Ultimately, the suspected non-polar contaminants and the potential targets determine which response(s) to measure and which test organism to use, but the method presented here is flexible and can accommodate other types of analysis and different test animals.

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854 **Figure Legends**

855 Figure 1. Location of SPMD deployment sites in Prince William Sound, Alaska.

856 Figure 2. EROD response to SPMD extracts from the oiled ANS sites used in this study. Sites
857 KN114A1, KN114A2, KN136A and KN109A are located on Knight Island; EL56C1, EL56C2
858 and EL56C3 are sites on Eleanor Island; DI67A1 and DI67A2 are on Disk Island (See Short et
859 al., 2008 for a detailed map). The field blank (FB), field control (FC) and positive control (β-
860 naphthoflavone; BNF) responses are included for comparison. Mean EROD activities are derived
861 from the logarithmic mean of (*n*) individuals, with ± 1 SE. White bars indicate 2 d response and
862 grey bars are 7 d. No data are available where noted.
863

864 Figure 3. EROD response to SPMD extracts from the human activity and salmon hatchery sites
865 (as noted) including Armin F. Koernig (AFK) hatchery. White bars indicate 2 d response and
866 grey bars are 7 d. Asterisks indicate significant increases (*t*-test $P < 0.05$).
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868 Figure 4. EROD responses to SPMD extracts from salmon streams (SS) and random sites (RS)
869 in both fall and spring. Responses from the peanut oil injections (PNUT) are included for
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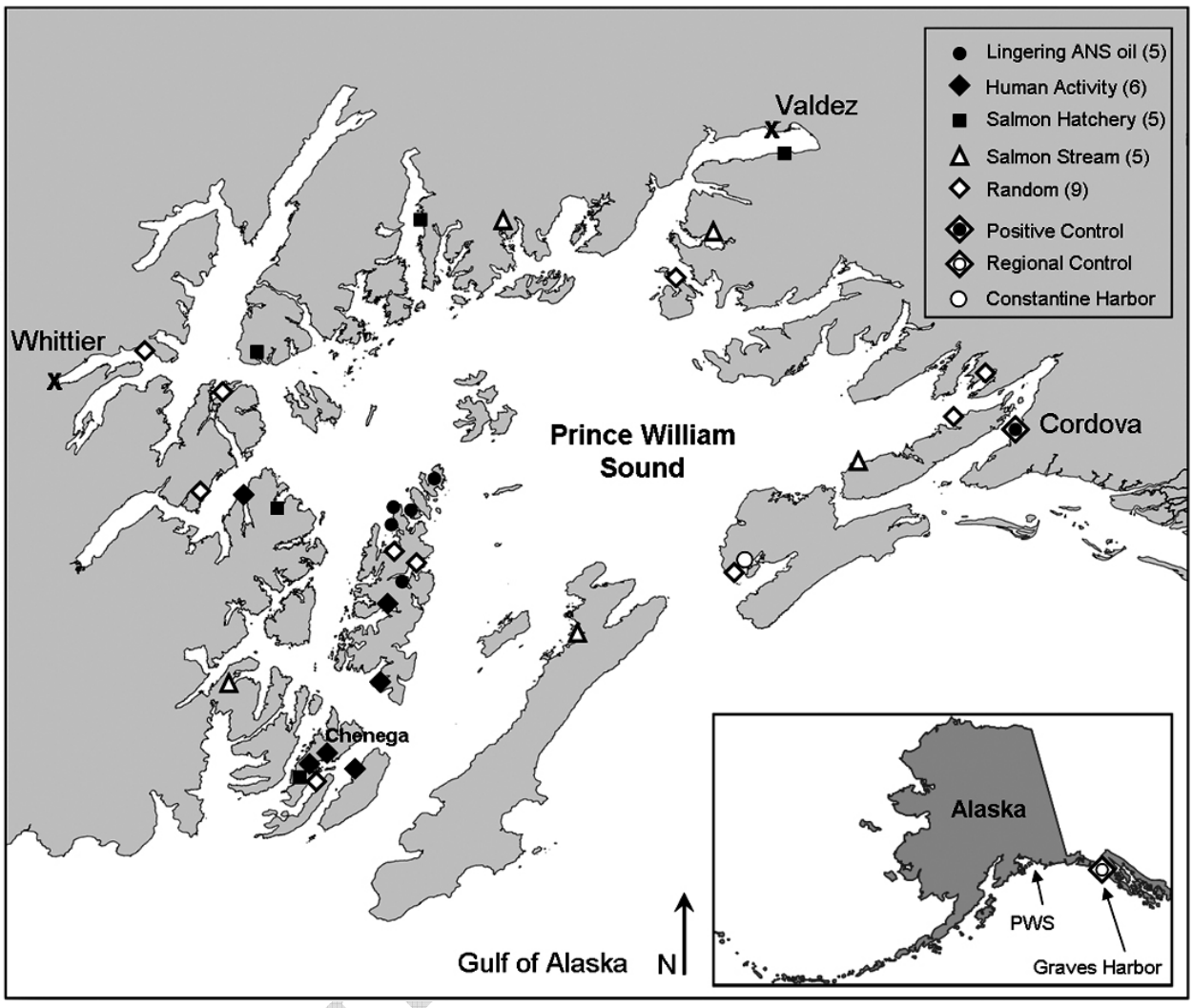


Figure 1. Location of SPMD deployment sites in Prince William Sound, Alaska.

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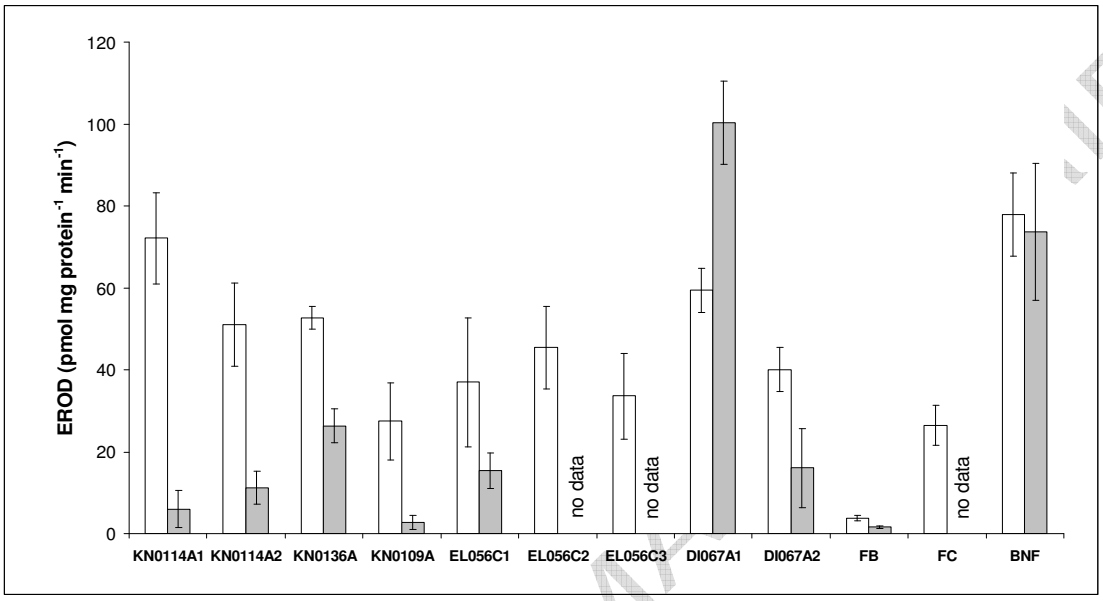


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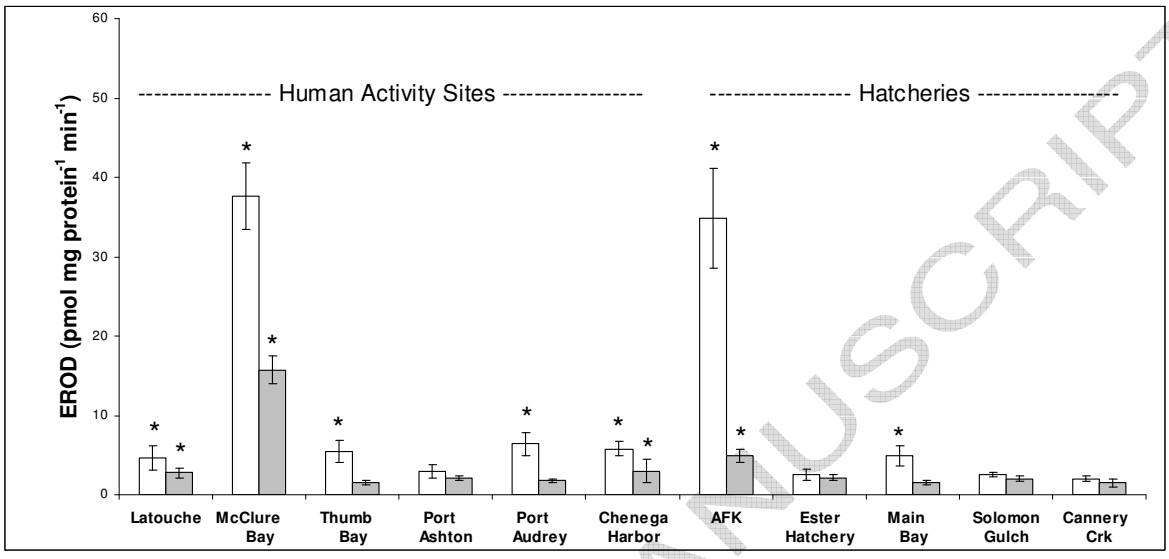


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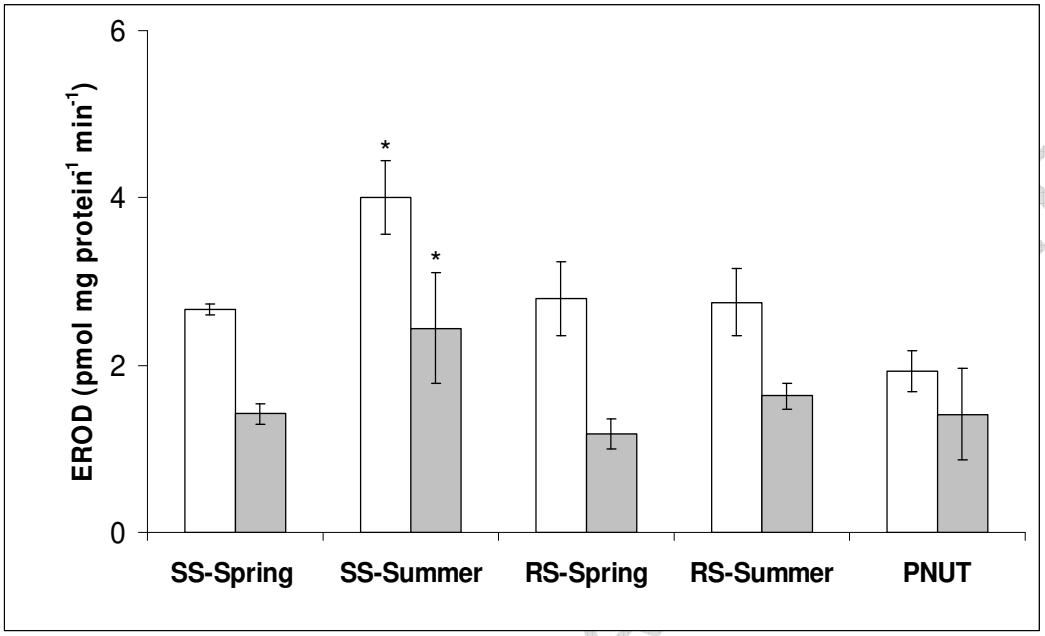


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Table 1. Basal EROD activity of juvenile rainbow trout (denoted "not injected") compared with EROD responses to negative and positive control treatments and responses from SPMD deployments at un-contaminated sites. Mean EROD activities are given as pmol $mg^{-1}$ $min^{-1}$, derived from the logarithmic mean of (*n*) individuals, with ± 1 SE in parentheses below.

| Site/Treatment Category | EROD Activity | |
|---|---|---|
| Not Injected | 0.932 (5)<br>(0.849 – 1.02) | |
| | 2-Day | 7-Day |
| Peanut Oil | 1.87 (5)<br>(1.66 – 2.10) | 1.13 (5)<br>(0.835 – 1.53) |
| Field Blanks | 3.78 (22)<br>(3.41 – 4.21) | 2.45 (20)<br>(2.07 – 2.90) |
| Un-Contaminated Sites | 2.24 (80)<br>(2.10 – 2.38) | 1.19 (78)<br>(1.11 – 1.27) |
| BNF | 75.3 (4)<br>(64.6 – 87.7) | 65.8 (5)<br>(51.5 – 84.0) |
| Cordova Harbor | 40.3 (10)<br>(34.9 – 46.5) | no data |

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