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Understanding the Chronic Impacts of Oil Refinery Wastewater Requires Consideration of Sediment Contributions to Toxicity

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Abstract Previous studies at an oil refinery in Saint John, New Brunswick, Canada, found a diminished fish community downstream of the effluent outfall that appeared to be associated with periodic low dissolved oxygen concentrations due to episodic discharges of contaminated transport vessel ballast water. This study was initiated after the ballast water was removed from the effluent to further investigate the potential causes of residual effects in the study stream, Little River. We used field caging of fish, laboratory bioassays, and chemical analysis of effluents and sediments from the field site to determine if the effluent or contaminated sediments were affecting the recovery of the fish community in Little River. The field studies suggested that exposed, caged fish were affected, displaying >40 % increases in liver sizes and increased liver detoxification enzyme activity (cytochrome P450 1A, CYP1A); however, similar responses were absent in laboratory exposures that used effluent only. Adding sediments collected from the vicinity of the refinery's outfall to the laboratory bioassays reproduced some of the field responses. Chemical analyses showed high concentrations of PAHs in sediments but low concentrations in the effluent, suggesting that the PAHs in the sediment were contributing more to the impacts than the effluent. Application of effects-based monitoring is suggested as beneficial to identify impacts to fisheries where refinery effluents of this type are involved.

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The Canadian Environmental Effects Monitoring (EEM) program was developed in the early 1990s to determine if discharge limits for pulp and paper mill effluents were sufficient to protect fish, fish habitat, and fisheries resources (Government of Canada 2010). The EEM program was then adapted for metal mines (Metal Mining Liquid Effluent Regulations 2008) and will be applied to municipal wastewater (Government of Canada 2012). However, oil refineries in Canada are not currently subject to EEM requirements, and effluent regulations are based on chemical end points and laboratory bioassays (Petroleum Refinery Liquid Effluent Regulation 2008).

Oil refinery effluents have been associated with decreases in phytoplankton abundance, lower reproduction in estuarine crustaceans and fish, and decreased growth in fish in their receiving environments (Buikema et al. 1981; Rowe et al. 1983a, b; Saha and Konar 1985). Other studies have also documented decreases in algal growth, fish reproduction, and induction of hepatic detoxification enzymes (cytochrome P450 1A, CYP1A) in fish (Roseth et al. 1996; Knudsen et al. 1997; Khan 1998).

A large oil refinery in Saint John, New Brunswick (NB), Canada, discharges its effluent to a small coastal stream, Little River. Previous research indicated that the fish community in Little River was impoverished compared with those at local and regional reference sites (Vallières et al.

2007). This research also indicated that periodic anoxia was occurring in Little River, coinciding with the release of untreated ballast water pumped from ships to the oil refinery and then into Little River (Vallières et al. 2007). This issue was corrected; however, the fish community did not recover, suggesting other causative factors, such as the accumulation of polycyclic aromatic hydrocarbons (PAHs), compounds known to be related to the toxicity petroleum products (Tatem et al. 1978), in sediments of Little River (SI Table 1). Owing to their hydrophobicity, some PAHs discharged in wastewaters adsorb to particles and are deposited in the sediments downstream of their point sources (Brenner et al. 2002). Increased concentrations of PAHs can be found in the vicinity of oil extraction facilities; however, the concentrations can also quickly decrease with increasing distance from the source (Law and Biscaya 1994).

We hypothesized that PAHs may be limiting recovery of the fish community in Little River. The objectives of this study were to determine if effects consistent with PAH exposure were found when fish were caged in the field and if those effects could be duplicated in laboratory bioassays using effluents only or both effluents and sediments from Little River. Finally, sediments and effluents were analyzed to determine their concentrations of PAHs.

Methods

Little River is located in the City of Saint John, NB, Canada, and flows into Saint John Harbour at the mouth of the Saint John River. The nearby Saint John oil refinery discharges, on average, 24,000 m^3/day of effluent, comprising ~12 % of Little River's volume at low flow (Vallières 2005). Two outfall locations have been used during the operation of the Saint John refinery. From 1960 to 1998, treated effluent was discharged into Little River at the old outfall location, which is located ~400 m upstream from the current outfall location (Fig. 1). In 1998, the effluent outfall was moved to the current location when the secondary treatment facility was completed.

Fish Studies

Fish Species

The field caging and laboratory bioassays used mummichog (*Fundulus heteroclitus*) collected from uncontaminated areas in the Saint John River estuary (45.16°N and 66.02°W, 45.21°N and 66.03°W) using minnow traps or from Shediac Bay, NB (46.20°N, 64.37°W) where they were collected with a beach seine. Fish for laboratory

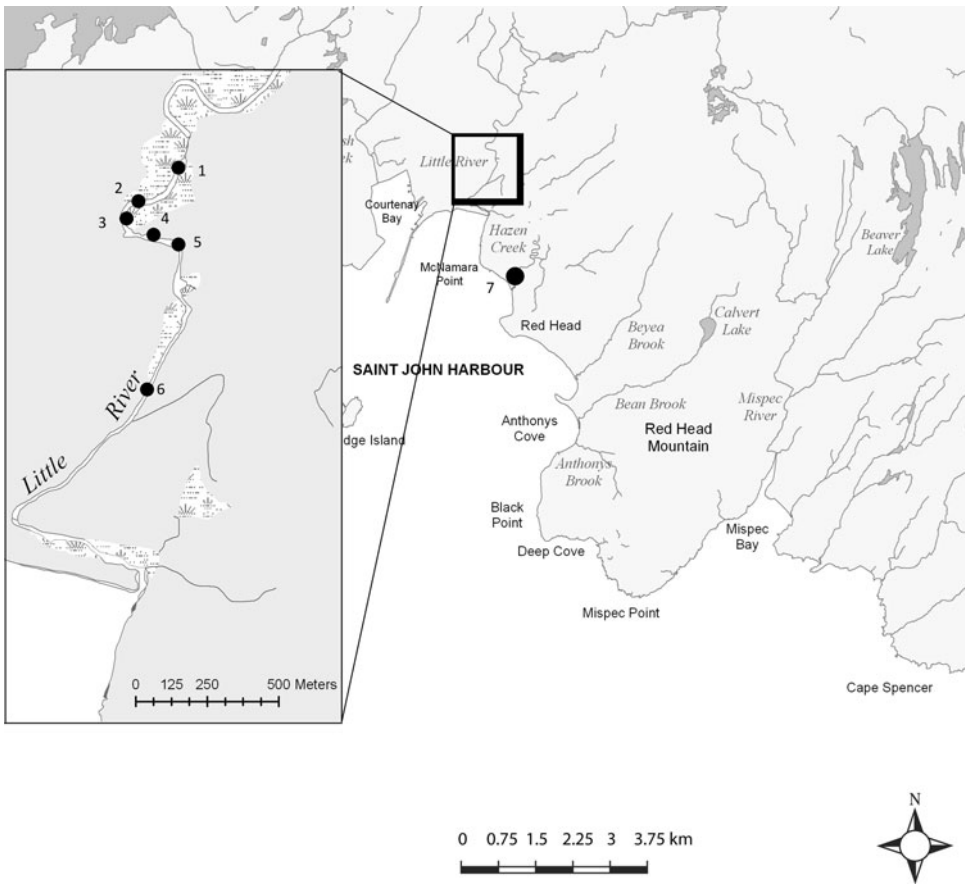

Fig. 1 Map of Little River draining into the Saint John Harbour, NB, Canada. (*closed circle*) indicate sites of interest: *1* Little River reference monthly surface sediment collection, reference caging site, *2* reference core site, *3* oil refinery older outfall location, old outfall core site, *4* current outfall location, *5* current outfall core, 10 m downstream caging site and 10 m downstream monthly surface sediment collection site, *6* 800 m downstream caging site, 800 m downstream monthly surface sediment collection, *7* Hazen Creek reference sediment site used in 2007 sediment–effluent bioassays

bioassays were acclimated to laboratory conditions in stock tanks for 2–3 weeks in 12–18 °C aerated water before distribution to experimental aquaria. Fish were acclimated to the experimental tanks (20-L glass aquaria) for 1 week before initiating the treatments. All fish collected for caged bioassays were held for at least 1 week before the start of the exposure.

Field Studies

The caging studies were performed in August 2006 and June 2007. Cages (30.8 × 30.8 × 16.5 cm^3) were constructed with a 1.9-cm polyvinyl chloride (PVC) pipe frame to support a cube sewn of aquaculture mesh (8 mm; Aquatic Ecosystems, Apopka, FL, USA). PVC pipe crosses, 7.5 cm in diameter, were installed inside the mesh cube to provide refuge from high-water velocities. Four cages were deployed per site, covering ~10 m, and the cages were used as the unit of replication for statistical analysis. They were suspended in the water column by a buoy and attached to an anchor. In each cage, four each of male and female fish were held for 14 days at three sites on Little River: a reference site (R; 45.29°N, 66.02°W) located upstream of the effluent outfall and two downstream sites located 10 m (45.16°N, 66.01°W) and 800 m (45.28°N, 66.02°W; Fig. 1), respectively, below the current effluent outfall. Water quality (salinity, conductivity, temperature, pH, and dissolved oxygen) was monitored every 20 min by simultaneous deployment of a YSI 6920 sonde (YSI Incorporated, OH, USA) at the reference site and 10 m downstream at the caging sites. Deployments were terminated if fish showed signs of stress.

Laboratory Studies

Effluent-Only Bioassays (2006 and 2009)

Mummichog were initially acclimated in 16 ppt saline water, and, if used in a freshwater experiment, the salinity was decreased gradually during the course of 1 week. Experimental aquaria, 20-L glass, held three females and three males each. Aquaria were labelled as exposed to oil refinery effluent or reference and reused for that concentration only. Exposed aquaria were randomly assigned concentrations and laboratory positions in each bioassay. An alkaline detergent (Contrad NF; Decon Laboratories, Inc., PA, USA) was used to purge organic build-up on aquaria glass between bioassays, although it is possible that some organics may have remained in the aquaria silicone. Treatments were randomly assigned four aquaria for 7 days in May 2006 and 14 days in October 2006. In May and October 2006, randomized exposures (all v/v %) at 0, 25, 50; 100 % (October only) with effluent were run static with daily renewal in 16 ppt saline water and freshwater, respectively (biomass loading = 3.39 g/L/day in May and 5.05 g/L/day in October). Dissolved oxygen, temperature, conductivity and salinity were monitored daily. Water quality during the May 2006 bioassay had similar temperature (17 ± 1 °C) and dissolved oxygen (>78 %) between concentrations; however, salinity and conductivity were different between concentrations (14.9 ± 1, 11.8 ± 1, and 8.9 ± 1 ppt salinity and 21.1 ± 0.2, 17.0 ± 0.5, and 12.9 ± 0.7 mS/cm conductivity for control [25 and 50 %, respectively]). Experimental conditions in October were similar between concentrations (>70 % dissolved oxygen, 15 ± 1 °C).

A freshwater bioassay was also performed in February 2009 and employed 0 and 50 % effluent concentrations, with static daily renewal, for 35 days (biomass loading = 2.44 g/L/day). Each tank was monitored for egg production by collecting eggs every second day for 14 days. A mesh screen made of aquaculture mesh (8 mm; Aquatic Ecosystems) placed on the bottom prevented fish from eating the eggs. On day 15, concentrations (all v/v %; 0 and 50 %) were randomly assigned to five aquaria for an additional 21 days with egg collections every second day. Dissolved oxygen, temperature, conductivity, and salinity were monitored daily and were similar between concentrations (>80 % dissolved oxygen, 18 ± 2 °C, 0.5 ± 0.9 mS/cm, and 0.5 ± 0.7 ppt).

Effluent–Sediment Bioassays (2007)

Sediment was collected from Little River and Hazen Creek (an uncontaminated reference stream) in December 2006 with precleaned Ekman dredges (13.2 × 13.2 × 13.2 cm^3; Halltech Environmental, Guelph, ON, Canada). The contents of the dredge were emptied into polyethylene-lined buckets; the liners were sealed and held for no longer than 2 h and frozen at −20 °C until used for the bioassays. Each aquarium was allocated a 500-g portion of sediment from either source.

Effluent–sediment bioassays used a flow-through system with a change-over rate of 20 L/day and were performed in randomized tanks at 16 ppt salinity in April 2007 (biomass loading per aquaria = 5.55 g/L/day) and in freshwater in July 2007 (biomass loading per aquaria = 1.47 g/L/day) for 14 days. Both bioassays employed 0, 25, and 50 % effluent concentrations in combination with either reference or effluent-exposed sediments. Dissolved oxygen, temperature, conductivity, and salinity were monitored daily and were similar between treatments (April = >77 %, 15 ± 1 °C, 22. 6 ± 1.1 mS/cm, and 16.9 ± 2 ppt; July = >80 %, 18 ± 1 °C, 1.3 ± 0.6 mS/cm, and 0.8 ± 0.4 ppt). Aquaria sediments were collected at the end of the experiment in polypropylene bags and frozen at −20 °C until analyzed for PAH content by the Research and Productivity Council in Fredericton, NB (based on

United States Environmental Protection Agency [USEPA] 3540C/8270C (2011); procedures used detection limits (0.01 mg/kg dw), method blanks (<0.01 mg/kg dw), and surrogates (% recoveries averaged 94 ± 4 % [range 70–112 %]) and a spike [% recoveries averaged 90 ± 2 %]). See SI Table 1 for a summary of bioassays.

Fish Sampling and End Points

Fish were anesthetized with MS-222 (Syndel Laboratories, Qualicum Beach, British Columbia; 50 mg/L), bled, and killed by spinal severance. Fish were measured for total length (±1 mm), wet weight (±0.01 g), liver weight (±0.001 g), and gonad weight (±0.001 g). Mummichog were bled by caudal puncture using 25-guage 5/8″ needles and 1-mL heparinized syringes; blood was kept on ice until centrifuged at 3,000×*g* at 4 °C for 12 min. The plasma was decanted and frozen at −20 °C for later analysis of testosterone, 11-ketotestosterone, and estradiol. Plasma samples were ether-extracted to separate steroids and steroid binding proteins (McMaster et al. 1992), and refrozen until measurement by radioimmunoassay using the methods described in MacLatchy et al. (2005).

In vitro steroid production by the mummichog was also measured. Gonads were removed, placed in Medium 199 buffer (McMaster et al. 1995; Sigma Aldrich, Oakville, ON, Canada), and held on ice until used in the in vitro bioassays. Briefly, an explant of gonadal tissue weighing between 18 and 22 mg was place in a 24-well polystyrene tissue culture plate containing 1 mL of Medium 199 buffer and kept on ice. When all wells of a plate held tissue, the Medium 199 buffer was replaced with fresh buffer, and the plate was incubated at 18 °C for 18 h. After incubation, the fluid in each well was collected and frozen at −20 °C until measured by RIA.

Livers were frozen at −80 °C until processed for CYP1A activity by ethoxyresorufin-*o*-deethylase (EROD) assay using the methods of Hodson et al. (1991); protein content of the S9 fraction was measured using Bio-Rad laboratory's microassay method and used to standardize CYP1A data.

Sediment and Effluent Chemistry

Effluent Chemistry

Three effluent samples were collected from the refinery in December 2006 in 1-L amber glass bottles to determine PAH concentrations. Dichloromethane (100 mL, DIG grade; Caledon Laboratories) was added to each sample of effluent to preserve the PAHs. The samples were chilled to 4 °C and packed with ice packs before being shipped to the Canada Centre for Inland Waters (Burlington, ON) for analysis (Environment Canada's National Laboratory for Environmental Testing method no. 03-3251). Effluent measurements included the 16 priority PAHs as per the USEPA (2011) as well as perylene, retene, dibenzothiopene, 1-methylnaphthalene, 2-methylnaphthalene, and tetrahydronaphthalene. Procedures used a method blank (<3.6 ng/L), a spike (% recovery average of 88 % [range 57–117 %], whereas recoveries of only indene, indeno(1,2,3,c,d)pyrene, and benzo(b)fluoranthene were <70 %), and surrogates (% recoveries 95 ± 9.83 % [range 39–227 %], whereas perylene-D12 recoveries were 41 ± 1 % and those of pyrene-D10 were 177 ± 20 % across the samples).

PAH Analyses

Although waterborne PAHs were initially suspected to be causing effects in fish, analysis of the effluent showed low concentrations ("Results" section). For this reason, sediment samples were collected from Little River from 2008 to 2010. In 2008, sediment samples were collected during May, July, and November. An Ekman dredge was used to collect a single sediment sample from a reference, a nearby field (10 m downstream of the outfall), and a distant field (800 m downstream of the outfall) site each month. These sites correspond to the sites used to cage fish. In 2009, sediment samples were collected in triplicate, once a month, from August to November at the same sites used in 2008. In 2010, triplicate sediment samples were also collected monthly from May to October at these three sites as well as an additional site corresponding to an older outfall location.

In addition, in September 2010 a sediment core was taken at each of the old and current outfall locations to measure alkylated PAHs and to determine if sediment PAH contamination was related to the original upstream outfall, the current discharges, or a combination thereof at the nearby field site. The percentage of alkylated PAHs and specific ratios were qualitatively compared between core sections to determine the source(s) of PAH contamination. Two of these ratios included the alkyl homologs of naphthalene and fluorene to the respective parent compound. A value exceeding two in these ratios indicates a petrogenic source (Lake et al. 1979). Two additional ratios—C_2-dibenzothiophene:C_2 phenanthrene and C_3 dibenzothiophene:C_3 phenanthrene—were used together to estimate any potential differences in petrogenic signatures between the old and current outfalls (Boehm et al. 1997).

Sediment cores were collected downstream of each outfall using a 50-cm hand corer. The core tubes were sealed while submerged and held upright until frozen in the laboratory. The frozen cores were then separated into 5-cm sections (corresponding to depths of 0–5, 5–10, 10–15, 15–20, and 20–25 cm below the stream bottom) with a hand

saw. The still-frozen sections were rinsed with distilled water to remove potential contamination in the upper and lower 0.1–0.5 cm. The saw blade was cleaned between sections. Sections were placed in amber glass jars and analysed for 16 priority PAHs at the University of New Brunswick Saint John (freeze-dried sediments were quantified using methods based on USEPA methods 3545/8270C—GC/MS; QA/QC procedures utilized method detection limits (MDL; 0.01 mg/kg dw and calculated by MDL = $t_{n-1,97.5} \times \sigma$), method blanks (<0.01 mg/kg dw), spikes (% recovery averaged 85 ± 1 % with a range of 70–97 %), certified reference materials (% recovery averaged 89 ± 1 % and ranged 37–125 %; where values <60 % occurred for naphthalene; $N = 5$), surrogates (% recoveries averaged 90 ± 1 % with a range 48–130 %, randomly distributed between naphthalene-D8, 2-fluorobiphenyl and *p*-terphenyl-D14 across samples) and duplicates (25 % average relative percent difference)). Sections were also analyzed for alkylated PAHs at the Axys Laboratory Services (ALS; Edmonton, Alberta) using USEPA methods 3540/8270C and a GC/MS (QA/QC procedures used detection limits (averaging 0.3 ± 0.004 mg/kg dw across individual compounds), method blanks (<0.04 mg/kg dw), surrogates (% recoveries averaged 89 ± 1 % [range 65–111 %]), and duplicates [9 % average relative percent difference]). On September 30, 2010, a second 25-cm core was collected at the current outfall site because the sample containing the surface section was compromised during transport. The top three 5-cm sections of this core were resubmitted to ALS. The average concentrations from the two repeated lower sections were used in data analysis. All sediment PAH concentrations were based on dry weight. See SI Table 2 for a summary of sediment sampling employed each year.

Data Analysis

Fish summary statistics were reported as mean ± SEM (standard error of the mean) or mean with lower and upper 95 % confidence intervals (CIs) if data were log-transformed. Fish length, body weight, liver weight, and gonad weight were analyzed by analysis of variance (ANOVA) with pairwise Tukey post hoc test. Fish condition factor [(body weight/(length^3)] × 100,000), gonadosomatic index [GSI; (gonad weight/body weight) × 100], and liver somatic index (LSI; [liver weight/body weight) × 100] were also calculated and analyzed by ANOVA with pairwise Tukey post hoc test. Biomarker data did not meet parametric assumptions after log transformation and were analyzed nonparametrically using Kruskal–Wallis test. The percentages of alkylated PAHs in core samples were compared with Chi square test. All analyses used a significance level of 0.05. Analyses were performed using Systat 11 software (Systat Software Inc., IL, USA). The 16 individual PAHs were summed to determine total PAH concentrations in a sample. Individual PAHs lower than detection limits were set to zero for further data analysis.

Results

Caged Bioassays

August 2006 male and female mummichog caged at the sites 10 and 800 m downstream of the current outfall had 66 and 42 % larger livers ($p < 0.001$ and $p < 0.001$, respectively) and 7 % decreased condition ($p = 0.007$ and $p < 0.001$) compared with reference fish (Table 1). Females at the 10 m downstream site also had 50 % smaller ovaries than the reference (R) site, whereas fish at the site 800 m downstream had ovaries that were intermediate in size ($p < 0.001$). There were no significant differences in plasma sex steroids in either males or females among sites (SI Table 3). However, mummichog caged at either downstream site had 2.7-fold more CYP1A activity compared with fish caged at R site ($p < 0.001$; Fig. 2a). Water-quality parameters were similar between sites with averages of 23.5 ± 1.2 °C, salinity 0.72 ± 0.56 ppt, conductivity 1.39 ± 1.05 mS/cm, pH 6.8 ± 0.10, and dissolved oxygen >70 % saturation.

During the June 2007 caging study, only female mummichog survived in sufficient numbers for statistical analysis. Similar to August 2006, there was a significant increase in liver size (54 %) in females at both downstream sites, but in June 2007 there were no differences in condition factor, gonad size, or sex steroid hormone concentrations among sites (Table 1; SI Table 3). CYP1A activity was significantly increased sixfold at both downstream sites compared with reference fish (SI Fig. 2). Water-quality parameters differed in temperature (20 ± 1 °C reference, 20 ± 1 °C reference; 24 ± 1 °C 10 m downstream), salinity (0.14 ± 0.00 ppt reference, 2 ± 0.01 ppt 10 m downstream), and conductivity (0.27 ± 0.00 mS/cm reference, 3.9 ± 0.02 mS/cm 10 m downstream); dissolved oxygen maintained levels were >70 % saturation, and pH was between 7 and 8 at both sites.

Laboratory Bioassays

There were no differences in fish summary statistics within sexes in either the May or October 2006 bioassays (Table 1). Female fish exposed to 50 % effluent during the May 2006 bioassay had 60 % lower testosterone levels ($p = 0.019$, SI Table 4). CYP1A enzyme activity was similar among treatments (female $p = 0.14$, male $p = 0.50$; Fig. 2b). The October bioassay had mortalities in 50 and 100 % effluent treatments beginning on day 5,

Table 1 Summary statistics for parameters from mummichog caged at Little River (field) or exposed to effluent in laboratory conditions (laboratory)

Location	Date	Sex	Site	*n*	Length (mm)	Weight (g)	GSI (%)	LSI (%)	Condition
Field	Aug	F	R	22	78.5 ± 1.0	5.16 ± 0.19[a]	1.06, 1.03, 1.15[a]	2.60, 1.10, 7.43[b]	1.06, 1.03, 1.15[a]
	2006		10 m	20	75.4 ± 1.1	4.23 ± 0.18[b]	0.97, 1.03, 0.98[b]	3.73, 1.10, 15.30[a]	0.97, 1.03, 0.98[b]
			800 m	13	76.8 ± 1.3	4.48 ± 0.22[ab]	0.98, 1.04, 0.99[b]	3.60, 1.14, 14.69[a]	0.98, 1.04, 0.99[b]
		M	R	10	75.9 ± 2.2	4.79 ± 0.44[a]	0.38, 1.42, 0.21	2.34, 1.12, 6.13[b]	1.07, 1.07, 1.23[a]
			10 m	8	74.4 ± 2.4	3.76 ± 0.27[a]	0.36, 1.53, 0.20	3.88, 1.14, 17.09[a]	0.91, 1.08, 0.89[b]
			800 m	8	74.1 ± 0.9	3.82 ± 0.17[a]	0.30, 1.48, 0.13	3.81, 1.14, 16.50[a]	0.93, 1.08, 0.94[b]
	Jun	F	R	11	71.4 ± 1.0	3.81 ± 0.20	3.97 ± 0.28	2.76 ± 0.29[b]	1.04 ± 0.03
	2007		10 m	5	73.4 ± 1.8	4.14 ± 0.30	4.01 ± 0.72	4.27 ± 0.36[a]	1.05 ± 0.07
			800 m	5	73.0 ± 1.6	3.80 ± 0.30	4.49 ± 0.55	4.27 ± 0.31[a]	0.97 ± 0.03
Laboratory	May 2006	F	0	11	84.3 ± 2.4[a]	10.03 ± 0.64	7.38, 1.28, 69.84	3.89, 1.20, 18.23	1.46, 1.20, 2.55[ab]
			25	10	97.1 ± 2.8[b]	9.27 ± 0.78	7.95, 1.45, 91.46	3.25, 1.32, 13.92	1.01, 1.31, 1.32[b]
	7 days		50	10	82.4 ± 2.8[a]	9.81 ± 0.97	7.67, 1.28, 75.43	3.76, 1.20, 17.03	1.49, 1.20, 2.67[a]
		M	0	8	82.0 ± 1.8[a]	8.69 ± 0.58	2.64 ± 0.18	2.72 ± 0.20	1.57 ± 0.06[a]
			25	11	92.6 ± 1.9[b]	7.96 ± 0.56	2.58 ± 0.16	2.31 ± 0.22	0.99 ± 0.04[b]
			50	10	79.7 ± 2.3[a]	8.52 ± 0.72	2.31 ± 0.14	3.39 ± 0.47	1.66 ± 0.05[a]
	October 2006	F	0	8	103.1 ± 1.3	12.85 ± 0.67	1.32 ± 0.08	4.34 ± 0.20	1.17 ± 0.04
			25	9	103.4 ± 1.4	12.50 ± 0.27	1.24 ± 0.08	4.24 ± 0.39	1.14 ± 0.04
	14 days		50[A]	7	101.1 ± 1.4	12.65 ± 0.60	1.12 ± 0.14	4.36 ± 0.13	1.22 ± 0.02
			100[A]	7	104.4 ± 1.4	14.14 ± 0.67	1.20 ± 0.11	4.47 ± 0.28	1.22 ± 0.04
		M	0	10	104.8 ± 1.4	13.80 ± 0.36	0.65 ± 0.10	3.79 ± 0.50	1.20 ± 0.03
			25	7	105.0 ± 2.1	14.13 ± 0.98	0.37 ± 0.08	4.39 ± 0.26	1.21 ± 0.03
			50[A]	11	104.3 ± 1.4	14.22 ± 0.41	0.47 ± 0.05	3.79 ± 0.29	1.26 ± 0.03
			100[A]	5	104.2 ± 2.9	13.46 ± 0.80	0.50 ± 0.05	3.24 ± 0.33	1.19 ± 0.03

R site = 10 m lower than the current outfall (10 m) and 800 m lower than the current outfall (800 m). Values are mean ± SE or mean, lower 95 % CI and upper 95 % CI for log-transformed data. Values with different superscript lower-case letters indicate statistical significance

[A] Values measured on ailing fish after 7-day exposure

and these treatments were terminated on day 7. CYP1A activity was not significantly different in male fish but were significantly decreased in female mummichog exposed to 50 and 100 % effluent compared with reference females ($p = 0.014$, SI Fig. 3).

Fish during the April 2007 bioassay were shorter and weighed less in the 25 % effluent concentration, but these differences did not affect condition factor among treatments (Table 2). In vitro production of testosterone was increased in females exposed to 50 % effluent and control sediment ($p = 0.029$; SI Table 5). However, male mummichog showed no differences in testosterone between treatments ($p = 0.79$, SI Table 5). CYP1A analyses indicated increased activity in females in the exposed-sediment treatments and in males in the exposed-sediment at 50 % effluent ($p < 0.001$, $p = 0.032$; Fig. 2c).

In July 2007, female fish had increased ovary sizes (69–186 %) in all treatments compared with females in the control sediment and control water ($p = 0.006$; Table 2). The largest ovaries, a 2.9-fold increase, were from fish in the exposed sediment and control water treatments. Male testes were increased in size with exposure to 25 % effluent regardless of sediment source ($p = 0.022$), where they were 44 and 93 % greater for control and exposed sediments, respectively.

There were no differences in the in vitro production of gonadal testosterone between treatments ($p = 0.22$) in males; however, female testosterone concentrations were increased by 2- to 3.5-fold in fish exposed to 25 % effluent with reference sediment and in treatments of Little River sediments with control water and 25 % effluent ($p = 0.013$; SI Table 5). Female mummichog also exhibited a threefold CYP1A induction in exposed sediment treatments ($p = 0.014$), although male mummichog had similar CYP1A activity among treatments ($p = 0.24$; Fig. 2d).

The February 2009 bioassay indicated differences in female weight and length and in male weight; however, fish condition between treatments were similar (SI Table 6). In vitro testosterone in male mummichog decreased by 1.4 times after exposure to 50 % effluent; however, there were no differences between treatments in 11-ketotestosterone compared with reference males (SI

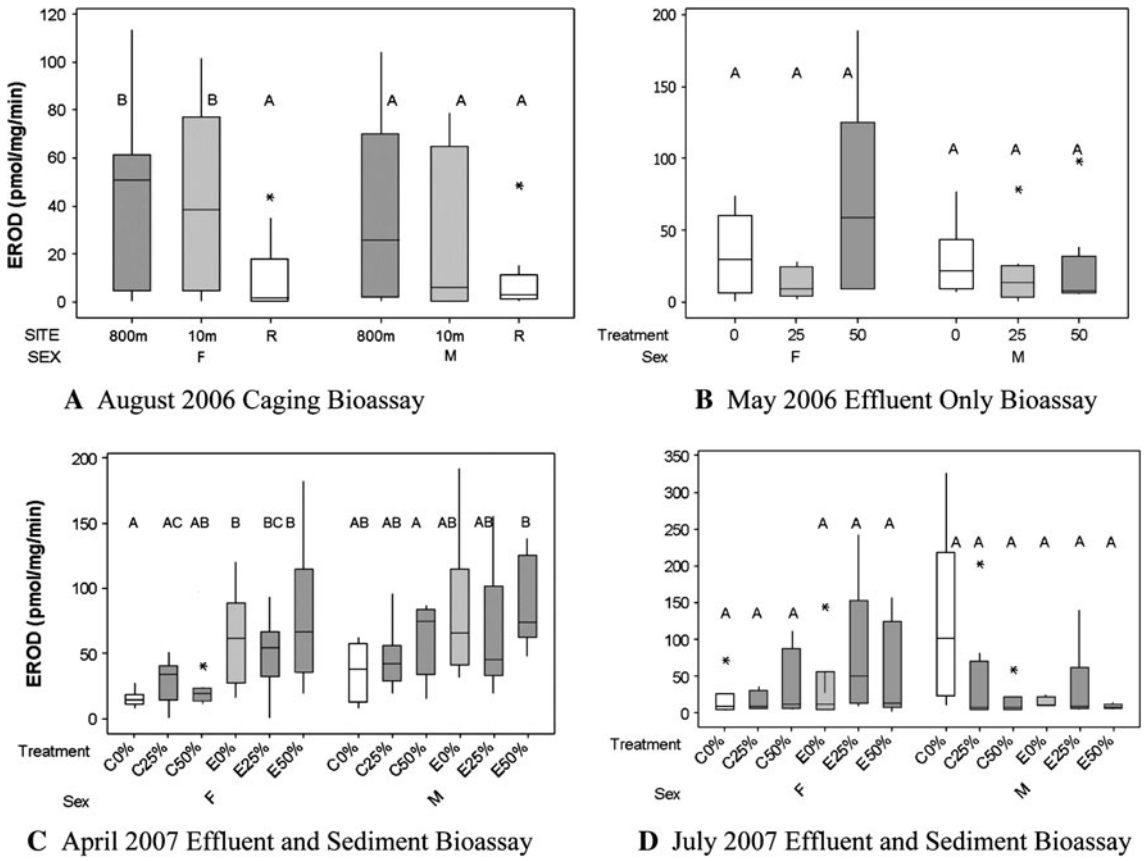

Fig. 2 EROD activity (pmol/mg/min) in female (F) and male (M) mummichog (1) exposed to oil refinery effluent in a bioassay for 7 days (no sediments; May 2006), (2) caged in the receiving environment (800 m, Little River 800 m below the current outfall; 10 m, Little River 10 m below the current outfall; and R site) for 14 days (August 2006), or (3) exposed to Little River sediments in a laboratory bioassay for 14 days (*C* Hazen Creek control sediment, *E* Little River near field sediments; April and July 2007). Treatments sharing a *letter* indicate similarity within a date and sex. *Extreme values

Table 7). In vitro testosterone in female mummichog increased by 2.5 times after exposure to 50 % effluent, but estradiol was similar between treatments (SI Table 7). There were no differences in CYP1A activity between treatments in liver of either sex ($p = 0.76$, $p = 0.74$ for females and males, respectively; SI Table 8). Egg production was similar between treatments ($p = 0.96$; SI Fig. 4).

Sediment and Effluent Chemistry

Sediments from Bioassays

Sediments used for the bioassay aquaria had 10- to 30-fold greater concentrations of total PAHs in Little River compared with Hazen Creek. For the two most dominant PAHs, fluorine, and pyrene, concentrations Little River sediments were greater in both April ($p = 0.013$) and July ($p = 0.014$). In April, both fluorene and pyrene concentrations were $\sim$20 and 52 times, respectively, greater in Little River sediment with 50 % effluent than those of Hazen Creek sediment without effluent ($p = 0.050$). In July, sediments were 34-fold greater in total PAH concentrations in Little River sediment and 25 and 50 % effluent groups compared with reference sediment and water ($p = 0.046$ both effluent groups), although these PAH concentrations were significantly lower than during the April exposures ($p < 0.001$). A comparison of liver CYP1A activity with sediment PAH content found a significant relationship for fish exposed in April 2007 ($r^2 = 0.79$, $p < 0.001$; SI Fig. 5).

PAHs in effluent

Only 14 of the 16 priority PAHs were detected (ng/L) in effluent samples, and four of these were lower than detection limits when the three samples were averaged. Two of the additional PAHs, 2-methylnaphthalene and tetrahydronaphthalene, were lower than detection limits. Total PAHs averaged 626 $\pm$ 28 ng/L and, of the individual PAHs, retene was the highest at an average of 186 $\pm$ 7 ng/L. The next highest of the 16 priority PAHs were chrysene

Table 2 Summary statistics for mummichog exposed to E or C sediment and oil refinery effluent for 14 days at 0, 25, and 50 % effluent in 16 ppt salinity water (April 2007) or 0 ppt saline water (July 2007)

Date	Sex	Effluent (%)	Sediment	*n*	Length (mm)	Weight (g)	GSI (%)	LSI (%)	Condition
Apr	F	0	C	9	111.8 ± 1.6^{ab}	19.68 ± 1.36^{a}	15.32 ± 2.14	4.08 ± 0.23	1.39 ± 0.04
		25	C	9	106.6 ± 1.3^{b}	17.33 ± 0.88^{ab}	13.46 ± 1.37	5.13 ± 0.20	1.43 ± 0.05
		50	C	7	109.3 ± 1.7^{ab}	18.01 ± 0.81^{ab}	21.63 ± 3.53	4.31 ± 0.43	1.38 ± 0.05
		0	E	8	114.4 ± 1.1^{a}	19.24 ± 0.94^{a}	15.88 ± 1.72	4.85 ± 0.53	1.28 ± 0.04
		25	E	9	106.2 ± 0.8^{b}	15.43 ± 0.38^{b}	15.92 ± 1.41	5.59 ± 0.41	1.29 ± 0.03
		50	E	8	108.9 ± 2.2^{ab}	18.58 ± 1.11^{ab}	15.27 ± 3.14	4.64 ± 0.34	1.44 ± 0.05
	M	0	C	9	104.3 ± 1.7	14.40 ± 0.72	2.60 ± 0.20	3.52 ± 0.30	1.26 ± 0.02^{a}
		25	C	8	104.9 ± 1.4	13.88 ± 0.76	2.99 ± 0.23	3.24 ± 0.23	1.20 ± 0.04^{ab}
		50	C	8	106.8 ± 1.6	14.36 ± 0.51	2.73 ± 0.13	3.57 ± 0.32	1.18 ± 0.03^{ab}
		0	E	8	105.8 ± 0.8	13.12 ± 0.32	2.35 ± 0.19	3.82 ± 0.19	1.11 ± 0.03^{b}
		25	E	8	103.8 ± 1.7	12.33 ± 0.60	2.67 ± 0.21	3.82 ± 0.19	1.10 ± 0.04^{b}
		50	E	9	103.7 ± 1.0	12.99 ± 0.37	2.43 ± 0.11	3.66 ± 0.28	1.17 ± 0.03^{ab}
Jul	F	0	C	7	74.6 ± 2.0	4.81 ± 0.40	4.39 ± 0.72^{a}	3.85 ± 0.52	1.15 ± 0.05
		25	C	4	77.5 ± 2.8	5.85 ± 0.43	7.53 ± 1.58^{ab}	4.77 ± 0.28	1.26 ± 0.05
		50	C	7	75.4 ± 1.3	5.53 ± 0.27	7.42 ± 0.92^{ab}	4.84 ± 0.23	1.29 ± 0.04
		0	E	6	75.2 ± 2.5	5.54 ± 0.51	12.57 ± 1.65^{b}	4.47 ± 0.48	1.29 ± 0.04
		25	E	8	72.9 ± 1.4	4.74 ± 0.30	8.53 ± 1.63^{ab}	4.86 ± 0.18	1.21 ± 0.02
		50	E	7	76.3 ± 1.2	5.51 ± 0.26	9.34 ± 1.21^{b}	4.45 ± 0.44	1.24 ± 0.04
	M	0	C	7	72.0 ± 2.0	4.25 ± 0.40	0.94, 1.31, 1.15^{ab}	4.28 ± 0.34	1.12 ± 0.04
		25	C	8	72.8 ± 1.5	4.60 ± 0.13	1.41, 1.29, 2.55^{a}	3.15 ± 0.37	1.20 ± 0.05
		50	C	7	70.6 ± 1.2	4.21 ± 0.21	1.20, 1.31, 1.88^{ab}	3.21 ± 0.25	1.19 ± 0.02
		0	E	6	74.5 ± 2.7	4.80 ± 0.51	0.79, 1.34, 0.83^{b}	3.88 ± 0.50	1.14 ± 0.02
		25	E	8	70.8 ± 1.6	4.01 ± 0.30	1.43, 1.29, 2.62^{a}	3.35 ± 0.22	1.12 ± 0.03
		50	E	9	72.4 ± 1.5	4.48 ± 0.29	1.23, 1.27, 1.93^{ab}	3.18 ± 0.21	1.17 ± 0.02

Values (mean ± SE or mean, lower 95 % CI, upper 95 % CI for log-transformed data) within a sex and time period sharing a lower-case superscript letter are not statistically different

E Little River sediment, *C* Hazen Creek sediment

(mean 138 ± 3 ng/L) followed by benzo(e)pyrene (61 ± 4 ng/L), pyrene (54 ± 5 ng/L), and fluoranthene (43 ± 2 ng/L).

2008 to 2010 Monthly Sediment Surveys

Total sediment PAH concentrations close to the refinery's current outfall ranged from 16.5 to 48.6 mg/kg dw in 2008 (SI Fig. 6). Reference and 800 m downstream sites were similar in total PAHs (<1.0 mg/kg dw). In 2009, total PAHs at reference sites averaged 0.85 ± 0.16 mg/kg dw (mean ± SE, $n = 12$); concentrations at 4 of 12 sites were lower than detection limits for total PAHs (0.39 mg/kg dw). The monthly sediment survey in 2009 indicated that the majority of Little River samples 800 m downstream were similar to the average reference concentration. Samples taken near the current outfall varied by 27-fold over time but showed no seasonal trends (SI Fig. 7).

In 2010, PAH concentration in sediment samples collected at the reference site was <5 mg/kg dw (average of 1.8 ± 0.23 mg/kg dw [range 0.6–4.2]; Fig. 3). Concentrations at both the current and old outfall were considerably greater on average (18.4 ± 2.4 and 20.0 ± 2.3 mg/kg dw, respectively) than the reference or 800 m downstream locations. The sediment PAH concentrations were, however, variable at the outfall locations; the concentration of PAHs ranged from 2.6 to 39.4 and 5.9 to 48.1 mg/kg dw at the current and old locations, respectively. The PAH concentration at the site 800 m downstream was similar to that of the reference site with an average of 2.2 ± 0.4 mg/kg dw (range 0.4–6.7).

Alkylated PAHs

Percent Alkylated

The percentage of alkylated PAHs to total PAHs in the surface sediments (0–5 cm) at the current outfall site was similar to that of the top four layers at the old outfall site (5–25 cm; $p \geq 0.14$). The surface section at the current

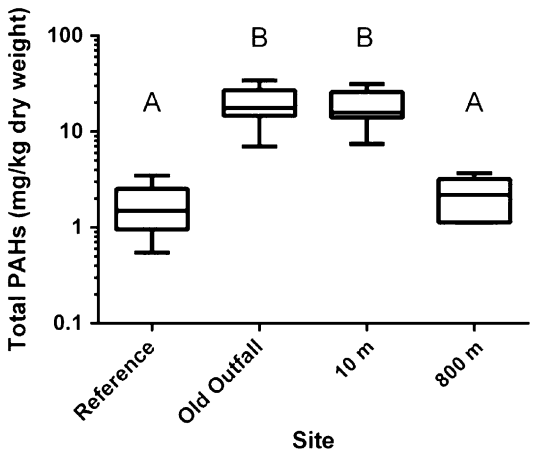

Fig. 3 Total PAH concentrations (mg/kg dw) in surface sediment samples measured monthly from May to October of 2010 in Little River at the reference site, old outfall, 10 m below the current outfall, and 800 m lower than the current outfall. Sites sharing a *letter indicate* similarity

Table 3 Percentage of alkylated PAHs to total PAHs, and naphthalene and fluorene parent-to-alkylated ratios, by sediment core depth extracted in 2010

Depth (cm)	Percentage of alkylated PAH (%)		Alkylated PAH metric			
			Parent-to-alkylated naphthalene ratio		Parent-to-alkylated fluorene ratio	
	Old outfall	Current outfall	Old outfall	Current outfall	Old outfall	Current outfall
0–5	88.75	91.96	95.88	116.66	123.82	67.14
5–10	94.29	49.26	43.92	3.71	96.36	6.48
10–15	90.43	52.30	27.84	5.31	77.89	5.71
15–20	69.93	40.25	11.63	7.95	13.72	1.97
20–25	52.02	39.13	2.90	5.62	12.00	2.24

Ratios <2 indicate pyrogenic

outfall site was, however, significantly increased in the percentage of alkylated PAHs compared with those of the deepest layer at the old outfall site (20–25 cm; $p < 0.001$; Table 3). Sections from the current outfall site <5 cm had a lower percentage of alkylated PAHs than those found in all but the deepest layers at the old outfall site ($p < 0.001$).

PAH Ratios

Ratios of C_2-dibenzothiophenes to C_2-phenanthrenes and of C_3-dibenzothiophenes to C_3-phenanthrenes showed distinct patterns when plotted as a "double ratio" (Boehm et al. 1997; Fig. 4). The current outfall core sections had the lowest values of both ratios. The samples from the old outfall site had greater ratios for both PAH pairs than the

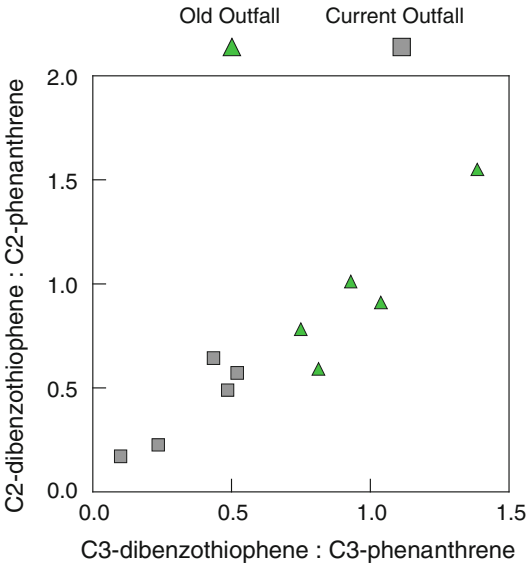

Fig. 4 Ratios of C2-dibenzothiophene to C2-phenanthrene and C3-dibenzothiophene to C3-phenanthrene from sediment core extractions at the old outfall and current outfall sites in September 2010

current outfall site. All of the samples from the sediment cores do, however, fall along a similar trajectory, suggesting a relationship between the sites (Fig. 4).

The ratio of the alkylated to the parent naphthalene showed high values in all surface sediments from all core samples (Table 3). At the current outfall site, the ratio decreased to <5 in the first subsurface section and remained low at all other depths. At the old outfall site, the ratio of alkylated to the parent naphthalene was >20 at all depths except at 15–20 and 20–25 cm; at 15–20 cm, the ratio decreased to >10, whereas at the deepest section (20–25 cm) at the old outfall site, the alkylated-to-parent ratio of naphthalene approached 2. In addition, a similar pattern to the alkylated-to-parent naphthalene was found for the fluorene ratio (Table 3). In the surface layers at all sites, the ratio far exceeded the petrogenic/pyrogenic threshold of the two. At the current outfall site, the ratio was close to 2 at all other sample depths. At the old outfall site, the ratio of alkylated to the parent fluorene was >75 in the top 3 sediment sections and was close to 2 in the two deepest sediment layers analyzed.

Discussion

Responses, such as liver size increases and CYP1A induction, that were seen in fish caged in the field at the oil refinery outfall were only observed in laboratory exposures that included PAH-contaminated sediments. Regardless of the presence of effluent, fish exposed to Little River sediments showed CYP1A induction that was

similar to levels measured in fish caged at the outfall site. Exposure to environmental PAHs has been linked to increases in hepatic CYP1A enzyme induction (Whyte et al. 2000), and Little River sediment PAHs were greater than reference site concentrations near the current and previous outfalls.

The concentrations of PAHs in the final effluent were below those that induce responses in fish, but the sediment contained concentrations of PAHs that were greater than effects thresholds. Individual PAHs measured in the effluent and sediment (SI Fig. 8) showed some PAHs in common between the two with a similar trend in the concentrations of greater molecular-weight PAHs (fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(e)pyrene, benzo(a)-pyrene, indeno(123 cd)pyrene and benzo(ghi)perylene), but levels were 2 orders of magnitude greater in sediments. The deposition of PAHs near effluent outfalls are common downstream of oil refineries as PAHs adsorb to particles in water (Le Dreau et al. 1997) and are subsequently deposited into sediments.

The sediment PAH bioassay effects threshold for CYP1A induction in the current study was estimated at 10 mg/kg dw. These levels were near the minimum PAH concentrations found downstream of the two outfalls in our study and were comparable with concentrations that induced CYP1A production in rainbow trout (*Oncorhynchus mykiss*) in Hamilton Harbour, Canada (Munkittrick et al. 1995), in corkwing wrasse (*Symphodus melops*) in Norway (Almroth et al. 2008), and in largemouth bass (*Micropterus salmoides*) in Lake Conestee, SC, USA (Schreiber et al. 2006). Although laboratory and field comparisons have been performed for pulp and paper mill effluents (Munkittrick et al. 2002) and mercury contamination (Hedayati and Safahieh 2011), there are few reports comparing fish field and laboratory bioassay responses to oil refinery effluent receiving environments (Wake 2005).

Core PAH concentrations can provide information on PAH formation through petrogenic, pyrogenic, or diageneic processes. Spikes of PAHs in sediments near both the current and historic outfall locations in 2010 had unique signatures of alkylated PAHs, suggesting that the petrogenic PAHs in these effluents were deposited close to the discharges. Comparisons among cores and among depths, to determine if a similar source lead to the deposited PAHs in the sediments at each outfall location, should be performed with caution. Although care was taken to look at profiles by depth, a dating technique was not employed on the cores, thus limiting comparisons among cores and depths.

If a diagenic formation was occurring, perylene concentrations would increase with increasing depth and/or the proportion of perylene would be >20 % of 5-ring PAHs (Hollerbach and Dehmer 1994; Tolosa et al. 2004). Perylene concentrations and proportion in cores from the current outfall core decrease with depth, indicating non-diagenic source of PAHs (SI Table 9). However, concentrations within the old outfall core were similar between the surface and the lowest section. Moreover, the proportion of perylene to 5-ring PAHs increases with depth, indicating diagenic formation of PAHS in the 15–20 and 20–25 cm sections (SI Table 9).

Although the effluent–sediment bioassays replicated the induction of CYP1A found in fish in the field study, LSI was greater in fish exposed to PAHs in the field but not in the laboratory. In the literature, researchers have found varying relationships between LSI and CYP1A induction (Whyte et al. 2000). Increased LSI with increased CYP1A induction has occurred with wild mummichog caught in Little River in past studies (Vallières 2005), in fish exposed to persistent organic pollutants, such as polychlorinated biphenyls (Leatherland and Sonstegard 1979), and in wild perch caught downstream of a pulp and paper mill effluent (Huuskonen and Lindström-Seppä 1995). We also observed increased CYP1A activity with increased LSI in female mummichog after a 2-week caging study performed during the spawning season (June 2007). Decreases in LSI with increased CYP1A activity have been reported by Khan (1998) in winter flounder (*Pleuronectes americanus)* caged in the vicinity of a petroleum refinery and in Mozambique tilapia (*Oreochromis mossambicus*) exposed to distillery effluent (Shailaja et al. 2006). However, links between CYP1A activity and greater level health effects from PAH exposure have not been established (Lee and Anderson 2005) and may be due to how PAHs in mixtures interact with each other (Billiard et al. 2006). During the study of Little River, it is unclear why the fish used in the field studies showed increased liver size; however, the laboratory fish did not, unless the time duration of exposure was insufficient for the response.

Effects of effluents and contaminated sediments on plasma sex steroids in mummichog varied across the bioassays. Fish in the effluent-only bioassay (2006; 16 ppt saltwater) had decreased circulating testosterone concentrations in females, whereas increased gonadal testosterone production was observed in females exposed to 50 % effluent in clean sediment and to 25 % effluent with either sediment treatment (2007; 16 ppt saltwater). Sun et al. (2011) found increased testosterone and decreased estradiol in the testes of false kelpfish (*S. marmoratus*) after a 50-day waterborne exposure to phenanthrene. Goldfish (*Carassius auratus*) caged in oil sands mining tailing water had decreased plasma testosterone and estradiol concentrations in males and females and decreased gonadal production of testosterone in both sexes (Lister et al. 2008). Juvenile turbot (*Scophthalmus maximus*) fed fuel oil had decreased plasma testosterone (Martin-Skilton et al. 2008), and

flounder (*Platichthys flesus*) fed 0.5–12.5 nmol of phenanthrene/g of food or 0.4 nmol of chrysene/g of food had decreased plasma estradiol (Monteiro et al. 2000).

The May 2006 bioassay (7 days in 16 ppt saline water) matches the trend of decreased plasma or gonadal produced testosterone in female fish noted by other investigators (Monteiro et al. 2000; Lister et al. 2008; Martin-Skilton et al. 2008); estradiol was not affected in this study. However, the 2007 and 2009 laboratory bioassays (April 2007 = 14 days in 16 ppt saline water; July 2007 = 14 days in freshwater; and February 2009 = 35 days in freshwater) all had at least one effluent treatment with increased gonadal produced testosterone in female mummichog. Only Sun et al. (2011) reported increased testosterone, although they used only male fish.

Differences between bioassays within this study may be due to differences within the effluent itself during different collect years. It is assumed that effluent quality was associated with mortality in the October 2006 bioassay because water-quality parameters were similar between treatments. Although detailed water chemistry was not performed at the time of the bioassay, the levels of regulated substances within the effluent by the onsite laboratory were within their limits and were not unusual at the time.

Laboratory bioassays with mummichog are commonly performed with salinities ≥16 ppt (Couillard 2002; Chandra et al. 2012; Ito et al. 2013). However, in this study, the receiving environment salinity was much lower, ranging between 0.1 and 2 ppt. Therefore, bioassays were repeated in freshwater to determine if differences in salinity affect the potential toxicity of oil refinery effluent on fish health. Through examining the effect of salinity on the rate of 17-α-ethynylestradiol (EE_2) uptake, Blewett et al. (2013). found that mummichog acclimated to 16 ppt saltwater had a threefold greater rate of uptake than mummichog acclimated to freshwater. These differences were thought to be due differences in the abundance of gill apical pores and tied to the different cells that are present in the gill epithelium under different salinities (Blewett et al. 2013). However, Meina et al. (2013) determined that fish exposed to EE2 in freshwater and 16 ppt saltwater during 14 days did not affect mummichog body, gonad, or liver weight.

Salinity did not affect plasma sex steroid levels, with the exception of females exposed to 250 ng/L EE_2 in 16 ppt saltwater. Differences were found between the two salinities in the in vitro gonadal production of sex steroids. Freshwater acclimated ovary produced more testosterone after fish were exposed to 250 ng/L EE_2. No effect was seen on estradiol production. A similar trend was seen in male fish, with no difference between control gonadal production of testosterone but a decrease in testosterone in fish exposed to 50 ng/L EE_2. There was no effect on 11-ketotestosterone production. Fish in the bioassays in this study did show differences in female GSI in the freshwater but not in the 16 ppt saltwater bioassay. It is not clear if salinity or differences in the reproductive cycle were contributing factors to this difference because the bioassays were performed 2 months apart. However, there was a consistent trend of increased testosterone production in both freshwater and 16 ppt saltwater acclimated fish exposed for a minimum of 14 days.

Concentrations of PAHs in the sediment of Little River were likely high enough to cause chronic reproductive effects. Johnson et al. (2002) concluded that sediment-quality guidelines for total PAH content should be set at 1 mg/kg dw to avoid health impacts (reproductive impairment, liver cancers, and impacts to liver DNA in English sole [*Parophrys vetulus*]). This is much lower than the threshold of 10 mg/kg dw total PAH for CYP1A induction in the current study and in the Munkittrick et al. (1995). Johnson et al. (2002) models would predict that the sediment concentrations observed in this study at the outfall sites would be associated with liver disease in 40 %, smaller female gonads in 27 %, and inhibited spawning abilities in 43 % of English sole.

Monthly fish surveys of Little River performed in 2005–2008, from April to October each year, indicated an abundant and species-rich fish community at the reference site; however, sites downstream of the outfall contained few species or individuals (SI Fig. 1). This compares with other local coastal streams, including one contaminated with raw sewage and creosote (Vallières 2005). With few fish found in Little River downstream of the effluent discharge, it would be difficult to assess if the PAH content in the sediments was affecting gonad or liver size of wild fish, adaptability of wild fish to PAHs (Meyer et al. 2002), or toxicity of the sediments to larval fish (Couillard 2002). Although we did not see reproductive effects in the laboratory bioassays, no sediments were used in the exposures that included egg production. Although Johnson et al. (2002) estimated effects levels in total PAHs, Puget Sound sediments contained significant concentrations of high molecular-weight pyrogenic PAHs (4- to 6-ring), whereas Little River sediments were primarily contaminated with 3- to 5-ring petrogenic PAHs (89 % of total PAHs).

Conclusion

Responses in caged fish were associated with contaminant(s) downstream of effluent outfall locations at the oil refinery. Laboratory bioassays and initial investigations with effluent and sediment chemistry suggested that fish responses were unrelated to present-day refinery operations and were more likely due to historical contamination of the riverbed. However, later investigations showed that the PAHs

originated from both the historical and current outfalls. PAHs are common constituents of refinery wastewater but were found in the current study at low concentrations. Accumulation of PAHs in the sediment of Little River was found to be the main factor affecting fish. Bioassays performed on refinery effluent should also consider sediments in the immediate vicinity of the facility's outfall. PAH concentration was low in effluent but increased in sediments around the historical and current outfalls.

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