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Polycyclic aromatic hydrocarbons in Pacific herring (*Clupea pallasii*) embryos exposed to creosote-treated pilings during a piling-removal project in a nearshore marine habitat of Puget Sound

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ABSTRACT

We used manually spawned, field-deployed embryos of a common marine fish species, Pacific herring (*Clupea pallasii*), to evaluate accumulation of polycyclic aromatic hydrocarbons (PAHs) associated with an incomplete creosote-treated piling (CTP) removal project. Embryos near undisturbed 100-year-old CTPs (before removal) accumulated higher PAHs and exhibited higher *cyp1a* gene expression than embryos from reference areas. Embryos incubated close to CTP debris after CTP removal showed PAHs 90 times higher than reference areas up to a year after CTP removal. *cyp1a* fold-induction correlated with total embryo PAHs in all three years. Patterns of individual PAH chemicals differed slightly between embryos, wood sampled from CTPs, and passive samplers. This study illustrates the importance of using appropriate techniques and procedures to remove CTPs in aquatic environments to prevent release of toxic chemicals. Of particular concern is that incomplete CTP removal could expose sensitive life stages of fishes to chemicals that may reduce their survival.

1. Introduction

Creosote treated pilings (CTPs) have long been recognized a potential source of toxic contaminants in the marine environment. CTP removal programs are active in many U.S. coastal states including California's San Francisco Bay (Werme et al., 2010) and Washington State's Puget Sound (Shoemaker, 2017). Creosote is a complex mixture of chemicals created by distilling tar, and one of its primary uses has been as a wood preservative for application in aquatic environments. The complex creosote mixture is dominated by polycyclic aromatic hydrocarbons (PAHs), which can account for 85% of its mass, but also includes phenols, N-, S- and O-heterocycles, dibenzofurans (Mueller et al., 1991) and nitrogen-containing aromatics such as acridine and carbazole (Malins et al., 1985). These CTP chemicals are of particular concern because of their known toxicity to aquatic organisms, and because several important Puget Sound fish species spawn in the nearshore habitats where CTPs have been extensively used.

Pacific herring (*Clupea pallasii*) are a common and abundant, small-bodied, schooling, pelagic planktivore found in temperate estuaries and marine nearshore waters of the northern Pacific Ocean. Herring spawn on nearshore vegetation such as seagrasses and macroalgae, and on nearshore structures including CTPs. Their use of nearshore habitats for spawning increases the importance of understanding the degree to which these habitats are contaminated by chemicals originating from CTPs, and other nearshore contaminant sources including terrestrial runoff, residues from oil spills and other point sources.

Developing herring embryos are particularly sensitive to low concentrations of polycyclic aromatic hydrocarbons (PAHs) in their incubation habitats (Carls et al., 1999; Incardona et al., 2015; Incardona and Scholz, 2016; Duncan et al., 2017). High mortality has been observed in herring embryos spawned directly on creosote-treated pilings, (Vines et al., 2000), and sublethal effects have been observed in

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developing herring embryos exposed to effluent from creosote-treated wood (Duncan et al., 2017). Moreover, West et al. (2014) reported accumulation of PAHs by herring embryos at several locations throughout Puget Sound, implicating PAHs as a potential risk to herring embryo health. Other lab and field studies have demonstrated links between petrogenic PAHs and sublethal effects in fish embryos, including cardiac edema and arrhythmia (Incardona et al., 2009; Incardona et al., 2004). Cardiac toxicity was also reported in herring embryos exposed to low concentrations of petrogenic PAHs following the 2007 Cosco Busan spill in San Francisco Bay (Incardona et al., 2012). In addition, these authors reported mortality and tissue necrosis related to background pyrogenic PAHs along urbanized shorelines. Moreover, exposure to ultraviolet light from sunlight is known to exacerbate the toxic effects of PAH exposure on fish embryos (Barron et al., 2003; Barron et al., 2005; Hatlen et al., 2010); thus, fish species such as Pacific herring that spawn in shallow nearshore waters may experience an increased risk of PAH-phototoxicity.

During a three-year study, we placed manually spawned herring eggs in a derelict CTP field which was scheduled for removal, to test for possible PAH effects on herring embryos over three exposure scenarios: (1) embryos near undisturbed CTPs before the CTPs were removed (2013), (2) just after the CTP removal (2014), and (3) one year after CTP removal (2015). The final sampling was initially designed to investigate the effects of possible residual PAHs one year after CTP removal. However, planned CTP removal procedures were not followed; pilings were cut off at the seafloor, resulting in exposed CTP stumps remaining at the seafloor, along with CTP debris and broken pilings left at the remediation site. Hence, the second and third year of sampling actually represented a condition of potential PAH contamination resulting from improper CTP removal procedures at the remediation site. Although unintentional, failure to completely remove CTPs from Quilcene Bay afforded us an unusual and unique opportunity to evaluate the PAH exposure of a sensitive life stage to freshly exposed surfaces of old, highly weathered CTPs in a field setting.

To assess PAH exposure of manually spawned herring embryos in the field we (1) measured PAH levels in embryos across a distance gradient from undisturbed CTPs prior to removal activities in 2013, (2) compared PAH levels in embryos from the undisturbed CTP field in 2013 with embryos incubated in the same area in 2014 and 2015 after CTPs had been removed, but also near unremoved piling fragments, (3) compared PAH patterns in embryos with wood collected from the removed CTPs, and (4) compared PAH patterns in embryos and CTP-wood with co-located passive PAH sampling devices. To further assess exposure to and potential biological impact of PAHs, we measured cytochrome P450 (*cyp1a*) mRNA induction by quantitative polymerase chain reaction across a wide range of embryo-PAH levels.

2. Methods

2.1. Study area

The CTP field selected for this study was an approximately 100-year-old derelict railway trestle in Quilcene Bay, (Washington, USA; Figs. 1 and 2). This location was targeted for three reasons: 1) its small size (approximately 300 CTPs) reduced the need for spatially extensive sampling, 2) its remote location was characterized by sparse land development and shoreline activities, which reduced the likelihood of other local PAH sources, and 3) the CTP field was located in an area known to be used by herring for spawning since at least 1975, when the Washington Department of Fish and Wildlife began spawn surveys in the region (Stick et al., 2014).

2.2. Collection of adults and manual spawning

Spawn-ready adults were collected using nets within 3 km of the Quilcene CTP study site. Nets were set at night, and they were continuously tended in order capture live fish. Ovaries and testes were resected from adults, placed in sealed plastic petri dishes, and chilled according to Dinnel et al. (2011), until fertilizations commenced. Fertilizations were conducted within 72 h of collecting adults, in shallow water tables with ambient seawater using methods adapted from Vines et al. (2000) and Incardona et al. (2011). Eggs were teased from excised ovaries using pre-cleaned stainless steel spatulas and placed in a Teflon beaker containing calcium- and magnesium-free seawater pre-mixed to match seawater salinity in the water table. Four composites of ovarian eggs were retained for PAH analysis in one year (2014) to evaluate possible maternal transfer of chemicals (Table 1). Water table salinity was set to one-half ambient seawater, approximately 16 ppt, by diluting with deionized water according to Dinnel et al. (2011). Mixed eggs were then distributed evenly using a pipette over submerged panels of 1.0 mm-square nylon mesh measuring up to 12 × 40 cm. As eggs were dripped into the seawater they immediately became adhesive and adhered to the submerged nylon mesh in the water table.

After unfertilized eggs had been evenly distributed over the submerged nylon mesh panels, milt was prepared by macerating together whole testes from six to 28 male fish in a pre-cleaned glass beaker containing herring ringer's solution (Yanagimachi and Kanoh, 1953). After draining the water level in the table to its lowest possible volume (while keeping eggs submerged), the water input was shut and the prepared sperm mixture was poured into the water table. The water was then gently swirled to ensure eggs contacted sperm. After approximately 2 h, the water in the table was drained while being simultaneously replenished by fresh seawater. During the first 12 to 24 h, subsamples of approximately 100 eggs were removed and assessed for fertilization rate. Fertilization rate was obtained by comparing the number of eggs that had undergone first cleavage (Hill and Johnston, 1997) with the total number of eggs. Fertilization rates ranged from > 60% to > 90% in all three years of the study.

Anti-predator cages were either extruded aluminum mesh cubes measuring 15 × 15 × 15 cm (2013 and 2014) or plastic-coated steel mesh cylinders measuring 23 cm long x 9 cm diameter (2015). An additional piece of nylon mesh (with no eggs attached) was used to line the inside of cages in 2014 and 2015 to better exclude predators. Caged embryo sampling units (CESUs) were prepared on the day of deployment, approximately 24 h after fertilization; each embryo-laden mesh panel was slipped into a separate plastic bag and placed in a cooler filled with ambient seawater. Holes were cut in bags to allow water circulation, and coolers were transported to the study site. Pre-cleaned cages were transported to the study site sealed in plastic bags. On the boat (with engine shut off), each cage was removed, a mesh panel inserted, and lid attached with nylon conduit ties. In 2013, cages were affixed to steel rods oriented horizontal to the seafloor, and attached to pilings by SCUBA divers to facilitate accurate placement from existing pilings at 0 cm, 30 cm, 100 cm, and 200 cm for the distance-gradient experiment (Fig. 3, Table 1). CESUs were all approximately 10 cm above the seafloor.

During deployment, all CESUs were submerged while inside plastic bags to avoid contact with potential contaminants in the sea surface microlayer. In 2014 and 2015, after CTPs had been removed, individual CESUs were affixed to epoxy-coated mesh panels with steel rods as ballast, and lowered to the seafloor from the boat. The mesh panels were bent at the edges to elevate the CESUs 10 cm above the seafloor (Fig. 4). The position of each CESU was marked with a small numbered float. At the end of each deployment day, three egg panels were retained as deployment controls to determine whether embryos were exposed to PAHs during the fertilization, transport and deployment procedures (Table 1).

In 2013, 20 CESUs were placed within an area of high density CTPs (Fig. 2 filled circles, and attached to or placed near five individual, existing CTPs (shown schematically in Fig. 3). In 2014, approximately two weeks after the last CTPs had been removed, seven CESUs were placed inside the area defined by the former CTP field and another eight

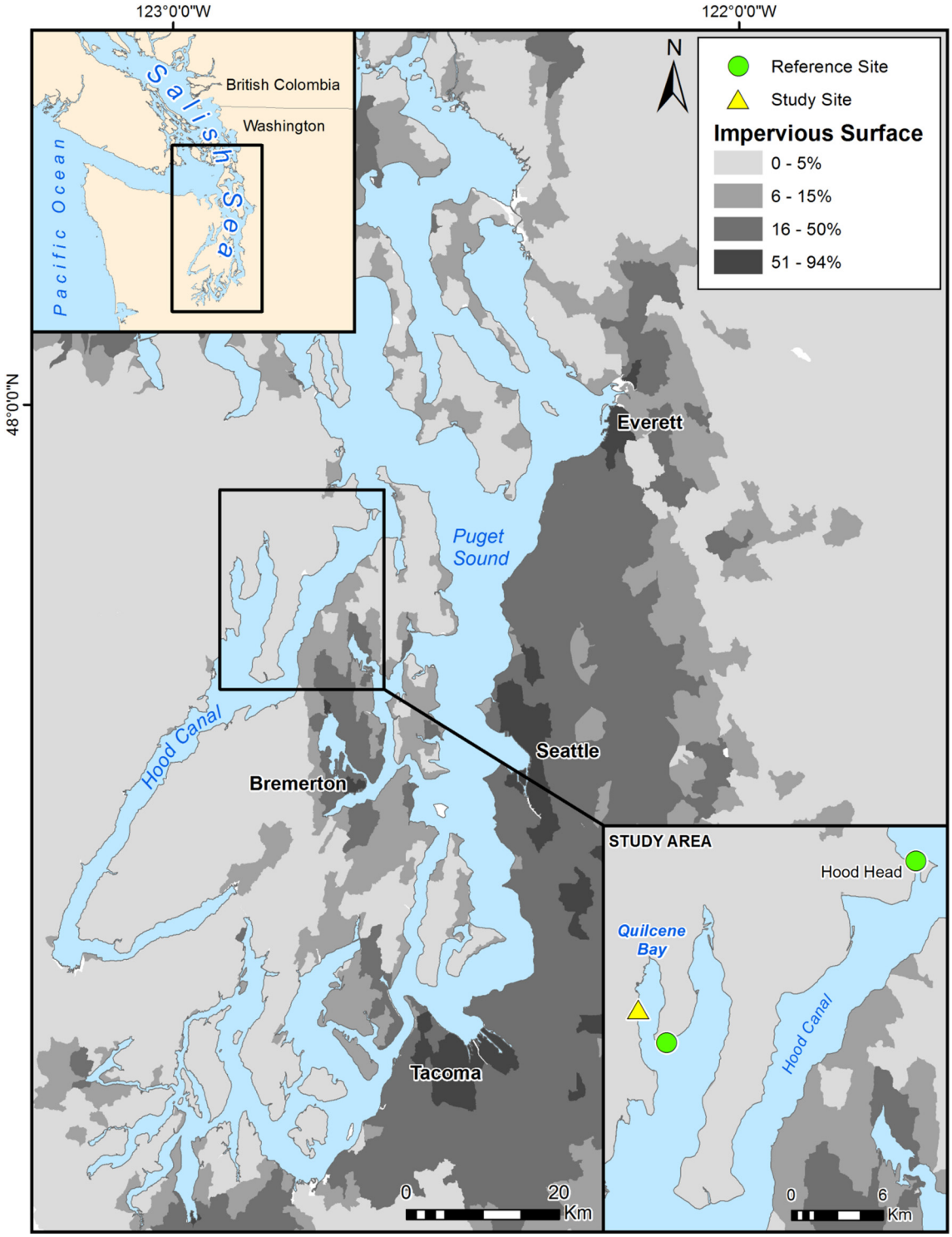

Fig. 1. Location of the Quilcene Bay creosote-treated piling study site, and two reference areas three to 45 km from the study site. Gray-scale shading indicates levels of impervious surface, a proxy for degree of land development, and potential polycyclic aromatic hydrocarbon sources.

were placed near (within 25 m of) the former CTP field (Fig. 2, filled squares). The same arrangement was repeated in 2015, with eight CESUs inside the former CTP field and six near to the former CTP-field footprint (Fig. 2, filled triangles). In each year, three to five CESUs were placed in reference areas three to 45 km from the study site. Reference areas were selected based on their isolation from putative contaminant sources, including CTPs, shoreline roadways, urbanization, and residential or other watershed development (see gray shading in Fig. 1).

CESUs remained in situ for 10 days, after which they were retrieved using the deployment method in reverse. Embryo-laden mesh panels were removed from the CESUs and placed temporarily in plastic bags for transport back to the laboratory where they were processed. Once in the laboratory, 5-27 g of embryos were scraped from the nylon mesh panels into pre-cleaned glass jars for tissue PAH analysis, and 12 to 20 embryos per treatment were preserved in RNA*later* for *cyp1a* quantification. In addition, in 2013, naturally occurring spawned eggs were collected from *Laminaria* and *Sargassum* macroalgae at the reference site using an iron rake designed for such purpose. Wood splinters were also

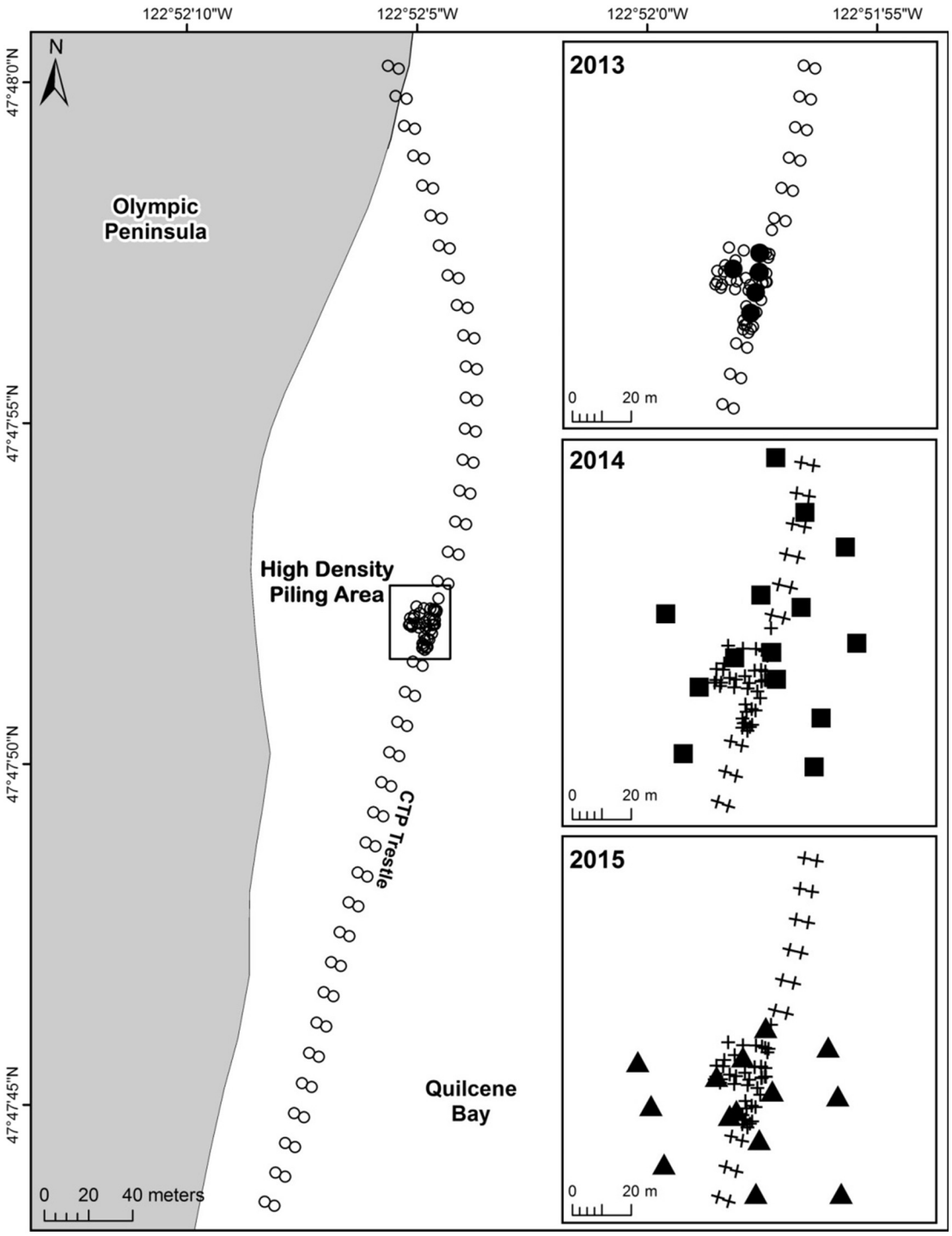

Fig. 2. Location of caged herring embryo sampling units (filled symbols) relative to creosote-treated pilings (CTP) in 2013 (open circles), and to the former CTP positions in 2014 and 2015 (crosses).

sampled by divers from five CTPs at the same depth as CESUs in 2013. These wood chips were placed in a sealed plastic bag and brought to the surface, where they were stored in pre-cleaned glass jars for later analysis.

2.3. Low density polyethylene strips (LDPSs)

A single embryo-to-LDPS comparison study was conducted in 2014. LDPSs were constructed using the same low-density polyethylene material as Carls et al. (2004) except in this case a single strip was used without a trapped lipid matrix, and LDPSs were attached directly to CESUs, rather than in a separate structure. To prepare for use, LDPSs were cleaned with methylene chloride and stored in sealed plastic bags until deployment with CESUs. During deployment, LDPSs measuring approximately 5 cm × 20 cm were attached to CESUs using nylon conduit ties where they remained for the entire 10-day incubation period. The strips were cut from each CESU immediately upon retrieval and placed in pre-cleaned glass jars for later analysis. Nine LDPSs were deployed inside and near to the former CTP field, as well as in a reference area (Table 1). In addition, three LDPS deployment controls were created by treating LDPSs to the full deployment activity, except they were retained just prior to submerging them in water, to evaluate

Table 1
Polycyclic aromatic hydrocarbons (PAHs) in herring embryos from caged embryo sampling units (CESUs), deployment controls, and reference areas, and PAHs from low-density polyethylene strips (LDPS) and wood from creosote-treated pilings (CTP). Mean frequency of detection and concentration of Σ_{32}PAH (ng/g wet weight) and mean concentration of lipids (%). Fertilization success for the three spawning years was 63% in 2013, and 92% in each of the following years.

	Treatment	Type	N	Det Freq	Σ_{32}PAH	Lipids(%)
2013: pre deployment	Deployment Control	CESU	3	0.051	0.51	0.040
	Reference Area (natural spawn on vegetation)	Embryo	6	0.061	0.75	0.094
	Reference Area	CESU	3	0.051	0.58	0.076
	Pre-removal CTP Field	CESU	20	0.17	2.7	0.15
	Pre-removal CTP Field	Wood	1	0.97	nr	na
2014: 1 month post-deployment	Ovarian eggs	Eggs	4	0.0076	0.065	0.098
	Deployment Control	CESU	1	0.030	0.25	0.15
	Reference Area[a]	CESU	5	0.042	0.48	0.48
	Post-removal, inside former CTP field	CESU	7	0.71	50	0.47
	Post-removal, near former CTP field	CESU	8	0.44	15	0.44
	Deployment Control	LDPS	3	0.40	88	na
	Reference Area	LDPS	3	0.41	71	na
	Post-removal, inside former CTP field	LDPS	3	0.91	2300	na
	Post-removal, near former CTP field	LDPS	3	0.78	472	na
2015: 1 yr post -deployment	Deployment Control	Embryo	1	0	< 0.22	0.17
	Reference Area (CESU)[b]	CESU	0	nr	nr	–
	Post-removal, inside former CTP field	CESU	8	0.67	74	0.56
	Post-removal, near former CTP field	CESU	6	0.36	16	0.50

[a] 100% mortality observed in 3 CESUs deployed at the Fishermen's Point reference area, and so not reported here. The five reported here were from the Hood Head reference area.
[b] TPAH not reported because of high embryo mortality.

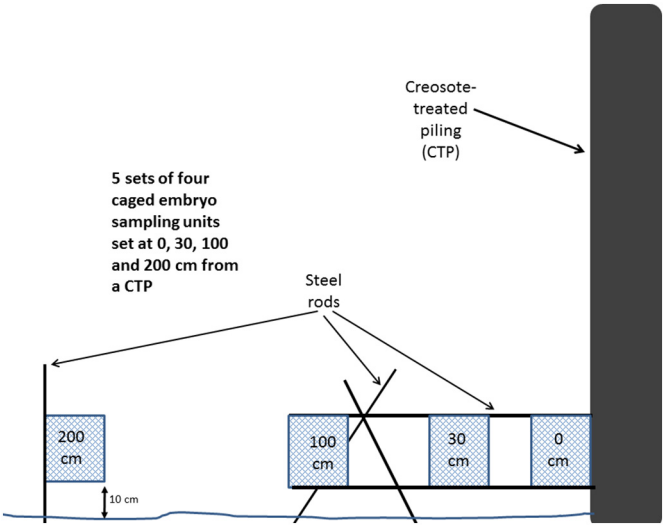

Fig. 3. Apparatus for fixing caged herring embryo sampling units at four prescribed distances from existing, undisturbed creosote-treated pilings, and 10 cm above the seafloor in 2013.

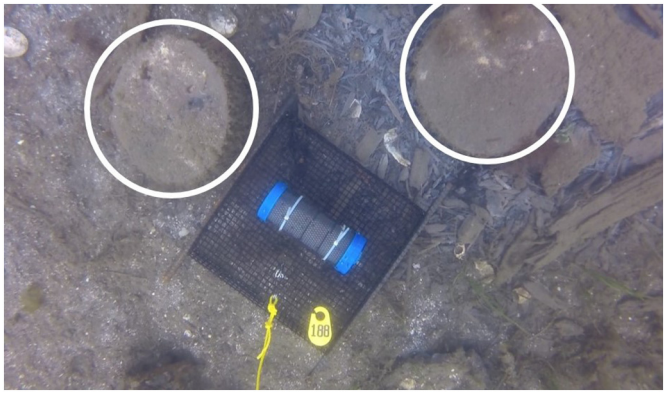

Fig. 4. Looking down from above, herring caged embryo sampling unit attached to its mesh platform elevated 10 cm above the seafloor, and situated adjacent to two creosote-treated pilings (CTPs) cut off approximately 10 cm above the seafloor (highlighted with white circles). Also visible are splintered wood fragments from CTPs.

potential PAH contamination resulting from the deployment process.

2.4. Polycyclic aromatic hydrocarbon analysis

Forty-two PAH analytes were quantitated in this study, consisting of 22 low molecular weight compounds and 20 high molecular weight compounds (Table 2), according to Sloan et al. (2014). In brief, wet samples were extracted with methylene chloride and accelerated solvent extraction, which provided an extract that was used for both PAH recovery and gravimetric lipid evaluation (reported herein as percent lipid). Recovered PAHs were subsequently cleaned up by silica/aluminum columns and size-exclusion high-performance liquid chromatography before final quantitation of aromatic hydrocarbons using gas chromatography/mass spectrometry with selected-ion monitoring.

In cases where a PAH compound was not detected in a sample, the value was reported as "less than the limit of quantitation", or " < LOQ". An LOQ concentration was calculated for each sample based on sample weight and instrument performance for each batch of samples (Sloan et al., 2014). PAH summations were based on detected values only. In cases where no PAHs were detected in a sample, the mean of all LOQ values, 0.21 ng/g wet weight (wet wt), was used for the PAH summation value in that sample.

Some PAHs were detected in solvent-blank samples. In cases where a blank-detected PAH was also detected in a field sample from the same batch, the value of the PAH in the blank sample was subtracted from the value of the field sample if the PAH value of the field sample exceeded three times the value of the blank-sample. If the PAH value of the field sample was less than three times the value of the blank-sample, the value of the PAH in the field sample was considered indistinguishable from the blank (i.e., the PAH originated from contamination unrelated to the experiment), and it was reported as < LOQ.

Forty-one of 42 quantitated PAH compounds or alkylated compound groups were detected in at least one sample over the three years of this study in any of the embryos, LDPSs and CTP wood (C_4Chrysenes were never detected; Table 2). Quantitation of nine PAH analytes, C_2, C_3 and C_4NPH, C_2 and C_3FLU, C_3DBT, C_3 and C_4PHN/ANT, and C_1CHR was complicated by interference from unknown compounds, observable as

Table 2
Detection frequency of the 42 PAH analytes detected in herring embryos, low density polyethylene strips, and wood from creosote-treated pilings. Nine compounds were excluded from analysis because of interference from unknown compounds (shown as *int* below).

LMW Compounds	Freq%	HMW Compounds	Freq%
Naphthalene (NPH)	18	Fluoranthene (FLA)	56
C_1NPH	12	Pyrene (PYR)	39
C_2NPH	*int*	C_1FLA/PYR	37
C_3NPH	*int*	C_2 FLA/PYR	26
C_4NPH	*int*	C_3 FLA/PYR	16
Acenaphthylene (ACY)	1.2	C_4 FLA/PYR	4.9
Acenaphthene (ACE)	63	Benz[*a*]anthracene (BAA)	26
Fluorene (FLU)	65	Chrysene (CHR)[a]	34
C_1FLU	28	C_1CHR	*int*
C_2FLU	*int*	C_2CHR	18
C_3FLU	*int*	C_3CHR	1.2
Dibenzothiophene (DBT)	27	C_4CHR	< LOQ
C_1DBT	16	Benzo[*b*]fluoranthene (BBF)	28
C_2DBT	16	Benzo[*k*]fluoranthene (BKF)[b]	26
C_3DBT	*int*	Benzo[*e*]pyrene (BEP)	24
C_4DBT	3.7	Benzo[*a*]pyrene (BAP)	24
Phenanthrene (PHN)	74	Perylene (PER)	12
Anthracene (ANT)	35	Dibenz[*a,h*]anthracene (DBA)[c]	2.4
C_1PHN/ANT	57	Indeno[*1,2,3-cd*] pyrene (IDP)	20
C_2PHN/ANT	35	Benzo[*ghi*]perylene (BZP)	9.8
C_3PHN/ANT	*int*		
C_4PHN/ANT	*int*		

[a] Coeluted with triphenylene.
[b] Coeluted with benzo[*j*]fluoranthene.
[c] Coeluted with dibenz[*a,c*]anthracene.

unidentifiable peaks on the gas chromatography chromatogram. Because of uncertainty in the contribution of the unknown compounds to mass of these nine analytes, they were removed from PAH summations and pattern analyses, and the total PAH was therefore calculated as a sum of 32 identified and detected PAH analytes (Σ_{32}PAH).

2.5. *cyp1a* analysis

Transcriptional responses of *cyp1a* in embryos were measured using quantitative polymerase chain reaction (qPCR; Edmunds et al., 2015; McIntyre et al., 2016), across the range of PAH concentrations measured in embryos from matched CESUs. Total RNA was extracted from embryos preserved in RNA*later* using three steps: 1) mechanical homogenization using a TissueLyser (Invitrogen Inc.) in TRIzol reagent (Invitrogen Inc.) with RNAse-free stainless steel beads (Qiangen Inc.), 2) phase separation, and 3) RNA phase purification and on-column DNAase-treatment using DirectZol spin-columns (Zymo Inc.). First-strand complementary DNA (cDNA) was then synthesized from a normalized quantity of total RNA using random hexamers (High Capacity RNA-to-cDNA Reverse Transcription Kit; Life Technologies Inc.). qPCR reactions (10 µL) were run in duplicate using optical 96-well plates (Applied Biosystems Inc.) containing Fast SYBR Green chemistry (Applied Biosystems Inc.), 100 nM primer, and 10 ng cDNA template on a Viia7 Real-Time qPCR Detection System (Applied Biosystems Inc.) under fast-cycling conditions (95 °C for 2 min and then 40 cycles at 95 °C for 1 s and 60 °C for 20 s). Single-product amplification was verified by generating dissociation curves as the terminal step of all qPCR reactions. Target (*cyp1a*) and reference gene (elongation factor 1 alpha; *ef1α*) primer pairs (*cyp1a*-F: ATCAGCAAGGAGGGTCTGTACC, *cyp1a*-R: GAACTCATCGCTCATGTTGACC; *ef1α -F:* CAAGAACGACCCACCTATGG, *ef1α -R:* CCTTCAGCTCATTGAACTTGC) demonstrated acceptable efficiencies (95–104%; Schmittgen and Livak, 2008). Relative expression of *cyp1a* was normalized to the expression of *ef1a* using Reference Residual Normalization (Edmunds et al., 2014).

cyp1a induction was reported as fold-increase in treatment embryos compared to reference embryos. Because of high unexplained mortality in embryos from one reference area (Fishermen's Point), we used the five embryo samples from the 2014 Hood Head as a PAH-unexposed reference for calculating fold change in treatment embryos for all three years.

2.6. Data analyses

All comparisons of Σ_{32}PAH concentration between treatments and reference groups were conducted using analysis of variance (ANOVA) of log10-transformed Σ_{32}PAH by groups, using $\alpha = 0.05$. *Post-hoc* pairwise comparisons between groups were conducted using Tukey's multiple range test. Linear regressions were performed on log10-transformed Σ_{32}PAH by independent variables. PAH patterns were illustrated using Principal Components Analysis on standardized and square-root-transformed PAH concentrations on a reduced set of ten most influential PAH compounds, which were identified using the BVSTEP routine in Primer v6 (Clarke and Gorley, 2006).

3. Results

3.1. Σ_{32}PAH concentration in embryos

We observed uptake of PAHs in embryos in all three years, compared to all field reference areas and deployment controls. Σ_{32}PAH from CESUs placed inside the former CTP field in 2014 and 2015, near CTP fragments and cut-CTP stumps (pooled across the two years), were 90 times greater than Σ_{32}PAH in CESUs from reference areas and 19 times higher than the CESUs placed in the undisturbed field prior to CTP removal in 2013 (ANOVA of log10-transformed Σ_{32}PAH by treatment; Fig. 5). Σ_{32}PAH from CESUs placed outside but near the former CTP field in 2014 and 2015 (pooled) were intermediate between the 2013 pre-removal condition and the inside-CTP-field treatments from 2014 and 2015 (Fig. 5).

It also appears undisturbed 100-year-old CTPs leached sufficient PAHs to be accumulated by embryos incubating nearby (within 200 cm). Embryos incubated inside the CTP field in 2013 accumulated significantly higher (four- to five-fold) Σ_{32}PAH concentration (2.5 ng/g wet wt) than the naturally spawned embryos (0.73 ng/g wet wt) and CESU embryos deployed in the reference areas (0.48 ng/g wet wt; Fig. 5). The four distance-to-CTP treatments in 2013 (0 to 200 cm) were pooled because Σ_{32}PAH was not significantly different between them, based on an ANOVA of log10-transformd Σ_{32}PAH by CTP-distance

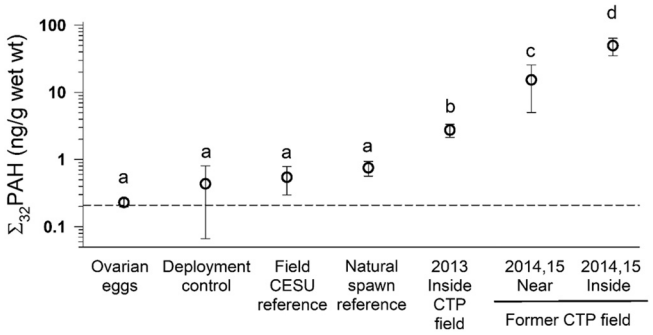

Fig. 5. Polycyclic aromatic hydrocarbons (PAHs) in herring embryos from three treatments, two reference areas, deployment controls, and maternal transfer controls (ovarian eggs). Geometric mean ± 95% confidence interval. Data collected in 2014 and 2015 were pooled for the Near treatment and for the Inside treatment because there was no significant difference in Σ_{32}PAH between the years for each location type (ANOVA of log10-transformed Σ_{32}PAH by year, $p > 0.05$ for both location types). Similar lowercase letters indicate no significant difference in Σ_{32}PAH concentration based on ANOVA of log10-transformed Σ_{32}PAH by sample type; $F_{65,6} = 66.2$, $P < 0.001$, Tukey's multiple pairwise comparison. Horizontal dashed line indicates mean LOQ for all samples.

($F_{19,3}$ = 0.182, p = 0.91) within that year.

Σ_{32}PAH occurred in low concentration and ranged narrowly, from 0.065 to 0.75 ng/g wet wt, in all control and reference samples (mean values) indicating PAHs measured in treatments were almost completely attributable to treatment effects. These include the two control sample types (ovarian eggs and deployment controls) indicating PAHs from maternal sources and from creation and deployment of CESUs was trivial. Moreover, low PAH concentrations in the two field reference sample types (field-deployed CESUs and natural spawn in reference areas) indicate trivial contribution of PAHs from ambient or background sources (Table 1, Fig. 5). Σ_{32}PAH in all control and reference samples resulted from low-level detects of only one to four analytes, near the mean LOQ (0.21 ng/g wet wt), and all were significantly lower than Σ_{32}PAH in all treatments (ANOVA of log10-transformed Σ_{32}PAH by treatment type, $F_{65,6}$ = 66.2, $P < 0.001$; Tukey multiple pairwise test; Fig. 5).

Overall, PAHs declined in embryos with increasing distance from the former CTP field. Although variability in embryo Σ_{32}PAH was high relative to the distance of former CTP positions in 2014 and 2015, a logarithmic model of embryo TPAH by distance of CESU to former CTPs was significant, and explained roughly 35% of Σ_{32}PAH variability (Fig. 6; linear regression of log-transformed TPAH by estimated distance to former pilings; p = 0.0012, adjusted r^2 = 0.35). Underwater remote-video surveys of the site following the completion of the CTP removal activities showed approximately 24 CTPs had been cut off at the seafloor, leaving freshly cut CTP surfaces, nine broken CTPs, and numerous CTP splinters remaining on the seafloor in the former CTP field (Fig. 4). Because of the great number and varying size of CTP fragments, it was impossible to quantify the distance of each CESU from all CTP fragments. However, all embryo samples with Σ_{32}PAH exceeding 100 ng/g wet wt were situated within 2 m of pilings that had been cut at the seafloor (example shown in Fig. 4; cut CTP stumps highlighted with white circles). Moreover, variability of Σ_{32}PAH concentration was especially high in CESUs placed near cut pilings, with values ranging from 11 ng/g to approximately 200 ng/g wet wt.

3.2. PAH patterns

Pattern analysis of individual PAH compounds showed some concordance and some differences among the matrices; CTP wood, LDPSs sampled in 2014, and embryos sampled from inside the former CTP field in 2014 and in 2015. This analysis was limited to samples that exhibited enough detected values for at least ten compounds, to achieve a robust pattern. All individual PAH data from 2013 embryos, as well as embryos outside the former CTP field in 2014 and 2015, were excluded from the PCA pattern analysis because the preponderance of < LOQ values in these samples precluded calculation of meaningful similarity matrices.

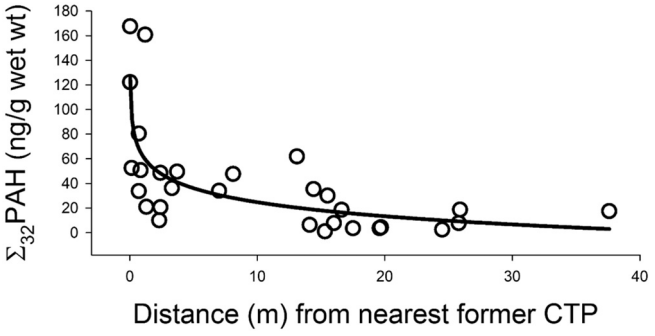

Fig. 6. Decline of polycyclic aromatic hydrocarbons (PAHs) in herring embryos with distance from former creosote-treated piling (CTP) positions after CTP removal; pooled samples from 2014 and 2015. Linear regression of log10-transformed Σ_{32}PAH by distance, P = 0.0004, adjusted r^2 = 0.35.

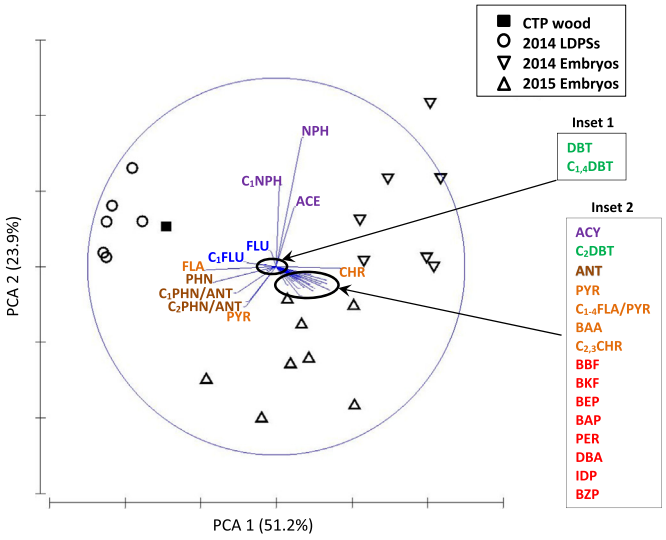

Fig. 7. Principal Component Analysis (PCA; Primer v6, Clarke and Gorley, 2006) of 32 polycyclic aromatic hydrocarbons (PAHs) in identifying PAH patterns from CTP wood (2013), LDPS (2014), and embryos from inside the former creosote-treated piling (CTP) field in 2014 and 2015. The 2013 embryos and samples from near the former CTP field in 2014 and 2015 were excluded from this analysis because the preponderance of < LOQ values for individual PAHs in these samples resulted in patterns that were driven based on unquantifiable values. See Table 2 for description of PAH abbreviations.

Overall, the relative abundance of the 32 PAH compounds appeared to result in three distinct groupings: (1) the two abiotic matrices, CTP wood (filled square) and LDPSs (open circles) together, (2) 2014 embryos (downward triangles), and (3) 2015 embryos (upward triangles – Fig. 7). The PAH pattern in LDPSs and CTP wood was separated from embryos primarily across the PCA1 axis, which explained 51% of the variance. The abiotic matrices were characterized by comparatively more abundant lower molecular weight, 2- and 3-ring compounds (FLU compounds, PHN compounds, and FLA), whereas all embryos (2014 and 2015 combined) exhibited comparatively more abundant higher molecular weight, 4-ring compounds (e.g., CHR, PYR, BAA) and eight 5-ring compounds along PCA1. The 2014 embryos were also separated from the 2015 embryos along the vertical axis (which explained 24% of PAH variation) by greater relative abundance of low molecular weight NPH, C_1NPH, and ACE compounds and fewer of some of the higher molecular weight PHNs and PYR. In total, the abiotic media exhibited the “lightest signal” with a dominance of low molecular weight compounds, followed by 2014 embryos with a wide range of molecular weights, and 2015 embryos with the “heaviest” signal (dominated by compounds with the greatest molecular weight).

3.3. Comparison of Σ_{32}PAH in LDPSs and embryos

LDPSs were co-located with nine CESUs across a wide range of CTP proximity in 2014, from a reference area to inside the former CTP field, resulting in pairings with embryo Σ_{32}PAH ranging from < LOQ to 84 ng/g wet wt. Although there were differences in the PAH pattern between LDPSs and embryos as noted above, the concentration of Σ_{32}PAH in LDPSs was highly correlated with embryo Σ_{32}PAH (linear regression of Σ_{32}PAH in LDPSs by Σ_{32}PAH in embryos, r^2 = 0.96, $p < 0.0001$; Fig. 8). PAHs were not detected in embryos from any of the three CESUs deployed in the reference area in 2014; thus, in these cases, the mean limit of quantitation of all PAHs (0.21 ng/g wet wt) was substituted for the Σ_{32}PAH value. This substitution resulted in high leverage near the origin for the regression model; however, any value below the LOQ produced a similar result and substituting any values less than the 0.21 ng/g wet wt LOQ had a trivial effect on the model.

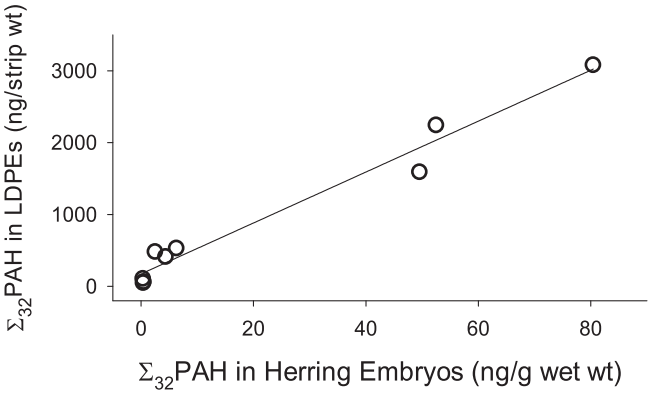

Fig. 8. Comparison of polycyclic aromatic hydrocarbons (PAHs) in low-density polyethylene strips co-deployed with embryos, incubated in situ for 10 days. Linear regression, $p < 0.0001$, adjusted $r^2 = 0.97$.

Σ_{32}PAH in LDPSs was expressed on a per-strip-gram basis; concentrations ranged from 46 to 3100 ng Σ_{32}PAH/g strip.

3.4. PAHs and embryo health

Expression of *cyp1a* increased with Σ_{32}PAH in embryos from 2014 and 2015, with five- to six-fold induction, compared to reference embryos (Fig. 9; logarithmic regression of fold-induction by Σ_{32}PAH; $p < 0.001$, adjusted $r^2 = 0.93$ in 2014; $p = 0.0004$, adjusted $r^2 = 0.51$ in 2015). Fold-induction of *cyp1a* also increased significantly ($p = 0.02$) in 2013, although only weakly (adjusted $r^2 = 0.18$). Fold induction was measured across a Σ_{32}PAH concentration gradient from < 1 ng/g wet wt to over 60 and 100 ng/g wet wt in 2014 and 2015, whereas the Σ_{32}PAH gradient in 2013 ranged from < 1 ng/g to only approximately 4 ng/g wet wt.

4. Discussion

The original intent of this study was to evaluate PAH exposure of herring embryos incubating near undisturbed, 100-year-old creosote-treated pilings (CTPs), and to compare PAHs in embryos from the same area after CTPs had been completely removed. Failure to fully remove CTPs afforded a unique, unanticipated opportunity to evaluate the exposure of, and possible effects on, herring embryos to CTP-derived PAHs in a situation where derelict pilings had been highly disturbed and not fully removed.

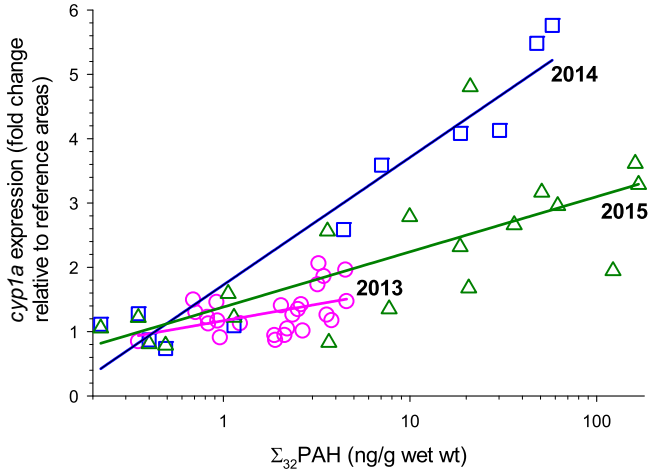

Fig. 9. Increase of *cyp1a* induction with summed concentrations of 32 polycyclic aromatic hydrocarbons (Σ_{32}PAH) in each year of the study. Regression of *cyp1a* expression by $\log_{10}$ transformed PAHs; adjusted r^2 0.18, 0.93, and 0.51 for 2013, 2014, and 2015, respectively. *P* values < 0.05 for all models.

Substantial effort has been put towards evaluating the lethal and sublethal effects on fish embryos exposed to oil, and PAHs from oil, in the context of assessing and predicting biological damage related to oil spills (Incardona, 2017). A few studies have compared health effects with TPAH concentration in embryos, however the number of PAH analytes included in TPAH summations varies slightly across studies. Hence, although comparisons of TPAH must be made cautiously with this variation in mind, it still may be instructive to review them. Incardona et al., 2009 reported cardiotoxic effects such as irregular heart contraction rate and rhythm, bradycardia, pericardial edema, and reduced cardiac chamber size associated with petrogenic total PAH tissue concentrations of 480 to 8100 ng/g wet wt, in lab-exposed embryos. Carls et al. (1999) reported embryo malformations and mortality across a lowest observable effects concentration (LOEC) range of 22–108 ng/g total PAH wet wt in embryo tissues. The TPAH concentrations calculated by Carls et al. (1999) are likely conservative values because they did not include 5-ring compounds, which were commonly detected in our samples and included in our TPAH summations. Incardona et al. (2015) reported subtle alterations in heart shape and reduced cardiorespiratory performance in juvenile herring raised from otherwise normal embryos exposed to PAHs at a tissue concentration of 29 ng/g total PAH wet wt. We observed a maximum Σ_{32}PAH of 170 ng/g in embryos from the post-removal CESU deployments (mean of 62 ng for 2014 and 2015 combined), which was within the Carls et al. (1999) LOEC range and expected to cause persistent adverse effects on heart development and function.

Petrogenic PAH patterns in the oil-exposed embryo studies were characterized by high concentrations of alkylated homolog groups relative to their parent compounds, as well as an overall dominance of lower molecular weight, tricyclic compounds. However, as oil weathers in the environment and the pattern shifts to a dominance of higher molecular weight PAHs (resembling more the creosote pattern we observed in this study), toxicity increases per unit mass (Carls and Meador, 2009). This realization led to studies focused on toxicity of higher molecular weight PAHs, which are more dominant in weathered oil and creosote sources. Carls et al. (1999) reported greater toxicity of PAH mixtures containing greater proportions of high molecular weight PAHs and concluded the composition of PAH mixtures is of prime importance to understanding PAH toxicity. Incardona et al. (2017) highlighted the importance of understanding the differences between toxicity of PAH mixtures, especially petrogenic versus pyrogenic sources, and suggested higher molecular weight compounds such as BEP, BAP, and BKF, which were moderately abundant in the Quilcene embryos, may exhibit synergistic toxic effects.

Although oil spills are obvious and easily observed events resulting in exposure of organisms to petrogenic PAHs, pyrogenic PAHs and those originating from creosote in many nearshore marine systems may be less obvious, and of potentially high concern. West et al. (2014) reported background PAHs exhibiting pyrogenic patterns in naturally spawned herring embryos at tissue burdens high enough to predict toxic effects in several locations across Puget Sound, including less urbanized shorelines. Greatest embryo PAH concentrations were reported from embayments with a history of residential or industrial wood burning and the presence of creosote treated pilings. The combination of West et al. (2014) observations with the current Quilcene Bay observations suggest that herring spawned near to CTPs and along developed embayment shorelines are at risk of exposure to ongoing, chronic background PAHs. Incardona et al. (2012) described the difficulty in resolving PAH source-exposures and effects when oil is spilled in urban areas with such high background PAHs.

The *cyp1a* induction we observed in the 2013 embryos suggests that creosote-derived PAHs activated detoxification metabolism in embryos with tissue concentrations as low as 3–5 ng/g wet wt for Σ_{32}PAH, well below the effects thresholds noted above. This may be related to the

presence of 5-ring compounds such as BKF, BAP IDP, some of the most potent *cyp1a* inducers in fish, along with other high-potency 5-ring compounds (Barron et al., 2004). Five-ring compounds, which can exhibit dioxin-like toxicity as well as AHR-mediated cardiotoxicity (Incardona, 2017; Incardona et al., 2011; Hatlen et al., 2010) and other effects, were observed in CTP wood, LDPSs, and embryos, however proportionally highest in the last. Although five-ring compounds occurred in both CTP wood and LDPSs, their relative abundance in embryos was approximately two times greater. These differences could reflect differential uptake or accumulation of PAH compounds by embryos; alternatively, embryos may be more capable of metabolizing lower molecular weight PAHs (4-ring and lower), which would increase the proportion of 5-ring compounds. Analysis of PAH metabolites in embryos may help to resolve this. Σ_{32}PAH concentration in LDPSs deployed in the former CTP field area from this study (mean, 2300 ng/g strip) were similar to LDPS results from similar field-deployed devices in three types of existing CTP piling fields in active marinas and harbors (Duncan et al. 2017; Fig. 6). Although these studies indicate LDPSs adequately represented PAH abundance from CTP wood sources, we observed significant differences in the relative abundance of PAHs in embryos. This suggests that a broad range of PAH compounds, including alkylated homologs, should be measured in field studies when using LDPSs as a proxy for embryos.

The presence of only a few PAH compounds at extremely low concentrations in ovarian eggs, deployment controls, and field controls confirm the source of PAHs in this experiment were related to the field treatments and not to maternal transfer, contamination of CESUs during deployment, or confounding background environmental sources. This last was expected because of the isolation of the Quilcene piling field and reference areas, a characteristic that made this site particularly appropriate for this type of study. Even though the derelict pilings in Quilcene Bay were highly weathered, having existed in the marine environment for over 100 years, embryos developing in close proximity to these undisturbed CTPs accumulated PAHs at concentrations that appeared to activate the cytochrome P450 metabolic detoxification pathway. This suggests that normal weathering of CTP pilings did not necessarily remove PAH risk to fish embryos even on a 100-year time scale, and that herring spawning near to CTPs in that area have been exposed to PAHs close to a century. Moreover, the natural weathering of derelict CTPs likely resulted in ongoing fragmentation and sinking of CTP-wood splinters and so creation of creosote-chemical hot spots in herring spawning habitats probably occurred during the lifespan of the CTP field.

Incomplete removal of CTPs during remediation efforts and the subsequent release of new creosote from interior wood surfaces resulted in increased PAH exposure to embryos. It is impossible to infer exactly which CTP removal activities were responsible for the increase in PAHs; however, the most likely sources included: 1) CTPs cut at the seafloor left the cut surface exposed, resulting in the release of otherwise sequestered PAHs from the interior wood of the CTPs, 2) new CTP wood splinters generated from the crushing force of the CTP-grasping machinery, which fell to the seafloor, 3) incomplete cleanup of broken piling sections, which could have been generated from previous natural processes or from removal activities, and 4) disturbance of contaminated sediments from pulling pilings out of the substrate. Although it is possible that sediment disturbance during the removal of CTPs may have released previously sequestered PAHs, PAH uptake and effects were particularly high and acute for embryos developing closest to the cut CTP surfaces, where sediments were not disturbed by the CTP extraction. The simplest explanation is that PAHs in the second and third years of the study originated from newly cut or fragmented CTP wood. In any case, this study illustrates the potential hazards associated with releasing creosote-related toxic contaminants from activities associated with incomplete removal of pilings or piling material.

5. Conclusions

This study sharpens the focus on even highly weathered creosote-treated pilings as a source of toxic contaminants in marine ecosystems. Although these results support removal of CTPs to reduce contamination, especially in fish spawning habitats, they also provide a caution to remove CTPs completely, and as intact as possible, to prevent release of previously sequestered chemicals. Moreover it highlights the sensistive nature of developing fish embryos, in this case Pacific herring, to PAHs from CTPs or other sources and raises questions about effects on other species in productive, nearshore, temperate marine habitats. Field-deployed LDPSs may serve as a proxy for fish embryos in marine waters because they seem to mimic uptake of PAHs by embryos, providing a similar time-integrated mechanism for concentrating low levels of aqueous PAHs. The differences in relative abundance of specific compounds, especially 5-ring PAHs, may be predictable, and therefore accountable when using LDPSs as a proxy for fish embryos. LDPSs are advantageous because they are (a) inexpensive, (b) not limited to a narrow window of fish-spawn timing and so can be deployed year-round, (c) not limited to nearshore fish spawning habitats and so could be used in deeper waters, (d) structurally and chemically uniform, and not affected by variation in egg quality and fertilization success, and (e) not dependent on collection of adults and successful fertilization of eggs. Although this and other studies (Carls et al., 2004; Duncan et al., 2017) have shown a high correlation between PAH uptake in embryos and LDPSs, more work may be required to establish a predictable calibration between the two. Finally, this study highlights the need to understand the effects of background chemicals such as PAHs that occur chronically in sensitive marine habitats such as fish spawning grounds, and raises questions regarding preventable loss of productivity of these important species.

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References

Barron, M.G., Carls, M.G., Short, J.W., Rice, S.D., 2003. Photoenhanced toxicity of aqueous phase and chemically dispersed weathered Alaska North Slope crude oil to Pacific herring eggs and larvae. Environ. Toxicol. Chem. 22 (3), 650–660. https://

doi.org/10.1002/etc.5620220326.
Barron M.G., R. Heintz, S.D. Rice. 2004. Relative potency of PAHs and heterocycles as aryl hydrocarbon receptor agonists in fish. Mar. Environ. Res. 58(2–5):95–100. https://doi.org/10.1016/j.marenvres.2004.03.001.
Barron, M.G., Carls, M.G., et al., 2005. Assessment of the phototoxicity of weathered Alaska North Slope crude oil to juvenile pink salmon. Chemosphere 60 (1), 105–110. https://doi.org/10.1016/j.chemosphere.2004.12.006.
Carls, M.G., Meador, J.P., 2009. A perspective on the toxicity of petrogenic PAHs to developing fish embryos related to environmental chemistry. Hum. Ecol. Risk Assess. Int. J. 15, 1084–1098. https://doi.org/10.1080/10807030903304708.
Carls M.G., S.D. Rice, J.E. Hose. 1999. Sensitivity of fish embryos to weathered crude oil: part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasi*). Environ. Toxicol. Chem. 18:481–493. https://doi.org/10.1002/etc.5620180317.
Carls M.G., L.G. Holland, J.W. Short, R.A Heintz, S.D. Rice. 2004. Monitoring polynuclear aromatic hydrocarbons in aqueous environments with passive low-density polyethylene membrane devices. Environ. Toxicol. Chem. 23:1416–1424.
Clarke, K.R., Gorley, R.N., 2006. Primer Version 6: User Manual/Tutorial. PRIMER-E Ltd., Plymouth, England.
Dinnel, P.A., Middaugh, D.P., et al., 2011. Methods for conducting bioassays using embryos and larvae of Pacific herring, *Clupea pallasi*. Arch. Environ. Contam. Toxicol. 60 (2), 290–308. https://doi.org/10.1007/s00244-010-9600-8.
Duncan, D.L., Carls, M.G., Rice, S.D., Stekoll, M.S., 2017. The toxicity of creosote treated wood to Pacific herring embryos and characterization of polycyclic aromatic hydrocarbons near creosoted pilings in Juneau, Alaska. Environ. Toxicol. Chem. 36 (5), 1121–1405. https://doi.org/10.1002/etc.3653.
Edmunds R.C., J.K. McIntyre, J.A. Luckenbach, D.H. Baldwin, J.P. Incardona. 2014. Toward enhanced MIQE compliance: reference residual normalization of qPCR gene expression data. J. Biomol. Tech. 25:54. https://doi.org/10.7171/jbt.14-2502-003.
Edmunds R.C., J.A. Gill, et al. 2015. Corresponding morphological and molecular indicators of crude oil toxicity to the developing hearts of mahi mahi. Sci. Rep., 5:17326. https://doi.org/10.1038/srep17326.
Hatlen, K., Sloan, C.A., et al., 2010. Natural sunlight and residual fuel oils are an acutely lethal combination for fish embryos. Aquat. Toxicol. 99 (1), 56–64. https://doi.org/10.1016/j.aquatox.2010.04.002.
Hill, J., Johnston, I.A., 1997. Photomicrographic atlas of Atlantic herring embryonic development. J. Fish Biol. 51, 960–977. https://doi.org/10.1111/j.1095-8649.1997.tb01535.x.
Incardona, J.P., 2017. Molecular mechanisms of crude oil developmental toxicity in fish. Arch. Environ. Contam. Toxicol. 73, 19–32. https://doi.org/10.1007/s00244-017-0381-1.
Incardona J.P., M.G. Carls, et al. 2009. Cardiac arrhythmia is the primary response of embryonic Pacific herring (*Clupea pallasi*) exposed to crude oil during weathering. Environ. Sci. Technol. 43(1):201–207. https://doi.org/10.1021/es802270t.
Incardona, J.P., Scholz, N.L., 2016. The influence of heart developmental anatomy on cardiotoxicity-based adverse outcome pathways in fish. Aquat. Toxicol. 177, 515–525. https://doi.org/10.1016/j.aquatox.2016.06.016.
Incardona J.P., T.K. Collier, N.L. Scholz. 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicol. Appl. Pharmacol. 196(2):191–205. https://doi.org/10.1016/j.taap.2003.11.026.
Incardona J.P., G.M. Ylitalo, et al. 2011. The 2007 *Cosco Busan* Oil Spill: Field and Laboratory Assessment of Toxic Injury to Pacific Herring Embryos and Larvae in the San Francisco Estuary. file:///C:/Users/westjew/Downloads/App%20D%20herring%20injury%20(1).pdf.
Incardona, J.P., Vines, C.A., et al., 2012. Unexpectedly high mortality in Pacific herring embryos exposed to the 2007 *Cosco Busan* oil spill in San Francisco Bay. Proc. Natl. Acad. Sci. 109 (2), E51–E58. https://doi.org/10.1073/pnas.1108884109.
Incardona, J.P., Carls, M.G., et al., 2015. Very low embryonic crude oil exposures cause lasting cardiac defects in salmon and herring. Sci. Rep. 5 (13499), 1–13. https://doi.org/10.1038/srep13499.
Malins, D.C., Krahn, M.M., Myers, M.S., Rhodes, L.D., et al., 1985. Toxic chemicals in sediments and biota from a creosote-polluted harbor: relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). Carcinogenesis 6, 1463–1469. https://doi.org/10.1093/carcin/6.10.1463.
McIntyre J.K., R.C. Edmunds, et al. 2016. Confirmation of stormwater bioretention treatment effectiveness using molecular indicators of cardiovascular toxicity in developing fish. Environ. Sci. Technol., 50(3), 1561–1569. https://doi.org/10.1021/acs.est.5b04786.
Mueller, J.G., Middaugh, D.P., Lantz, S.E., Chapman, P.J., 1991. Biodegradation of creosote and pentachlorophenol in contaminated groundwater: chemical and biological assessment. Appl. Environ. Microbiol. 57, 1277–1285.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative Ct method. Nat. Protoc. 3 (1101). https://doi.org/10.1038/nprot.2008.73.
Shoemaker, M., 2017. Creosote Removal. www.dnr.wa.gov/programs-and-services/aquatics/restoration/creosote-removal.
Sloan, C.A., Anulacion, B.F., et al., 2014. Northwest fisheries science center's analyses of tissue, sediment, and water samples for organic contaminants by gas chromatography/mass spectrometry and analyses of tissue for lipid classes by thin layer chromatography/flame ionization detection. In: NOAA Technical Memorandum NMFS-NWFSC-125, . https://repository.library.noaa.gov/view/noaa/4626 (81 pp.).
Stick, K.C., Lindquist, A., Lowry, D., 2014. 2012 Washington state herring stock status report. In: Washington Department of Fish and Wildlife: Report No. FPA-14-09, 107pp. http://wdfw.wa.gov/publications/01628/.
Vines, C.A., Robbins, T., Griffin, F.J., Cherr, G.N., 2000. The effects of diffusible creosote-derived compounds on development in Pacific herring (*Clupea pallasi*). Aquat. Toxicol. 51, 225–239. https://doi.org/10.1016/S0166-445X(00)00107-7.
Werme, C., Hunt, J., et al., 2010. Removal of creosote-treated pilings and structures from San Francisco Bay. In: Prepared for California State Coastal Conservancy. Contribution No. 605. San Francisco Estuary Institute, Oakland, California. www.sfei.org/sites/default/files/ReportNo605_Creosote_Dec2010_finalJan13.pdf.
West, J.E., O'Neill, S.M., et al., 2014. An evaluation of background levels and sources of polycyclic aromatic hydrocarbons in naturally spawned embryos of Pacific herring (*Clupea pallasii*) from Puget Sound, Washington, USA. Sci. Total Environ. 499, 114–124. https://doi.org/10.1016/j.scitotenv.2014.08.042.
Yanagimachi, R., Kanoh, Y., 1953. Manner of sperm entry in herring egg, with special reference to the role of calcium ions in fertilization. J. Fac. Sci. Hokkaido Univ. 11, 7. http://hdl.handle.net/2115/27132.