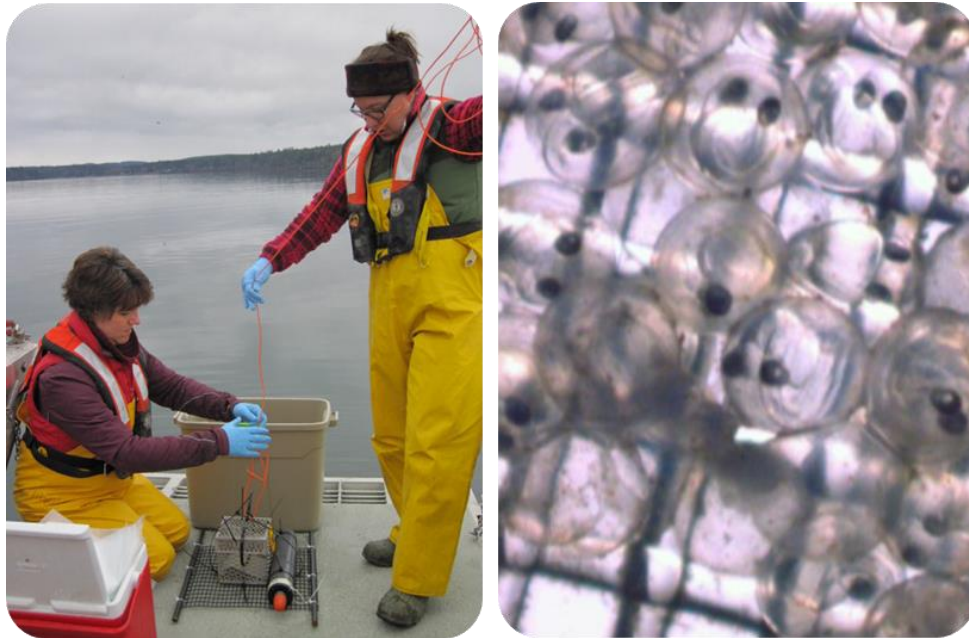


Toxic contaminants in embryonic and adult Pacific Herring (*Clupea pallasii*) from Port Gamble Bay, Washington: extent and magnitude of contamination by polycyclic aromatic hydrocarbons (PAHs) and other toxic contaminants

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ABSTRACT

In this study, we deployed manually spawned herring embryos in Port Gamble Bay to evaluate polycyclic aromatic hydrocarbon (PAH) contamination and the potential related health effects related to exposure of developing herring eggs to PAHs. We confirmed the suspicion from previous studies that PAHs are the most widespread and abundant of potentially harmful chemicals spawned embryos may encounter in the bay. In particular, PAHs were measured in greatest concentration in herring embryos from the two Mill Sites and the Former Lease Area, areas which had previously been identified as PAH-contaminated areas. High PAH levels and the induction of cytochrome P4501A (an enzyme associated with PAH exposure) in some of the embryos from these locations suggests that eggs spawned in these areas face a greater risk of lethal and sublethal effects than eggs spawned elsewhere in Port Gamble Bay. Although contaminant levels and hatching success were not correlated in this study, herring embryos survived significantly better outside Port Gamble Bay than inside the bay. Deployment, retrieval, and analysis of the polyethylene membrane devices (PEMDs) were highly successful. PAH profiles in PEMDs were largely congruent with their co-deployed eggs, with a few exceptions. The concentration of PAHs in PEMDs correlated well with PAHs in embryos, although PAH concentrations were approximately 30 times greater than embryos. Further lab-controlled studies are needed to more accurately calibrate this relationship, and evaluate its full potential. Although some other (non-PAH) chemical contaminants such as PCBs, PBDEs, DDTs, dioxins, and furans occurred in some embryo samples, their levels were largely low enough to conclude lower risk regarding embryo health. The relative abundance, or fingerprint, of PAHs suggested a pyrogenic or highly weathered petroleum signal. This is consistent with the putative sources of PAHs in the bay, namely creosote pilings, and a long history of burning wood in the area. The contaminant levels described herein have provided an *in situ*, broad-spatial-scale, and real-time observation of the potential chemical exposure naturally spawned herring may experience in Port Gamble Bay. These results can be used as a baseline for monitoring the success of cleanup or remediation efforts in Port Gamble Bay.

INTRODUCTION

The study presented here examined the exposure and effects of contamination by toxic chemicals on the health of Pacific herring (*Clupea pallasii*) in Port Gamble Bay, Washington. Port Gamble Bay is the site of historic wood-mill operations, wood chipping, log rafting, burning wood, and related industrial activities, which contaminated the bay with a number of chemicals. Of primary concern is a class of pyro- and petrogenic compounds called polycyclic aromatic hydrocarbons, or PAHs, some of which have been shown to be highly toxic to the development of fish embryos. The primary purpose of the study was to identify the extent to which spawned herring eggs may be exposed to PAHs (and some other persistent organic pollutants) within Port Gamble Bay, and evaluate the potential effects of such exposure on embryo health. Secondary goals were to (a) evaluate the utility of passive sampling devices (polyethylene membrane devices, or PEMDs) as an abiotic proxy for herring embryos for measuring contamination, (b) compare contaminant concentration in embryos with contaminant levels in co-located sediment samples^a, and (c) to evaluate contaminant levels of adult herring known to spawn in the bay. These results are meant to be used as a baseline of conditions for evaluating changes that might occur during planned habitat remediation activities in the bay, as well as the long-term effectiveness of remediation efforts.

Prior to 2000, the Port Gamble Bay herring stock was considered one of the larger spawning stocks in Puget Sound, and since that time the spawning biomass has steadily declined from 2,459 tons to 404 tons in 2012 with an extreme low of 208 tons in 2008 (Stick et al., 2014). This decline is unexplained, is cause for concern, and provided motivation for this study. The Port Gamble S'Klallam Tribe (PGST) have identified this species as high value in the ecosystem and are particularly concerned with their spawning success in Port Gamble Bay. PGST members have reported declines in spawning usage of the bay by herring over the past 100 years.

Unusually high rates of embryo mortality (> 20%) have been observed in Port Gamble Bay since the early 1980s (WDFW, unpublished data), and since that time efforts have been made to (1) assess embryo mortality and its possible link to contamination (Kocan et al., 1987), (2) compare PAH concentrations and embryo health with other spawning stocks (Hershberger et al., 2005), and (3) use blue mussels (*Mytilus galloprovincialis*) as a proxy for PAH exposure in herring embryos (Applied Biomonitoring, 2009). These studies, combined with a recent assessment of PAH concentrations in herring embryos from five spawning stocks in Puget Sound, (not including Port Gamble Bay, West et al., 2014a) have implicated PAH exposure as a risk to herring embryo health in Puget Sound. Moreover, lab and field studies have demonstrated links between aqueous PAH and sublethal effects in fish embryos such as cardiac edema and arrhythmia (Incardona et al., 2009; Incardona et al., 2004). Cardiac toxicity was reported in herring embryos exposed to oil from the 2007 Cosco Busan oil spill in San Francisco Bay (Incardona et al., 2012). These authors also reported mortality and tissue necrosis related to background pyrogenic PAHs.

^a The Port Gamble S'Klallam tribe carried out a separate field survey to collect and analyze sediment samples for the same set of contaminants measured in embryos.

PAH exposure of embryos from creosote-treated pilings has also been identified as a potential source of mortality in developing herring embryos. Vines et al. (2000) demonstrated toxicity of diffusible creosote-derived compounds on herring embryos using a variety of health effects endpoints including cardiac function, embryo movement, hatching success, and larval morphology. Toxicological effects of PAHs on herring embryo health can also be exacerbated with addition of ultraviolet light from sunlight (Barron et al., 2003; Barron et al., 2005; Hatlen et al., 2010).

These studies, combined with a long history of wood burning, extensive use of creosote-treated pilings, and PAH conditions in Port Gamble Bay sediments form a weight of evidence that elevates suspicion of PAH contamination as a cause of herring embryo mortality in Port Gamble Bay.

For this study, embryo exposure to PAHs and other contaminants was estimated by measuring tissue residue concentrations in manually spawned and deployed embryos, incubated *in situ*, in eight areas within Port Gamble Bay and one reference area outside the bay (Figure 1; Appendix A: Cage Locations for Phase One, Two, and Three). The eight sampling areas were selected based on available sediment PAH distribution and roughly corresponded to the Sediment Management Areas (SMAs) identified as Port Gamble Bay Remedial Investigation (Ecology 2012), with additional sites not originally identified as SMAs. Embryos were deployed during two spawning seasons, the winters of 2014 (with co-deployed PEMDs) and 2015 (without PEMDs). Contaminants (or their metabolites) in adult herring were similarly measured as tissue residues in fish that were collected during their spawning activities in the bay. In addition, the activity of the CYP1A gene was compared in embryos across locations. This gene codes for the production of cytochrome P450 1A enzymes which detoxify PAHs by converting them to water soluble, excretable derivatives (see Incardona et al. 2005), and its activity is commonly used as an indicator of exposure to PAHs in vertebrates.

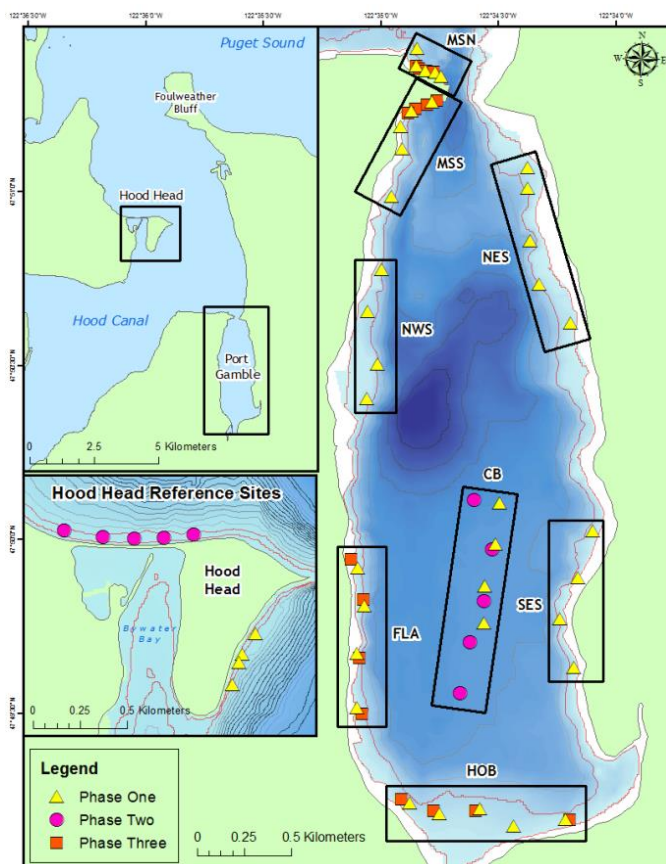


Figure 1. Location of caged embryo sample sites for Phase One (February 2014), Two (March 2014), and Three (March 2015).

This report documents and summarizes PAH and other contaminant levels in embryos, PEMDs, and adult herring, compares contaminants in embryos with sediments and PEMDs, compares contaminant levels with embryo health-effects endpoints, and discusses the implications of the exposures observed herein.

MATERIALS AND METHODS

Sampling and analytical methods in this study followed operating procedures detailed in the Quality Assurance Project Plan written for this work (West et al., 2014b), and an addendum to the QAPP for the addition of PEMDs to the study design (West 2014c). In brief, the study relied on transplanting manually spawned, developing herring embryos into the eight pre-selected locations inside Port Gamble Bay and one reference outside the bay. Embryos were created by manually fertilizing herring eggs using gonads collected from pre-spawning adults. Adhesive eggs were placed on nylon mesh inside protective cages, and deployed during the normal spawning window for Port Gamble Bay herring. Embryos remained *in situ* for 10 days, after which they were retrieved, and processed for chemical analyses and health endpoints. Deployments were conducted in two phases in the winter of 2014 and one phase in the winter of 2015.

Overall, the study was completed in close compliance with the QAPPs. Notable exceptions were related to unforeseen problems encountered in obtaining enough adult fish from Port Gamble Bay, and during the deployment/incubation phase of the study. Despite significant effort from this study, and from a related, concurrent study on the genetic population structure of Port Gamble herring, spawning adult herring were difficult to find in Port Gamble Bay. To remedy this, we used adult herring from the nearby Quilcene/Dabob Bay spawning stock to obtain gametes for egg deployments in Phase Two and Three.

High embryo predation from invertebrates small enough to pass through the cage mesh resulted in low embryo tissue weight during the first deployment in late February 2014 (Phase One). Embryo cages were subsequently fitted with small-mesh (1-mm square) nylon screen to exclude small predators, and two additional smaller spawnings/deployments were performed in March 2014 (Phase Two) and March 2015 (Phase Three). In addition, four deployed cages were stolen from the Hood Head Reference area during Phase One, so those deployments were repeated in Phase Two.

Because of the high numbers of embryos involved in the study and difficulties maintaining adequate incubation conditions for retrieved herring embryos, it became unfeasible to conduct both hatching success (counts of larvae) and survival counts on all samples. As a compromise, we counted pre-hatched embryos after retrieval, at 12 day post fertilization (dpf). Counts were conducted either on fresh samples as they were available in the lab, or on samples preserved in Stockard's solution. The total number of embryos that had clearly developed to the 12 day stage was compared with the number of eggs that had clearly been fertilized, and had developed to at least 3 dpf, but had died. The proportion of pre-hatching embryos was calculated from the total of these two embryo types.

Field Activities

Adult Herring Collection and Gonad Resection

Adult herring used for manual spawning were collected at various locations in Port Gamble Bay and Quilcene Bay. Adult male herring from Port Gamble Bay not used for spawning were retained for analysis of contaminants for that portion of the study, according to PSEMP protocols for long-term

monitoring of contaminants in adult herring. In Phase One, 18 female and 11 male herring were collected from seven gill net hauls in Port Gamble Bay on the night of February 19, 2014 (Figure 2, yellow areas). Adult herring used for manual spawning in Phase Two and Three were collected from the Quilcene/Dabob herring stock, off the south end of the Bolton Peninsula in Dabob Bay. Sixty-nine spawning adult herring were collected from night-sampling on March 26, 2014 (Phase Two, pink areas; Figure 2), and 200 spawners on March 19, 2015 (Phase Three, orange areas; Figure 2).

Manual Fertilizations

All fertilizations were conducted at Environ Laboratories, in Port Gamble Bay WA. Fertilization success was measured approximately 2 hours post fertilization (hpf), in sub-samples of embryos collected from random pieces of 17 mesh panels. Fertilization rates at 2 hpf were > 90% for the three Phases in this study. Development of embryos was then examined approximately 12 hpf, just before mesh panels were processed for deployment; rates ranged from 79 to 100% (Table 1).

During Phase Two, one composite sample of eggs was set aside for chemical analysis and used to examine maternal transfer of contaminants between female and eggs.

Data Analysis

Data summaries are presented herein primarily for contaminant class totals. In the case of PAHs, totals were calculated by summing all 42 detected PAH parent groups and their alkylated homologs as Σ_{42} PAHs. The remaining organics were summarized as follows:

- estimated total PCBs (TPCBs, calculated after Lauenstein and Cantillo 1993, from an algorithm using 18 commonly detected congeners),
- Sum_{11} PBDEs (the sum of 11 PBDE congeners), and
- Sum_6 DDTs (the sum of all 6 DDT isomers).

Dioxins and furans were summarized as individual congeners, as well as homolog group totals. Seventeen dioxin and furan congeners were measured in 18 field samples from six of the nine sampling areas, Mill Site North, Mill Site South, Former Lease Area, Central Bay, Head of Bay, and Hood Head Reference, and in ovaries from females used for manual spawning.

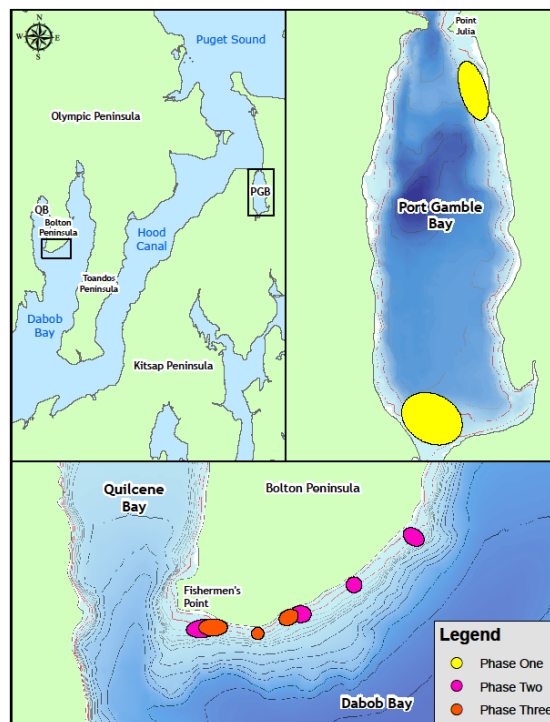


Figure 2. Areas in Quilcene Bay and Port Gamble where adult herring were collected during Phase One (February 2014), Phase Two (March 2014), and Phase Three (March 2015) of the study

All data are presented as wet weights, unless otherwise indicated. Toxic Equivalency Quotients (TEQs) were not summarized for herring embryos because the toxic equivalency factors provided by the analytical laboratory were based on human health criteria.

Table 1. Quantification and calculation of fertilization success (percent fertilization) performed prior to cage deployment for all three phases of the study. The date the counts were performed and the numbers of hours post fertilization (hpf) are listed in the Phase column.

Phase	Random Sample #	# Fertilized Eggs	# Unfertilized Eggs	Total Counted	Percent Fertilization (%)
One 2/20/14 18.5 hpf	1	27	7	34	79
	2	34	3	37	92
	3	31	3	34	91
	4	36	3	39	92
	5	53	5	58	91
	6	57	7	64	89
	7	54	6	60	90
	8	46	9	55	84
	9	41	5	46	89
	10	36	2	38	95
	Total	415	50	465	89
Two 3/24/2014 20 hpf	1	35	8	43	81
	2	59	6	65	91
	3	42	4	46	91
	4	25	6	31	81
	5	35	3	38	92
	6	39	3	42	93
	7	70	1	71	99
	8	55	4	59	93
	9	49	2	51	96
	10	94	0	94	100
	Total	503	37	540	93
Three 3/20/15 19.5 hpf	1	56	4	60	93
	2	60	8	68	88
	3	70	10	80	88
	4	79	6	85	93
	5	63	1	64	98
	6	72	10	82	88
	7	56	7	63	89
	8	67	6	73	92
	9	87	9	96	91
	10	75	3	78	96
	Total	685	64	749	91
	Overall Total	1603	151	1754	91

All summations were made using only detected values; non-detects were treated as zero in summations. If all analytes in a group were not detected, a value of the greatest LOQ of any single analyte in the group was used as the total. All the remaining chlorinated pesticides were analyzed individually because detects of any of these compounds were rare and easily described individually in terms of their frequency of detection. Some individual PAHs analytes were presented when they were useful to illustrate pattern differences between sampling locations or matrices (e.g., embryo vs PEMD vs sediments). All raw data are summarized in supplemental tables in Appendix B: Chemistry Summary Statistics.

A small amount of contamination of samples by some chemicals from external sources occurred in the laboratory during the analysis phase and in the field during the deployment phase. All PAH and PCB data were corrected for analyte concentrations detected in solvent (or PEMD) blanks as follows. If the sample concentration for a given analyte exceeded 5x the detected concentration in the blank from the same batch run, the sample concentration was accepted and adjusted by subtracting the blank concentration. If the sample concentration was less than 5x the blank concentration, the sample analyte was considered not detected, and its value reported as zero. In the case of PEMDs, both a solvent blank and a cleaned, undeployed PEMD blank were analyzed, and the larger of the value for the two blanks was used for the comparison with sample concentrations.

In most cases, the goal of the study was to report the contaminant increment in field samples attributable to environmental exposure (i.e., removing the portion attributable to maternal transfer, and contamination during transport and deployment of field samples). To account for these anticipated sources, the two sampling media (embryos and PEMDs) were corrected for initial conditions in each medium. In all but one analyte in one analytical batch, the field deployment controls had higher concentration than ovaries. Field controls were evaluated, using a random sample of fertilized eggs from cages retained in the field, prior to deployment, to control for potential contamination related to the fertilization, transport, and deployment activities. In the case of PEMDs, field controls were handled the same as deployed PEMDs until the point of deployment, when they were retained. In the case of both eggs and PEMDs, the largest control concentration for the entire experiment was subtracted from sample analyte concentrations.

In the comparisons of PAH concentrations with cytochrome P4501A induction, we did not correct for maternal transfer or deployment contamination because the measured effects would have been related to total body burden of contaminants, regardless of the source. In all cases, however, these sources were small for \sum_{42} PAH concentrations at the higher end of the scale, exceeding 20 ng/g wet weight.

Statistical comparisons of contaminant concentrations were conducted primarily using Analysis of Variance (ANOVA). Assumptions of data normality and homoscedasticity were typically met with log-10 transformations of contaminant data. Proportion (survival) data were arcsine-square-root transformed to achieve homoscedasticity.

RESULTS

Herring Embryos

Σ_{42} PAHs, TPCBs, Σ_{11} PBDEs, and Organochlorine Pesticides (OCPs) were analyzed in 51 embryo samples in Port Gamble Bay and the Hood Head Reference area from Phases One through Three (Table 2). Dioxins and furans were analyzed in a subset of 18 samples (Table 2). PAHs were the most commonly detected and most abundant contaminant measured in the study, followed by TPCBs, Σ_{11} PBDEs, Σ_6 DDTs, dioxins, and furans (Figure 3). All other chlorinated pesticides including hexachlorobenzene, hexachlorocyclohexanes, chlordanes, dieldrin, endosulfans, mirex, and aldrin were either never detected in any sample, or were detected rarely, and at concentrations near to the limit of quantitation.

Table 2. Sample size by station location for collection (number of cages deployed) and chemical analyses (organics and dioxins). For all three phrases, fertilization success ranged from 79-100% (measured on day of deployment) and exposure duration was 10 days.

Phase	Important Dates	Site	# deployed/# analyzed for organics/# analyzed for dioxins	
			Eggs	PEMDs
One	2/19/14 ^a , 2/20/14 ^b , 2/21 – 3/3/14 ^c	Condition Control	1/1/0	2/2/0
		Hood Head Ref. site	4/0/0	0/0/0
		Mill Site North	5/3/0	5/5/0
		Mill Site South	5/3/0	5/5/0
		Northwest Shore	4/4/0	4/0/0
		Former Lease Area	4/3/0	4/3/0
		Head of Bay	5/3/0	5/3/0
		Center of Bay	4/0/0	4/0/0
		Southeast Shore	4/4/0	4/0/0
		Northeast Shore	5/4/0	5/3/0
Two	3/26/14 ^a , 3/27/14 ^b , 3/28 – 4/7/14 ^c	Hood Head Ref. site	5/5/3	3/3/0
		Center of Bay	5/5/3	3/3/0
Three	3/19/15 ^a , 3/20/15 ^b , 3/21 – 3/30/15 ^c	Mill Site North	4/4/3	-
		Mill Site South	4/4/3	-
		Former Lease Area	4/4/3	-
		Head of Bay	4/4/3	-
		<i>Phase One subtotal</i>	<i>41/25/0</i>	<i>33/21/0</i>
		<i>Phase Two subtotal</i>	<i>10/10/6</i>	<i>6/6/0</i>
		<i>Phase Three subtotal</i>	<i>16/16/12</i>	<i>-</i>
		Overall Total	67/51/18	39/27/0

^aadult herring collection and gonad resection, ^bfertilization, ^cexposure in Port Gamble Bay

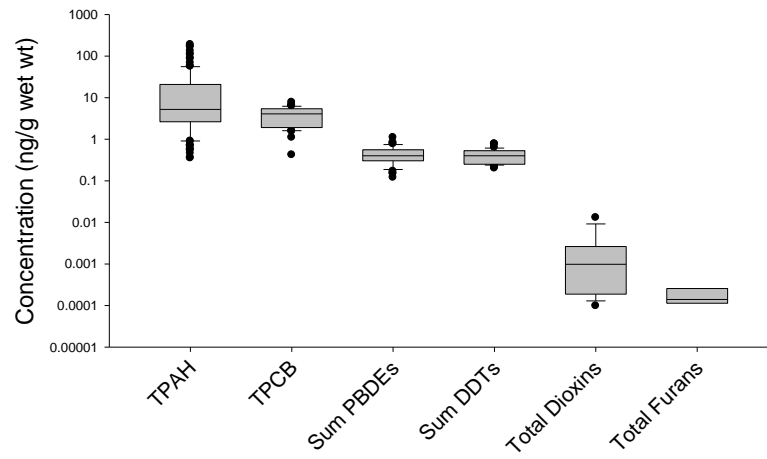


Figure 3. Box plots of four organic contaminants (n = 51), dioxins, and furans (n = 18) concentrations (ng/g wet weight) measured in herring embryo samples during Phases One through Three of the study

PAHs in Embryos

The greatest concentrations of Σ_{42} PAHs were observed in embryos deployed in the Mill Site North and Mill Site South areas, with concentrations ranging from 4.38 to 88 ng/g, wet wt. (North) and 2.8 to 110 ng/g, wet wt. (South). Variability in Σ_{42} PAH concentrations was high in both Mill Site areas (Figure 4), suggesting heterogeneous distribution of PAHs in these locations. Σ_{42} PAHs in both Mill Sites were significantly greater than Northwest Shore, Head of Bay, Southeast Shore, and Hood Head Reference areas (ANOVA of log-10 transformed Σ_{42} PAH by area, $p < 0.001$, $F_{43,35} = 6.76$, with a slight violation of the assumption of normality at $\alpha = 0.05$;

Tukey's pairwise multiple comparison test at $\alpha = 0.05$). Four of the 13 Mill Site samples (31%) exhibited Σ_{42} PAHs exceeding the 22 ng/g wet wt. no observable effects concentration (NOEC) suggested by Carls et al. (1999; Figure 5); these levels were also in the range of PAH concentrations where high embryo mortality was observed in another nearby Puget Sound embayment (West et al., 2014a). Several other samples from the Mill Site North, Former Lease Area, and Center of Bay areas had Σ_{42} PAHs between 10 and 20 ng/g wet wt., and all other samples from all other Port Gamble Bay sites exhibited Σ_{42} PAH less than 4.0 ng/g wet wt. Σ_{42} PAHs in four of the five Hood Head Reference area samples were not detected, and one Hood Head Reference sample had a Σ_{42} PAH concentration of 3.8 ng/g wet wt.

These results show contamination of embryos with PAHs primarily in the two Mill Sites, with sporadic, lower contamination in the Former Lease Area and Center of Bay. Embryos developing in these areas may exhibit sublethal effects from exposure to PAHs. Embryos from all other Port Gamble Bay areas appeared to be exposed to low levels of PAHs, with Σ_{42} PAH concentrations ranging from the LOQ to 4 ng/g wet wt. Most of these levels represent the summation of a number of detected values near to the LOQ, and these concentrations were indistinguishable from the reference area.

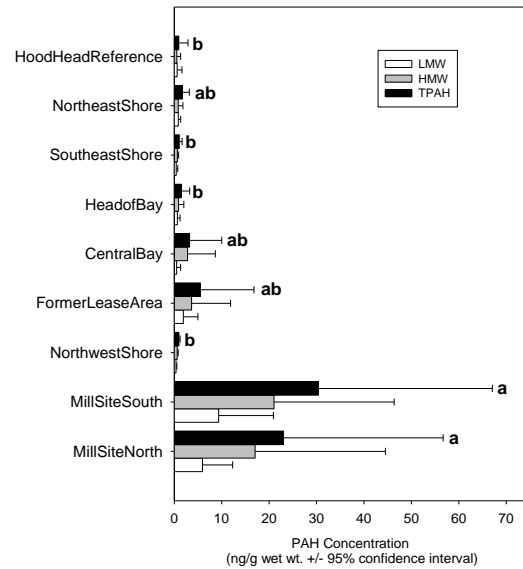


Figure 4. Mean low molecular weight (LMW), high molecular weight (HMW), and total PAH (TPAHs, or Σ_{42} PAH) concentrations (ng/g wet weight) measured in herring embryos during the three phases of the study. Similar lowercase letters signify no significant difference ($p > 0.05$) in Σ_{42} PAH (black bars) from the pairwise comparison results (ANOVA and Tukey's *post hoc* pairwise multiple comparisons).

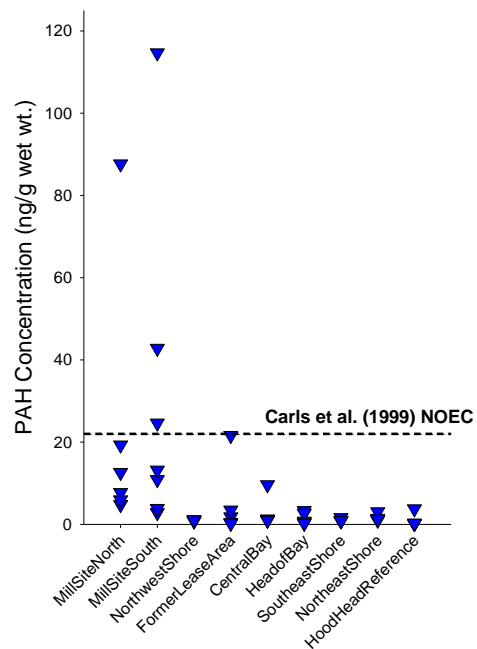


Figure 5. Σ_{42} PAHs concentrations (ng/g wet wt.) for all individual samples sites compared to Carls et al. (1999) No Observable Effects Concentration threshold of 22 ng/g wet wt. Four of the 13 Mill sites (31%) samples exceeded the NOEC.

PAHs in all embryo samples were dominated by three-ring phenanthrenes and anthracenes, 4-ring fluoranthenes, pyrenes and chrysenes, and several 5 and 6 ring compounds (Figure 6). Naphthalenes and fluorenes were rare in all samples except for both Mill Sites, and dibenzothiophenes were rare throughout. Within homolog groups, the greatest concentrations typically occurred at the parent analyte, with concentrations declining as degree of alkylation increased (i.e., parent > C₁ > C₂ > C₃ > C₄). The declining concentration of alkylated PAH analytes from parent through C₄ suggests that the source of PAHs observed in Port Gamble Bay was from combustion of fossil fuels or organic matter, or from highly weathered oil (Lima et al., 2005; Payne et al., 2003; Tobiszewski and Namieśnik, 2012).

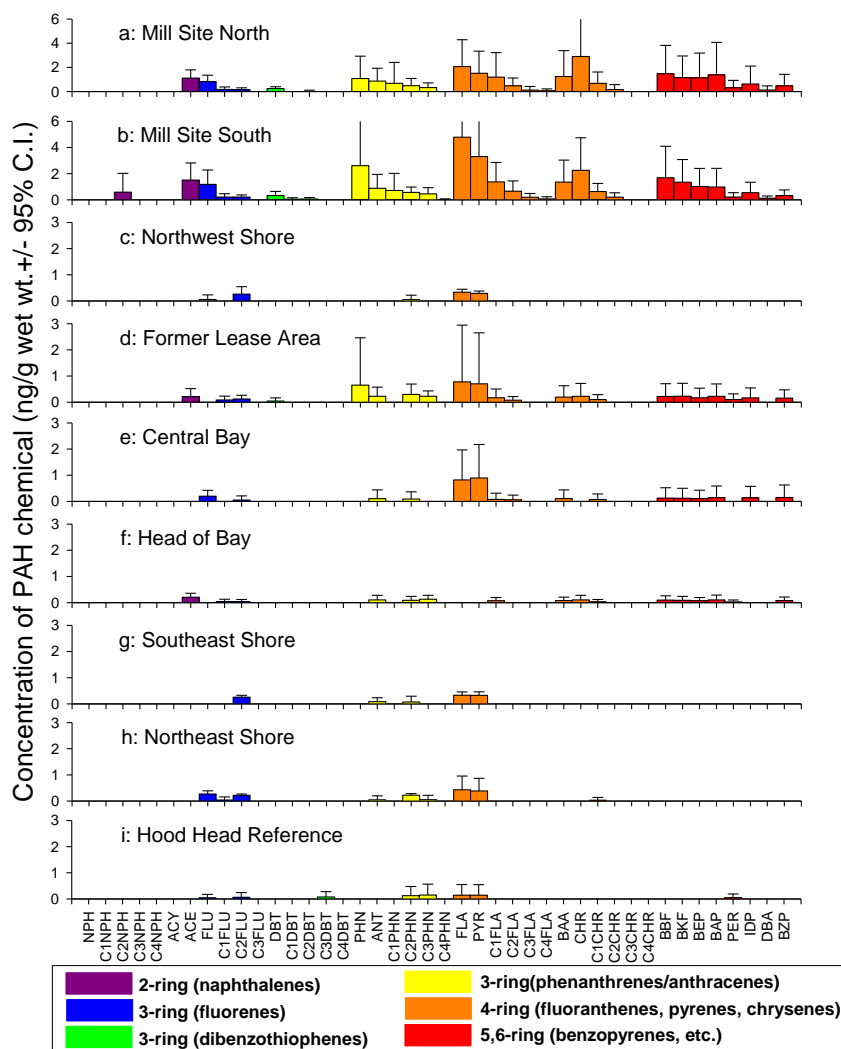


Figure 6. Mean PAH analytes ($\pm 95\%$ confidence intervals; ng/g wet weight) detected in herring embryos from eight areas within Port Gamble Bay and a reference area located at Hood Head. Bars are color coded by PAH analyte parent groups (see Table 3 for the names of the PAH acronyms used along the x-axis) with the low molecular weight PAHs on the left, increasing to the high molecular weight PAHs on the right.

Table 3. Sum of 42 PAHs measured in herring embryos.

Sum 42 PAHs

Low Molecular Weight	High Molecular Weight
Naphthalene (NPH)	Fluoranthene (FLA)
C1-naphthalenes (C1NPH)	Pyrene (PYR)
C2-naphthalenes (C2NPH)	C1-fluoranthenes/pyrenes (C1FLA)
C3-naphthalenes (C3NPH)	C2-fluoranthenes/pyrenes (C2FLA)
C4-naphthalenes (C4NPH)	C3-fluoranthenes/pyrenes (C3FLA)
Acenaphthylene (ACY)	C4-fluoranthenes/pyrenes (C4FLA)
Acenaphthene (ACE)	Benzo[<i>a</i>]anthracene (BAA)
Fluorene (FLU)	Chrysene (CHR) ^a
C1-fluorenes (C1FLU)	C1-benzanthracenes/chrysenes (C1CHR)
C2-fluorenes (C2FLU)	C2-benzanthracenes/chrysenes (C2CHR)
C3-fluorenes (C3FLU)	C3-benzanthracenes/chrysenes (C3CHR)
Dibenzothiophene (DBT)	C4-benzanthracenes/chrysenes (C4CHR)
C1-dibenzothiophene (C1DBT)	Benzo[<i>b</i>]fluoranthene (BBF)
C2-dibenzothiophene (C2DBT)	Benzo[<i>k</i>]fluoranthene (BKF) ^b
C3-dibenzothiophene (C3DBT)	Benzo[<i>e</i>]pyrene (BEP)
C4-dibenzothiophene (C4DBT)	Benzo[<i>a</i>]pyrene (BAP)
Phenanthrene (PHN)	Perylene (PER)
Anthracene (ANT)	Indeno[1,2,3- <i>cd</i>]pyrene (IDP)
C1-phenanthrenes/anthracenes (C1PHN)	Dibenz[<i>a,h</i>]anthracene (DBA) ^c
C2-phenanthrenes/anthracenes (C2PHN)	Benzo[<i>g,h,i</i>]perylene (BZP)
C3-phenanthrenes/anthracenes (C3PHN)	
C4-phenanthrenes/anthracenes (C4PHN)	

^acoelutes with triphenylene

^bcoelutes with benzo[*j*]fluoranthene

^ccoelutes with dibenz[*a,c*]anthracene

PCBs, PBDEs and Organochlorine Pesticides in Embryos

PCBs were detected in all samples, at low concentrations, with TPCBs ranging from approximately 0.75 (Southeast Shore) to 4.5 ng/g wet wt. (Hood Head Reference; Figure 7). The mean limit of quantitation (LOQ) for individual congeners was 0.25 ng/g wet wt. TPCB in embryos from both Mill Sites, Northeast Shore, and Central Bay were statistically indistinguishable from the Hood Head Reference area (ANOVA of log₁₀-transformed TPCB by area, $p < 0.001$, $F_{43,35} = 6.81$, with a minor violation of the normality assumption; Tukey's pairwise multiple comparison test, $\alpha = 0.05$). TPCB concentrations in embryos from all other locations were intermediate to the Hood Head Reference and Southeast Shore areas.

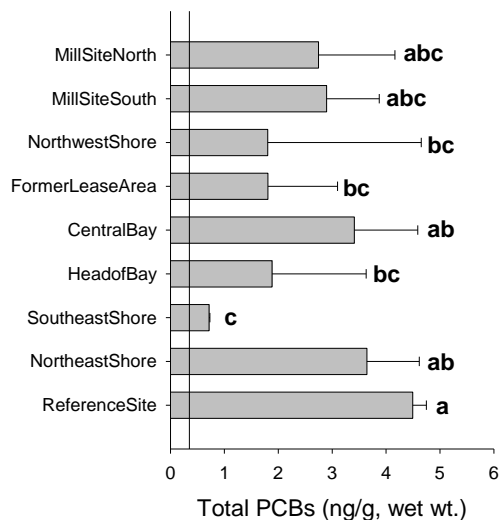


Figure 7. Mean total PCBs ($\pm 95\%$ confidence intervals) measured in herring embryos from all three phases of the study. Pairwise comparisons (ANOVA with Tukey's post hoc pairwise multiple comparison) are shown to the right of the error bars. Similar letters signify no significant difference ($p > 0.05$). The solid vertical line indicates the mean limit of quantitation for individual PCB congeners

Sum₆DDTs were detected in only 24 of 44 samples, and were low in all samples, with mean concentrations ranging from the LOQ (mean of 0.11 ng/g wet wt.) to 0.55 ng/g wet wt. Sum₆DDTs were statistically indistinguishable across all areas (Kruskal Wallis ANOVA of ranked Sum₆DDTs by area, $p = 0.048$, with Dunn's Multiple Pairwise Comparison, $p > 0.05$ for all pairwise comparisons; Figure 8).

PBDEs were detected in 26 of 44 samples, at low concentrations (from the mean LOQ of 0.11 to 0.38 ng/g wt. wt.), there was a pattern of higher concentrations at the Mill Sites (mean Sum₁₁PBDEs, 0.37 and 0.34 ng/g, wet wt. at the North and South sites, respectively compared to the Northeast Shore), with the remaining sites statistically indistinguishable from the Hood Head Reference area, with Σ_{11} PBDEs less than 0.2 ng/g wet wt.; ANOVA of log₁₀-transformed Σ_{11} PBDEs by Area, $p=0,002$, $F_{43,35}=3.90$; Tukey's pairwise multiple comparison test, $\alpha=0.05$; Figure 9).

Dioxins and Furans in Embryos

Dioxin and furan chemicals were rarely observed in embryo samples, and occurred in low concentrations when they were detected. No dioxin or furan congeners were detected in the ovary sample, suggesting that maternal transfer of these compounds to eggs did not occur. The areas sampled included the most contaminated areas (Mill Sites), three other locations distributed across Port Gamble Bay, and the Hood Head Reference area. Of the seventeen quantitated dioxin and furan congeners, only one (2,3,7,8-TCDD) was detected without censorship, in one egg sample (Table 4), from the North Mill Site area. The single uncensored analyte detected is the most toxic of this group, although its concentration was low (0.74 pg/g wet wt.). Seventeen other occurrences of detected congeners were censored with "J" flags, indicating that the chemical was

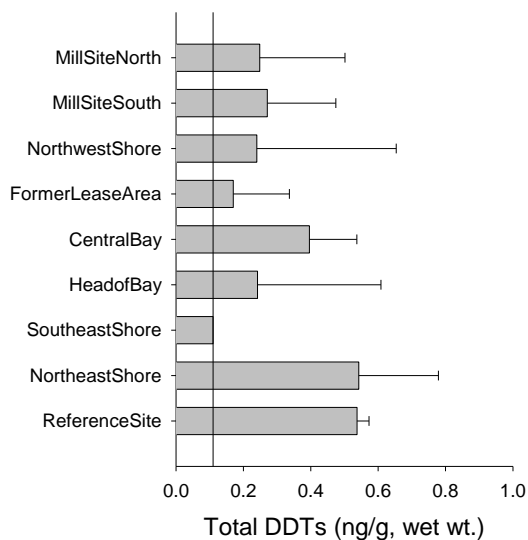


Figure 8. Mean Sum₆DDTs (Total DDTs; $\pm 95\%$ confidence intervals) measured in herring embryos from all three phases of the study. Pairwise comparisons are not shown because Sum₆DDTs were indistinguishable across all areas ($p \geq 0.05$). The solid vertical line indicates the mean limit of quantitation for individual DDT isomers.

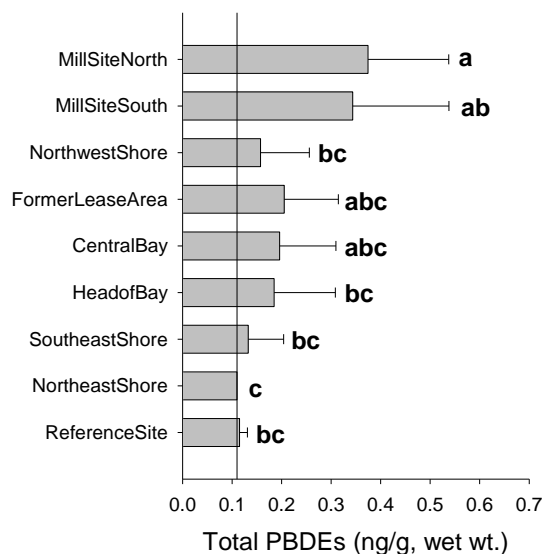


Figure 9. Mean Σ_{11} PBDE ($\pm 95\%$ confidence intervals) measured in herring embryos from all three phases of the study. Mean concentrations with different lowercase letters indicate significant differences, based on ANOVA of log₁₀-transformed Σ_{11} PBDE by Area ($\alpha = 0.05$), with Tukey's pairwise comparisons. The vertical solid line indicates the mean LOQ for individual PBDE congeners.

present, with a value greater than the quantitation limit (mean of 0.11 pg/g wet wt. for all congeners) and less than the lower method calibration limit. The concentration of J-censored values ranged from 0.11 to 3.0 pg/g wet wt. (Table 4).

Table 4. Dioxin and furan data from a total of 18 samples collected during Phase One and Two of the study

Class	Chemical	Number of Uncensored Detects	Number of J-flagged Detects	Range of concentration (pg/g wet wt.)
Dioxin	1,2,3,4,6,7,8-HPCDD	0	6	0.19-1.5
Dioxin	1,2,3,4,7,8-HXCDD	0	0	--
Dioxin	1,2,3,6,7,8-HXCDD	0	0	--
Dioxin	1,2,3,7,8,9-HXCDD	0	0	--
Dioxin	1,2,3,7,8-PECDD	0	0	--
Dioxin	2,3,7,8-TCDD*	1	1	0.11-0.74
Dioxin	OCDD	0	5	0.34-3.0
Furan	1,2,3,7,8-PCDF	0	0	--
Furan	1,2,3,4,6,7,8-HPCDF	0	1	0.026
Furan	1,2,3,4,7,8,9-HPCDF	0	0	--
Furan	1,2,3,4,7,8-HXCDF	0	0	--
Furan	1,2,3,6,7,8-HXCDF	0	0	--
Furan	1,2,3,7,8,9-HXCDF	0	0	--
Furan	2,3,4,6,7,8-HXCDF	0	0	--
Furan	2,3,4,7,8-PCDF	0	0	--
Furan	2,3,7,8-TCDF	0	3	0.12-0.20
Furan	OCDF	0	1	0.14

*detected at the North Mill Site

At least one total homolog group was detected in 14 of the 18 field samples, with at least one homolog total detected in each of the six sampled areas. All five dioxin groups (tetra-, penta-, hexa-, hepta-, and octa-chlorodibenzodioxins), and four furan groups (tetra-, penta-, hepta-, and octa-chlorodibenzofurans) were detected at least once. Six occurrences were censored as “J” values, with concentrations ranging from 0.099 to 0.14 pg/g wet wt. All uncensored concentrations occurred below 1.0 pg/g wet wt., except for two; one occurrence of total hepta-chlorodibenzodioxins each, at Mill Site South (13 pg/g wet wt.) and Mill Site North (2.8 pg/g wet wt.).

The concentration of the single detected occurrence of 2,3,7,8-TCDD, the most highly toxic of the dioxins tested (0.74 pg/g wet wt.) was more than 200 times lower than a lowest no observable effects concentration reported for this chemical in the early development of seven fish species (Elonen et al. 1998). Based on the low detection, low concentrations, and limited distribution of dioxins in herring embryos from Port Gamble Bay, these chemicals likely pose little risk to their health.

Survival to Near Hatching

Mean embryo survival to near hatch was 100% at the Hood Head Reference area, and was significantly greater than the eight pooled Port Gamble sampling areas, which exhibited a median 80% survival

(Mann Whitney Rank Sum Test, $p=0.009$). High survival at the reference area validates its utility as a comparison for the Port Gamble Bay locations. Survival within Port Gamble Bay areas ranged from 15% from one Northeast Shore site, to 100% from one Center of Bay site. Survival at the Center of Bay Areas (100%) was statistically indistinguishable from Hood Head Reference, and all other areas exhibited significantly lower survival, ranging from mean area estimates of 62% at the Northeast Shore, to 81% at Mill Site South (ANOVA of arc-sine-square root-transformed Survival by Area; $p < 0.001$, $F_{51,43} = 10.44$, Tukey's *post hoc* pairwise multiple comparison; Figure 10). Although both Mill Sites exhibited elevated PAHs and cytochrome P4501A induction, embryos from those locations exhibited similar survival to other Port Gamble areas. Embryos from the Northeast Shore area exhibited extremely high variability in survival, notably related to a single replicate where most embryos died (survival of 14%). Excluding that sample resulted in a survival of 74% for that area.

Cytochrome P4501A (CYP1A) Embryo Analysis

Mild induction of cytochrome P450 (mean Fold Change of CYP1A between two- and three-times the Hood Head Reference Area) was observed in embryos from three Port Gamble locations, Mill Site South, Mill Site North, and Former Lease Area (dark grey bar, Figure 11). Variability was high in the CYP1A response, resulting in little statistical significance to the differences reported here. CYP1A in these three locations was significantly greater than only the Center of Bay area (white bar, Figure 11), while CYP1A was statistically indistinguishable between all other areas (ANOVA of Fold Change by Area, $p < 0.001$, $F_{44,37} = 6.918$).

CYP1A induction increased with PAH exposure in embryos from one of the three areas where mean Fold Change was greater than 2 (linear regression of Fold Change by log-10 transformed TPAH for

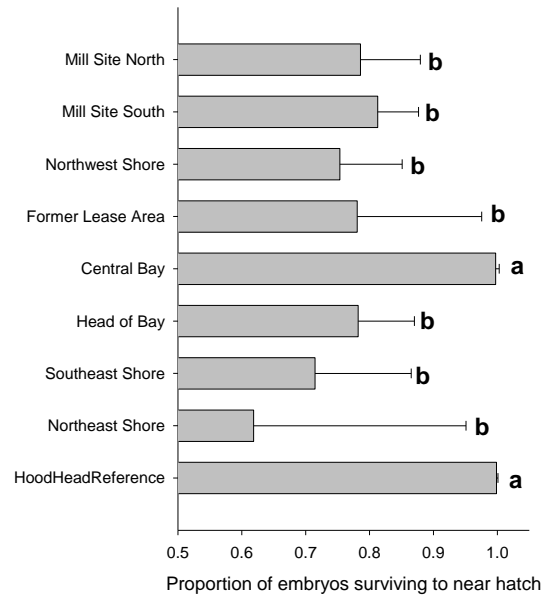


Figure 10. The mean proportions of embryos that survived to near hatch by cage location areas for all three phases of the study. Pairwise comparisons (ANOVA and Tukey's *post hoc* pairwise multiple comparisons) are shown to the right of the error bars. Similar letters signify no significant difference ($p > 0.05$).

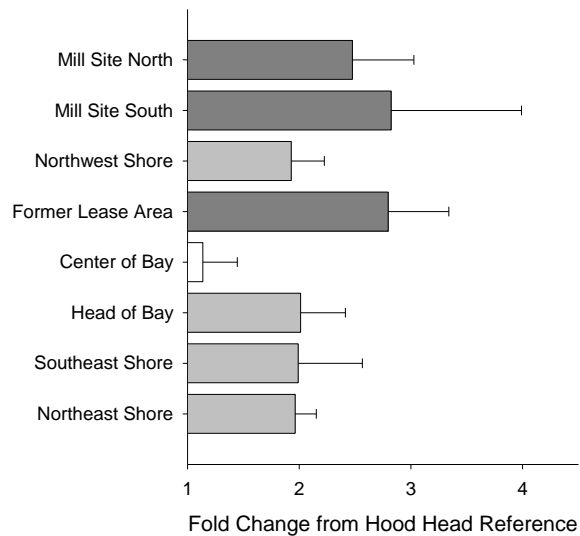


Figure 11. Mean Fold Change of CYP1A induction (±95% confidence intervals) measured at eight areas within Port Gamble Bay relative to the Hood Head reference area (y axis origin) during Phase One of the study. Dark grey bars signify the highest induction, light grey bars signify an intermediate induction, and the white bar signifies the lowest induction of CYP1A.

Mill Site South, $p = 0.0009$, $r^2 = 0.91$; filled stars in Figure 12). Embryos from Mill Site North (open squares) and Former Lease Area (filled triangles) and all the areas with lower Fold Change and TPAH exposure (filled circles) failed to exhibit a relationship between CYP1A induction and PAH exposure.

PEMDs

Overall, the geographic distribution of Σ_{42} PAHs in PEMDs was similar to Σ_{42} PAHs in embryos, with significantly greater concentrations observed at the Mill Sites than most other areas (ANOVA of log₁₀-transformed Σ_{42} PAHs by Area, $p < 0.001$, $F_{24,18} = 11.432$; Figure 13). The mean concentration of Σ_{42} PAHs ranged from 31 to 77 ng/g wet wt., at the Reference, Former Lease Area, Central Bay, Head of Bay, and Northeast Shore areas to 470 and 510 ng/g wet wt. at the South and North Mill Sites. Σ_{42} PAHs at both Mill Site areas were characterized by high variability, and Σ_{42} PAHs in embryos from individual locations within these two sites ranged from 120 to 1300 ng/g wet wt.

Σ_{42} PAH concentration in PEMDs was strongly correlated with Σ_{42} PAHs in co-deployed embryos, all of which were exposed for ten days *in situ* (linear regression of Σ_{42} PAHs in embryos versus PEMDs; $r^2 = 0.94$; slope coefficient = 0.023, $p < 0.001$; intercept not significant, $p = 0.61$; Figure 14a). The regression model results were based on combining fourteen PEMDs and embryo pairs from the current study with seven pairs that were deployed concurrently at another location, Quilcene Bay, approximately 45 km away in Hood Canal (West et al., in prep). The Quilcene Bay samples were deployed at a location where creosote-treated pilings had been recently removed, and resulted in substantially higher Σ_{42} PAH concentrations in embryos and PEMDs (up to 80 and 3,600 ng/g

wet wt., respectively), whereas the greatest Σ_{42} PAHs in embryos from Port Gamble Bay was approximately 25 ng/g wet wt. Quilcene Bay results were

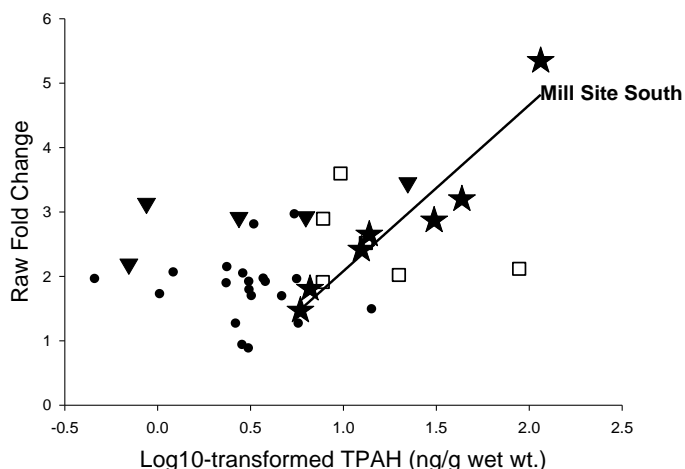


Figure 12. Comparison of CYP1A induction (x-axis, raw Fold Change) to the total PAHs (ng/g wet wt.) measured in the herring embryos during Phase One of the study. Linear regression of raw Fold Change by log₁₀-transformed Σ_{42} PAH concentration for the Mill Site South Area (star symbols).

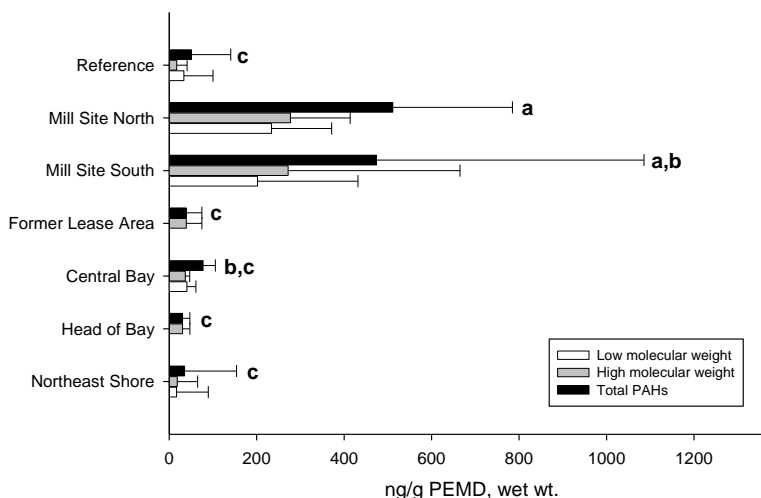


Figure 13. Mean concentration of low molecular weight, high molecular weight, and total PAHs (Σ_{42} PAHs; ng/g wet weight) measured in PEMDs from Phase One of the study. Pairwise comparisons results (ANOVA and Tukey's *post hoc* multiple pairwise comparisons) are shown to the right of the error bars. Similar letters signify no significant differences ($p > 0.05$)

included here to increase sample size, however, the three values with greatest Σ_{42} PAHs in embryos and PEMDs were from Quilcene Bay (Figure 14a), and greatly influenced the slope coefficient resulting from this linear regression (0.023).

Removing Quilcene Bay samples from the model still resulted in a positive correlation between Σ_{42} PAHs in PEMDs and embryos, however the model explained less variation ($r^2 = 0.65$; Figure 14b), and more variation was left unexplained without the Quilcene data. Moreover, the restricted regression model exhibited a 50% reduction in the slope (0.013). In this latter Port Gamble Bay-only model, the relationship between PEMDs and embryos was more strongly influenced by variability in low concentrations nearer to the limits of quantitation.

The paired PEMD and embryo samples exhibited some notable differences in the relative abundance of individual PAH analytes, as illustrated at three areas of high (Mill Site South), medium (Mill Site North), and low (Hood Head Reference area) PAH concentration. The proportion of each analyte relative to Σ_{42} PAH is shown in Figures 15a –f). No PAHs were detected in embryos from the low-concentration area (Hood Head reference, Figure 15f), suggesting that overall, PEMDs may be more sensitive attractors than embryos in a field setting (or, alternatively, that embryos are able to metabolize small burdens of PAH).

Two-ring naphthalene compounds (i.e. NPH and C1-4NPH) were detected only in PEMDs (not embryos) from the three sites (Figure 15a, c, and e). Three-ring PAH compounds were common in both embryos and PEMDs (except for Hood Head Reference embryos); fluorenes were more abundant in embryos than PEMDs, dibenzothiophenes were rare in all samples, and phenanthrenes were relatively more abundant in PEMDs than embryos. Both PEMDs and embryos showed similar proportions of four-ring compounds (fluoranthenes, pyrenes, and chrysenes) in locations with the highest overall Σ_{42} PAHs (Mill Site South Figure 15a and b), however the proportion of these compounds was notably higher in PEMDs than

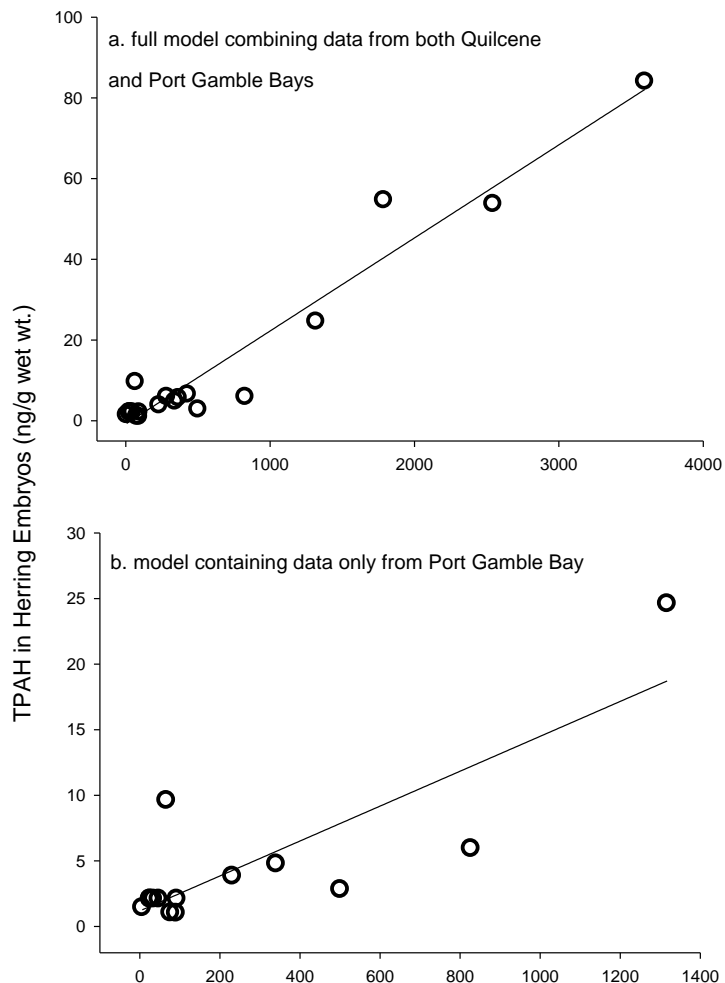


Figure 14. Σ_{42} PAHs measured in herring embryos compared to Σ_{42} PAHs in PEMDs (ng/g wet weight) exposed at (a) locations within Port Gamble Bay and a concurrent study that took place in Quilcene Bay and (b) within Port Gamble Bay only.

embryos at locations with intermediate Σ_{42} PAH levels (Mill Site North; Figure 15c and d). Embryos exhibited a greater proportion of 5- and 6-ring compounds than PEMDs at both Mill Sites.

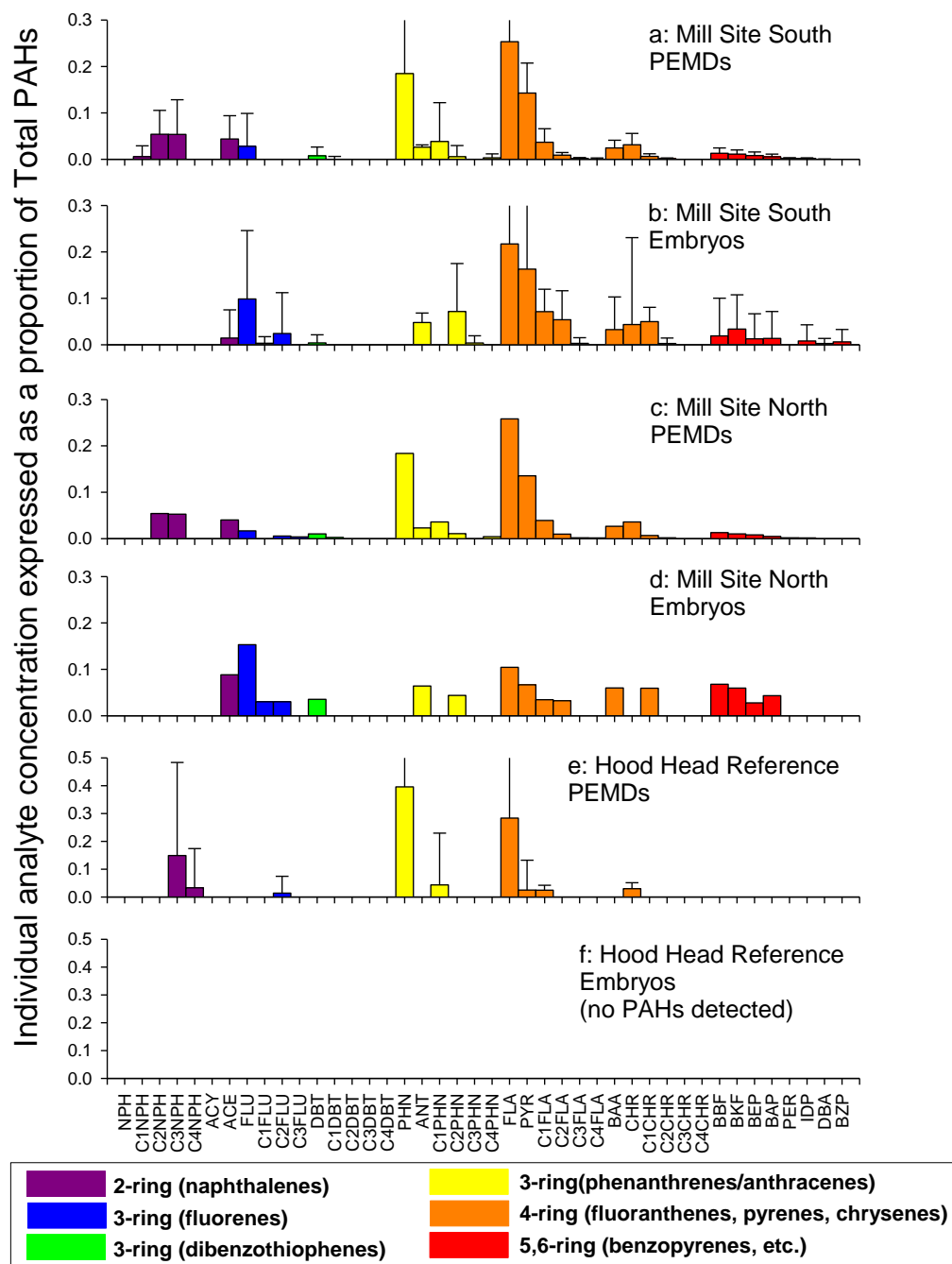


Figure 15. Relative abundance of individual PAH chemicals in both embryos and PEMDs from three selected areas representing high, medium, and low concentration of PAHs overall. Bars are color-coded by their ring structure (see color legend). Table 3 shows names of the PAH acronyms used along the x-axis. Molecular weight increases in PAH compounds from left to right along the x axis.

Σ_{42} PAH concentrations in PEMDs and in herring embryos were positively, and moderately well correlated with TPAH in sediments, as indicated by linear regressions of log-10-transformed PEMD and embryo data by sediment PAHs (Figure 16a and b). Both models had highly significant correlations ($p = 0.0003$ and 0.0002 , respectively), and explained a moderate amount of variation in the PEMDs and embryos (57% and 74%, respectively).

PEMDs and embryos were deployed for only a 10 day period and were meant to mimic the potential exposure time of developing herring embryos, whereas sediments exhibit conditions that may have been integrated over a much longer time period. In addition, sediment samples were taken approximately four months after the PEMDs were deployed, and so they may not have represented entirely equivalent conditions. Given these differences in exposure timing it is not surprising to see some variability in the relationship shown here.

Contaminants in Adult Herring

We compared the concentration of TPCBs, Sum_6 DDTs, and Sum_{11} PBDEs in adult male herring from the Port Gamble Bay spawning stock with same-age male herring from two other stocks funded in this study (Quilcene Bay and Holmes Harbor), and four stocks routinely measured biannually by PSEMP. The PSEMP herring stocks were sampled in 2012 and herring from the current study in 2014. In addition we compared the presence of biliary metabolites of two PAH compounds, phenanthrene and benzo[a]pyrene, in herring representing six Puget Sound herring stocks, including Port Gamble Bay stock.

Herring from the Port Gamble Bay stock exhibited the lowest concentration of TPCBs and Sum_{11} PBDEs among all seven stocks (ANOVA of log-transformed PCBs or PBDEs by Stock, $p < 0.001$, for both comparisons, $F_{60,54} = 132$ and 113 , respectively), with mean PCB and Σ_{11} PBDE concentrations of 28 and 4.5 ng/g wet wt., respectively (Figure 17a and c). TPCBs and Sum_{11} PBDEs were roughly 9 and 13 times higher in adult herring from the main Puget Sound basins (Port Orchard and Squaxin) than other stocks,

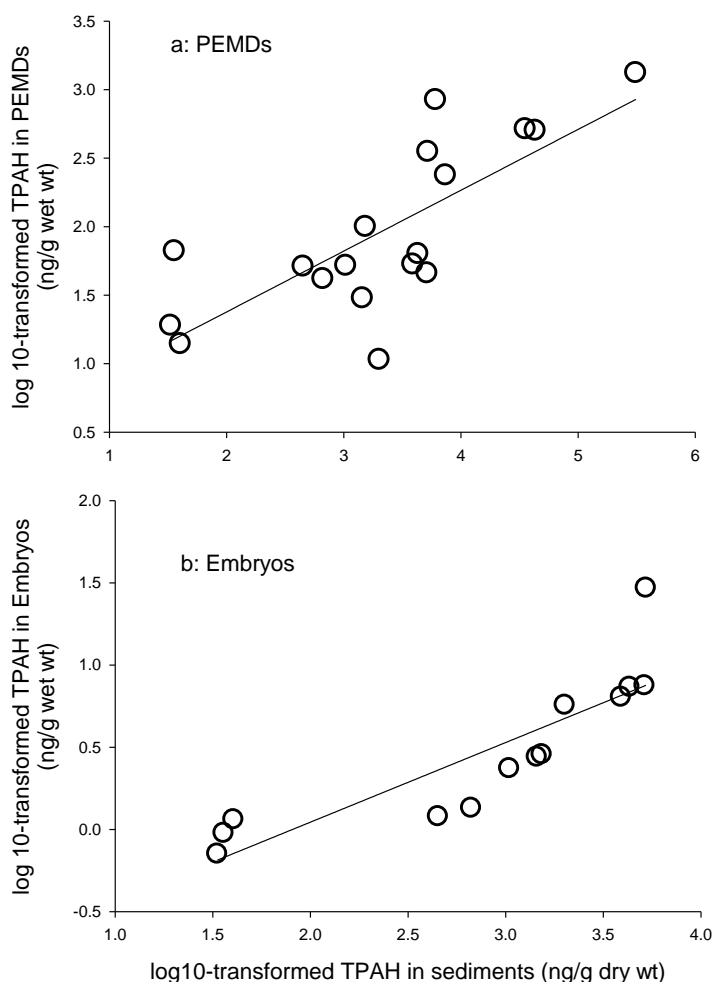


Figure 16. Σ_{42} PAH concentrations (ng/g wet wt.) measured in PEMDs (a) and herring embryos (b) compared to Σ_{42} PAH (ng/g wet wt.) measured in sediment at the same locations from Phase One and Two of the study

including Port Gamble Bay stock. Σ_6 DDTs were also low in herring from Port Gamble Bay (6.2 ng/g wet wt.), although statistically indistinguishable from two other stocks, Quilcene Bay and Cherry Point (7.3 and 7.1 ng/g, wet wt., respectively). Sum_6 DDTs in herring from Port Gamble Bay were roughly one-third the concentration of Port Orchard and Squaxin fish (Figure 17b).

Because PAHs are rapidly transformed by metabolic activities in most vertebrates (including fish), it is difficult to measure PAHs directly in fish exposed to these chemicals. Metabolites of phenanthrene- and benzo[a]pyrene-like compounds, commonly referred to as PHN and BaP equivalents, are often used as an indicator of PAH exposure in fish. PHN and BaP equivalents were detected in the bile of all adult herring sampled in this study (Figure 18), indicating exposure to PAHs in all

herring sampled in this study. The health implication of these exposures is difficult to evaluate, however these results show that herring from Port Gamble Bay stock experienced lower exposure (roughly one-half) to both PHN and BaP equivalents, than Port Orchard herring, and they were statistically indistinguishable from other herring stocks with the lowest PHN and BaP exposures (ANOVA of PHN or BaP equivalents by Area, $p < 0.001$ for both tests, with $F_{75,71} = 26.9$ and 24.1 for PHN and BaP; Tukey's multiple pairwise comparison used *post hoc* to evaluate the statistical significance of area differences in PHN and BaP equivalents).

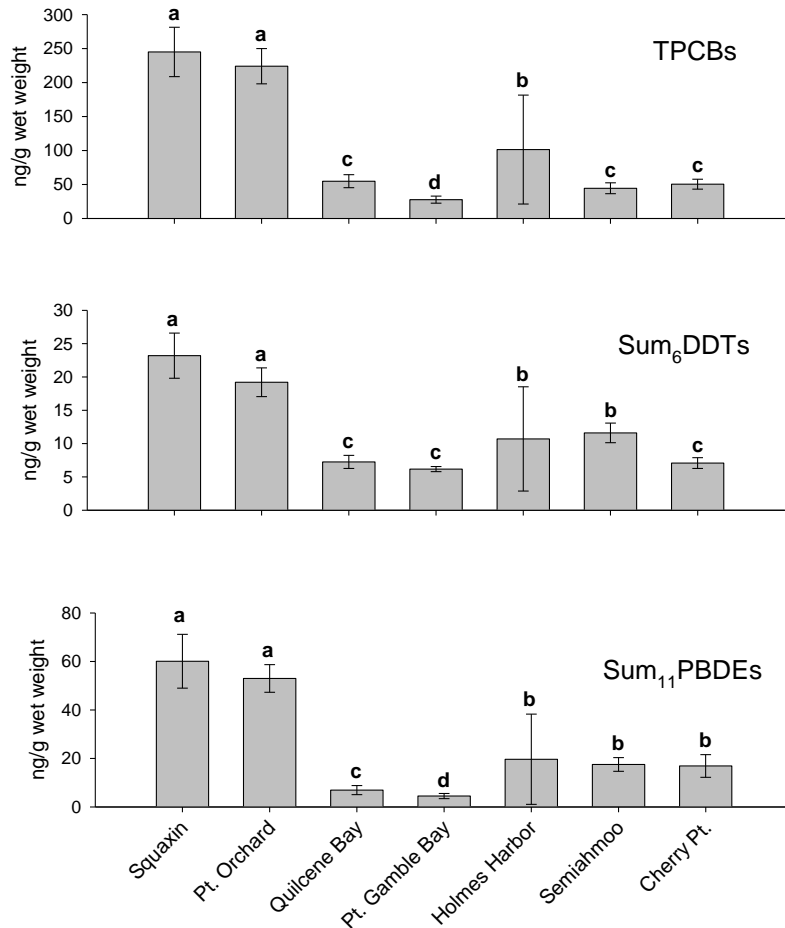


Figure 17. Mean concentrations ($\pm 95\%$ confidence intervals) of (a) TPCBs, (b) Σ_6 DDTs, and (c) Σ_{11} PBDEs (ng/g wet wt.) measured in whole body male herring collected from seven different Puget Sound stocks. Pairwise comparison results (ANOVA and Tukey's *post hoc* multiple pairwise comparisons) are shown above the error bars. Stocks with similar letters are statistically indistinguishable from each other ($p > 0.05$).

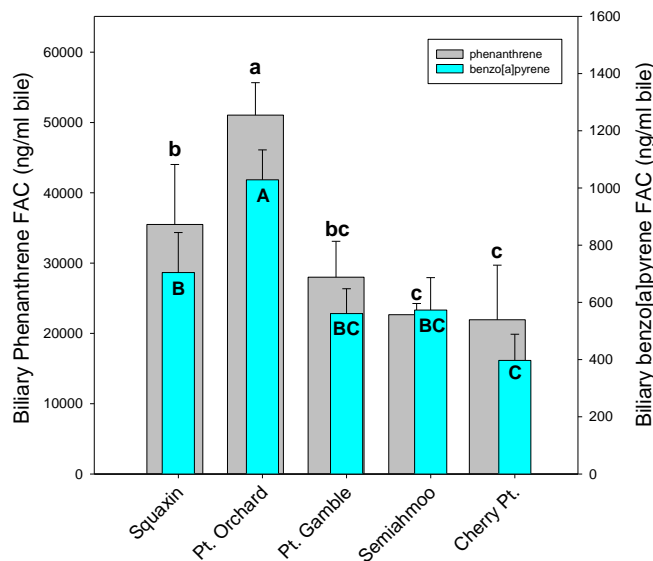


Figure 18. Mean concentrations (ng/ml bile; $\pm 95\%$ confidence intervals) of phenanthrene and benzo[a]pyrene measured in bile collected from adult herring. Results of pairwise comparisons (ANOVA and Tukey's *post hoc* multiple pairwise comparisons) between herring stock locations are located above the gray bars for phenanthrene and within the blue bars for benzo[a]pyrene. Similar letters signify no significant difference ($p > 0.05$).

CONCLUSIONS

Overall, we were successful in meeting the goals and objectives of this study. In particular, we used manually spawned herring embryos to provide an *in situ*, broad spatial scale, and real-time observation of the potential chemical exposure naturally spawned herring may experience in Port Gamble Bay. We confirmed the suspicion from previous studies that PAHs are the most abundant of potentially harmful chemicals spawned embryos may encounter in the bay. The contaminant levels described herein can be used as a baseline against which success of cleanup or remediation efforts be evaluated.

In particular, PAHs were most widely detected and measured in greatest concentration in herring embryos from the two Mill Sites, and the Former Lease Area, areas which had been identified as contaminated with PAHs (based on previous sediment sampling). PAH levels in some of the embryos from these locations exceeded a health effects threshold, so one might reasonably conclude that eggs spawned in these areas face a greater risk of lethal and sublethal effects than eggs spawned elsewhere in Port Gamble Bay. The induction of cytochrome P4501A, the enzyme notably associated with PAH exposure, indicated that the metabolic detoxification system in embryos from the South Mill Site, in particular was activated. Although this does not in itself imply harm to the animals, it indicates their detoxification systems recognized the chemicals, and they were actively processing them. The high spatial variability of PAHs in embryos within these sites suggests contamination is heterogeneous within these areas.

Hatching success was largely inconclusive across the spatial scale of the area replicate cages, with respect to contaminant exposure; there were no differences in success between the eight areas within the bay, and there were no significant correlations between survival and contaminant exposure. However, the high (100%) survival of the embryos from the reference area outside Port Gamble Bay indicate that (a) the manual spawning and deployment methods are valid and robust, and (b) there was a Reference Area versus Bay effect (slightly lower survival in the Bay) that we could not attribute to contaminants. That is, embryos survived slightly better outside the Bay than inside, but the difference was slight.

Although transplanting manually spawned herring embryos to desired locations provides a great degree of control in field studies, and provides biologically relevant information on exposure and effects in the animal of interest, this method is limited by several key factors. Successful deployment requires success in obtaining sufficient gametes of adequate quality, manual spawning with a high fertilization rate, and lack of inclement weather for deployment and retrieval activities. In addition, using manually spawned embryos as field subjects limits the timing of field work to the natural herring spawning window.

In this study, we evaluated the efficacy of PEMDs as a proxy for developing herring embryos, for estimating PAH exposure of embryos *in situ*. Deployment, retrieval, and analysis of the polyethylene membrane devices (PEMDs) were highly successful. We recovered all PEMDs, and their PAH profiles were largely congruent with their co-deployed eggs, with a few exceptions noted herein. The concentration of PAHs in PEMDs correlated well with PAHs in embryos, although PAH concentrations were approximately 30 times greater than embryos. Further lab-controlled studies are needed to more accurately calibrate this relationship and evaluate its full potential.

Although some other (non-PAH) chemical contaminants such as PCBs, PBDEs, DDTs, dioxins, and furans occurred in some embryo samples, their levels were largely low enough to conclude lower risk regarding embryo health.

The relative abundance, or fingerprint, of PAHs suggested a pyrogenic, or highly weathered petroleum signal. This is consistent with the putative sources of PAHs in the bay, namely creosote pilings, and a long history of burning wood in the area. There was little evidence in embryos or PEMDs to implicate petrogenic sources, such as recent oil spills.

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Appendix A: Cage Locations for Phase One, Two, and Three

Table A 1. Locations of cages deployed during Phase One of the study. Cages were deployed 2/21/2014 and retrieved 3/3/2014.

Site Name	Cage #	Latitude	Longitude
Reference Site 2	255	47.8834	-122.6168
Reference Site 3	253	47.8820	-122.6181
Reference Site 4	252	47.8814	-122.6184
Reference Site 5	254	47.8799	-122.6190
Mill Site North 1	217	47.8574	-122.5807
Mill Site North 2	218	47.8566	-122.5807
Mill Site North 3	215	47.8563	-122.5801
Mill Site North 4	214	47.8563	-122.5794
Mill Site North 5	213	47.8561	-122.5790
Mill Site South 1	239	47.8548	-122.5796
Mill Site South 2	235	47.8544	-122.5810
Mill Site South 3	237	47.8536	-122.5817
Mill Site South 4	240	47.8525	-122.5816
Mill Site South 5	238	47.8502	-122.5822
Northwest Shore 2	243	47.8467	-122.5828
Northwest Shore 3	242	47.8447	-122.5837
Northwest Shore 4	241	47.8422	-122.5829
Northwest Shore 5	244	47.8405	-122.5836
Former Lease Area 2	270	47.8324	-122.5839
Former Lease Area 3	269	47.8306	-122.5834
Former Lease Area 4	272	47.8283	-122.5838
Former Lease Area 5	273	47.8257	-122.5837
Head of Bay 1	264	47.8212	-122.5798
Head of Bay 2	266	47.8207	-122.5777
Head of Bay 3	267	47.8210	-122.5748
Head of Bay 4	263	47.8202	-122.5724
Head of Bay 5	265	47.8206	-122.5687
Southeast Shore 1	227	47.8345	-122.5673
Southeast Shore 2	231	47.8322	-122.5683
Southeast Shore 3	234	47.8302	-122.5695
Southeast Shore 4	233	47.8279	-122.5684
Central Bay 1	230	47.8357	-122.5740
Central Bay 2	258	47.8337	-122.5742
Central Bay 3	245	47.8317	-122.5749
Central Bay 4	260	47.8299	-122.5749
Northeast Shore 1	248	47.8518	-122.5726
Northeast Shore 2	250	47.8508	-122.5726
Northeast Shore 3	251	47.8483	-122.5723
Northeast Shore 4	247	47.8462	-122.5716
Northeast Shore 5	246	47.8444	-122.5693

Table A 2. Locations of cages during Phase Two of the study. Cages were deployed 3/28/2014 and retrieved 4/7/2014.

Site Name	Cage #	Latitude	Longitude
Reference Site 1	83	47.8903	-122.6235
Reference Site 2	86	47.8900	-122.6265
Reference Site 3	85	47.8899	-122.6295
Reference Site 4	88	47.8899	-122.6328
Reference Site 5	89	47.8903	-122.6368
Central Bay 1	72	47.8358	-122.5758
Central Bay 2	81	47.8335	-122.5745
Central Bay 3	70	47.8310	-122.5749
Central Bay 4	69	47.8290	-122.5758
Central Bay 5	78	47.8265	-122.5765

Table A 3. Locations of cages during Phase Three of the study. Cages were deployed 3/21/2015 and retrieved 3/30/2015.

Site Name	Cage #	Latitude	Longitude
Mill Site North	297	47.8565	-122.5807
Mill Site North	287	47.8564	-122.5806
Mill Site North	292	47.8563	-122.5800
Mill Site North	274	47.8563	-122.5795
Mill Site South	299	47.8549	-122.5792
Mill Site South	283	47.8547	-122.5799
Mill Site South	296	47.8545	-122.5807
Mill Site South	261	47.8543	-122.5812
Former Lease Area	286	47.8328	-122.5844
Former Lease Area	285	47.8309	-122.5835
Former Lease Area	284	47.8281	-122.5837
Former Lease Area	289	47.8254	-122.5834
Head of Bay	288	47.8214	-122.5804
Head of Bay	278	47.8209	-122.5781
Head of Bay	279	47.8209	-122.5751
Head of Bay	281	47.8206	-122.5684

Appendix B: Chemistry Summary Statistics

Table B 1. Summary of organic contaminants (ng/g wet weight) and mean lipids (%) measured in embryos exposed at each of the eight areas within Port Gamble Bay and the reference site at Hood Head. The total number of samples analyzed (n) is reported in parentheses beneath each location. In cases where all analytes for a summed group were less than the limit of quantitation (LOQ), the summation value used was the highest reported LOQ for any analyte for the area. Ref. = Hood Head reference site, MSN = Mill Site North, MSS = Mill Site South, NWS = Northwest Shore, FLA = Former Lease Area, HOB = Head of Bay, CB = Central Bay, SES = Southeast Shore, NES = Northeast Shore

	(n)	Ref. (4)	MSN (6)	MSS (7)	NWS (4)	FLA (5)	HOB (5)	CB (5)	SES (4)	NES (4)
Lipids (%)	Mean	0.47	0.51	0.44	0.35	0.37	0.47	0.33	0.26	0.26
TPCBs	n detects	4	6	7	4	5	5	5	3	4
	Minimum	4.4	1.6	1.6	0.56	1.1	0.72	2.1	0.25	3.0
	Maximum	4.7	4.8	4.4	4.5	3.6	4.3	4.5	0.72	4.5
	Mean	4.5	2.7	2.9	1.8	1.8	1.9	3.4	0.60	3.6
	Std. Dev.	0.16	1.4	1.1	1.8	1.0	1.4	0.95	0.24	0.61
Sum ₆ DDTs	n detects	4	3	5	1	1	1	5	0	4
	Minimum	0.52	0.052	0.060	0.11	0.11	0.11	0.28	0.11	0.43
	Maximum	0.57	0.61	0.56	0.63	0.41	0.77	0.53	0.11	0.76
	Mean	0.54	0.25	0.27	0.24	0.17	0.24	0.40	0.11	0.54
	Std. Dev.	0.022	0.24	0.22	0.26	0.13	0.30	0.11	0	0.15
Sum ₁₋₁₁ PBDEs	n detects	1	6	5	2	4	4	3	1	0
	Minimum	0.11	0.22	0.11	0.11	0.11	0.11	0.11	0.11	0.11
	Maximum	0.13	0.56	0.64	0.24	0.31	0.35	0.33	0.20	0.11
	Mean	0.12	0.38	0.34	0.16	0.21	0.19	0.20	0.13	0.11
	Std. Dev.	0.010	0.16	0.21	0.062	0.088	0.10	0.092	0.045	0

Table B 2. Summary of PAHs (ng/g wet weight) and mean lipids (%) measured in embryos exposed at each of the eight areas within Port Gamble Bay and the reference site at Hood Head. The total number of samples analyzed (n) is reported in parentheses beneath each location. In cases where all analytes for a summed group were less than the limit of quantitation (LOQ), the summation value used was the highest reported LOQ for any analyte for the area. Ref. = Hood Head reference site, MSN = Mill Site North, MSS = Mill Site South, NWS = Northwest Shore, FLA = Former Lease Area, HOB = Head of Bay, CB = Central Bay, SES = Southeast Shore, NES = Northeast Shore

		Ref.	MSN	MSS	NWS	FLA	HOB	CB	SES	NES
	(n)	5	6	7	4	5	5	4	4	4
Lipids (%)	Mean	0.48	0.51	0.44	0.35	0.33	0.44	0.37	0.26	0.26
LMWPAHs	n detects	2	6	7	4	4	4	3	4	4
	Minimum	0.23	1.5	0.89	0.21	0.20	0.23	0.22	0.25	0.57
	Maximum	2.1	18	36	0.49	6.2	1.3	1.3	0.72	1.3
	Mean	0.61	5.95	9.4	0.37	1.9	0.65	0.51	0.41	0.85
	Std. Dev.	0.82	6.1	12	0.12	2.5	0.47	0.53	0.21	0.32
HMWPAHs	n detects	1	6	7	4	2	2	4	4	4
	Minimum	0.25	2.5	1.9	0.47	0.25	0.25	0.77	0.56	0.45
	Maximum	1.7	70	79	0.74	15	2.0	8.3	0.9	1.8
	Mean	0.54	17	21	0.62	3.7	0.90	2.8	0.65	0.85
	Std. Dev.	0.65	26	27	0.13	6.6	0.90	3.7	0.17	0.61
TPAHs	n detects	2	6	7	4	4	4	4	4	4
	Minimum	0.23	4.8	2.8	0.78	0.25	0.25	0.99	0.83	1.0
	Maximum	3.8	88	110	1.1	22	3.3	9.6	1.6	3.1
	Mean	0.95	23	30	0.99	5.5	1.5	3.2	1.1	1.7
	Std. Dev.	1.6	32	40	0.16	9.1	1.4	4.3	0.38	0.93

Table B 3. Summary of PAHs (ng/g wet weight) measured in PEMDs exposed at six areas within Port Gamble Bay and the reference site at Hood Head. The total number of samples analyzed (n) is reported in parentheses beneath each location. In cases where all analytes for a summed group were less than the limit of quantitation (LOQ), the summation value used was the highest reported LOQ for any analyte for the area. Ref. = Hood Head reference site, MSN = Mill Site North, MSS = Mill Site South, FLA = Former Lease Area, HOB = Head of Bay, CB = Central Bay, NES = Northeast Shore

	(n)	Ref. (3)	MSN (5)	MSS (5)	FLA (3)	HOB (3)	CB (3)	NES (3)
LMWPAHs	n detects	3	5	5	0	0	3	1
	Minimum	14	120	45	0.50	0.50	31	0.50
	Maximum	64	390	490	0.50	0.50	48	51
	Mean	33	230	200	0.50	0.50	40	17
	Std. Dev.	27	110	180	0	0	8.6	29
HMWPAHs	n detects	3	5	5	3	3	3	3
	Minimum	12	152	74	22	25	34	6
	Maximum	28	430	830	47	38	41	40
	Mean	17	280	270	39	30	37	18
	Std. Dev.	9.2	110	320	14	6.8	3.8	19
TPAHs	n detects	3	5	5	3	3	3	3
	Minimum	26	270	120	22	25	66	6
	Maximum	92	830	1300	47	38	89	91
	Mean	51	510	470	39	30	77	35
	Std. Dev.	36	220	490	14	6.8	12	48