The 2007 *Cosco Busan* oil spill: Assessing toxic injury to Pacific herring embryos and larvae in the San Francisco estuary

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Summary

On November 7, 2007 the container ship *Cosco Busan* collided with a tower supporting the San Francisco Bay Bridge spilling roughly 54,000 gallons of bunker fuel into the Bay. The spill contaminated the shoreline adjacent to North Central Bay areas expected to be major spawning grounds for Pacific herring in the following months, based on the preceding decade of surveys. Based on experience following the 1989 *Exxon Valdez* spill, it was anticipated that contamination of the intertidal and shallow subtidal zones with *Cosco Busan* bunker oil could result in toxic injury to early life history stages of Pacific herring. Because of the relative ease of collecting herring spawn samples and a strong scientific understanding of the impacts of oil to herring embryos, this species was also chosen for study as a surrogate for other ecologically important fish species that utilize the intertidal and shallow subtidal for spawning. The aims of this study were to (1) assess and compare the biological responses of herring embryos and larvae that incubated adjacent to oiled shorelines with those incubated adjacent to reference non-oiled sites in the North Central Bay; (2) characterize the exposure of herring embryos to polycyclic aromatic hydrocarbons (PAHs) potentially derived from *Cosco Busan* oil.

To assess the biological responses of herring embryos, two approaches were taken. The primary approach was to sample herring embryos that were naturally deposited and fertilized and attached to marine vegetation along oiled shorelines. Because it was unknown where or even whether herring would spawn at oiled sites in the 2007-2008 season, a second approach used laboratory-fertilized caged embryos incubated on moorings in the shallow subtidal zones adjacent to pre-selected oiled and reference sites. Embryos were examined for morphological and functional defects, with a focus on known cardiac impacts of oil-derived PAH exposure. To determine PAH exposure, a large suite of PAHs was measured in samples of both naturally spawned and caged embryos, and in passive sampling devices that bind aqueous PAHs that were co-incubated with caged embryos. In general, markedly abnormal biological responses were observed widely in samples of embryos naturally deposited in the intertidal zone at oiled sites. Less severe effects were observed in caged embryos incubated farther from shore in the shallow subtidal zones of oiled sites. As expected in a heavily urbanized area, both oil- and combustion-derived PAHs were widely detected at relatively low levels in embryos from oiled and reference sites. Despite the prominent urban background, higher levels of the most abundant petrogenic PAHs in *Cosco Busan* oil were detected in both embryos and passive samplers from the oiled site least likely to have large PAH inputs from urban or other maritime sources.

Section 1: Background

1.1 Introduction. This report summarizes the design, implementation, and results of an assessment of potential injuries to Pacific herring (*Clupea pallasi*) undertaken by the NRDA fish injury workgroup, as part of the overall injury assessment for the *Cosco Busan* oil spill. The study design, implementation, analysis, and reporting was performed principally by the NOAA Northwest Fisheries Science Center and the University of California Davis Bodega Marine Laboratory, in cooperation with the natural resource trustee representatives and representatives for the responsible party.

Figure 1-1: Satellite overview of Central San Francisco Bay with SCAT estimates of shoreline oiling

Considering the locations affected (Figure 1-1) and the nature of the released fuel oil, the Trustees consulted resource managers and reviewed existing information on the fisheries in San Francisco Bay and the coastal ocean environment nearby, and developed an initial list of fish species to consider for assessment. The entire impacted area is designated as an essential fish habitat (EFH), and San Francisco Bay (SFB) is a habitat area of particular concern (HAPC) under the Magnuson-Stevens Fishery Conservation and Management Act. In the first several days following the spill, fish species under consideration for potential assessment included Pacific herring, green sturgeon, several species of salmon, tidewater goby, northern anchovy, jack mackerel pacific sardine, English sole, starry flounder, several species of rockfish, striped bass, California halibut, Pacific sanddab, lingcod, sand sole, leopard shark, spiny dogfish, big skate, pacific whiting (hake), soupfin shark, curlfin sole, bocaccio, and cabezon. The Trustees also considered investigating potential impacts to Dungeness crabs and other bottom dwelling macroinvertebrates, and to drift algae communities present along the coast outside of San Francisco Bay.

After considering all these species and communities and the characteristics of this spill the Trustees narrowed the focus of injury assessment to Pacific herring as a proxy for nearshore spawning fish species. (The Trustees also made preparations to assess potential injuries to grunion, a fish species that has been observed in recent years spawning on sandy beaches in San Francisco Bay from March through late spring. Although monitored, no grunion spawning was observed in San Francisco Bay during 2008.)

Among finfish, the potential for injury to Pacific herring (*Clupea pallasi*) is of particular concern. As forage fish, herring are a cornerstone of the pelagic food web. They therefore play an influential role in the ecology of the estuary. Herring and their spawned eggs also constitute the only remaining commercial fishery in San Francisco Bay, and the shoreline of the Central Bay serves as one of the largest spawning locations for herring in the state of California (detailed in Section 1.3). Visible oiling of herring spawning habitat, as indicated by the presence of spawn in recent years, ranged from non-detectable to heavy. The heaviest oil was observed between Keil Cove and Horseshoe Cove near the base of the Golden Gate Bridge. The season for herring spawning typically spans November to March, with peak spawning in December and January. Thus, in the winter and early spring of 2007/2008, herring were expected to spawn on eelgrass, seawalls, rip-rap, and other surfaces that were contaminated to varying degrees with *Cosco Busan* oil.

Due to both spawn timing and proximity to oiled substrates, early life stages of herring were likely to be disproportionately impacted by the *Cosco Busan* spill relative to most other finfish species in the Central Bay. In this respect, threats to herring paralleled those following the *Exxon Valdez* spill, which oiled herring spawning habitats in Prince William Sound, Alaska in 1989. Numerous studies following the latter spill have shown that herring embryos are highly sensitive to the toxicological effects of oil. This toxicity can arise from (but does not require) direct contact with particulate oil (e.g., droplets) or exposure to dissolved-phase oil constituents in surrounding seawater (detailed in Section 1.6). This raises the possibility of developmental defects and embryo mortality in locations that did not have visible shoreline oiling after the *Cosco Busan* spill.

This injury assessment characterized the toxicological responses of herring embryos to *Cosco Busan* oil under both natural exposure conditions and in artificially spawned embryos that were outplanted and incubated at oiled sites, as well as at areas where no visible oiling of the shoreline had occurred. The objective was to provide a scientific basis for estimating the oil-induced loss of individual herring larvae from the 2008 year-class. In preliminary discussions with the Trustees (Nov. 14th, 2007), this was identified as the highest priority in terms of assessing injury to fish. However, because of the relative ease of collecting herring spawn samples and a strong scientific understanding of the impacts of oil to herring embryos, this species was also chosen for study as a surrogate for other ecologically important fish species that utilize nearshore areas for spawning. These include, for example, the California grunion. Spawning grunion have been observed in San Francisco Bay in recent years, albeit later than the herring run (typically beginning in March). Eggs remain on the beach in the sand for approximately two weeks and therefore may be at risk for residual oil exposure. Other forage fish that spawn in the Central Bay nearshore include northern anchovy, topsmelt, and jacksmelt.

The study did not directly address oil exposure and potential injury to other species of fish in the San Francisco Bay. These include, for example, salmonids, leopard shark, white sturgeon, striped bass, midshipmen, rockfish, staghorn and prickly sculpin, threespine stickleback, white croaker, shiner perch, bay goby, California halibut, English sole, and starry flounder. In addition, this assessment will not provide a basis for monitoring longer-term exposures to oil or recovery from injury over time for species other than herring. Certain species, such as white croaker, English sole, and starry flounder, have been monitored at various times since the 1980s as sentinels for hydrocarbon exposure in San Francisco Bay

(e.g., as part of the National Benthic Surveillance Project) and may therefore be useful in terms of assessing any lingering impacts of *Cosco Busan* on fish in the estuary.

Table 1-1: Comparisons between crude, residual, and IFO cutting oils

	ANSCO[a]	*Exxon Valdez* oil[b]	No. 2 Diesel[a]	Residual fuel oil[a]	*Cosco Busan* oil
density (15˚C)	0.87	NA	0.83	0.99	0.95[c]
percent aromatics	15	5	10	29	NA
TPAH (μg/g oil)	10600	13300	27000	29000	39000[d]

a, reference 3; b, NOAA Auke Bay Lab, unpublished; c, at 12.8˚C, from OSPR; d, NOAA NWFSC this study; NA, data not available

1.2 Properties of *Cosco Busan* bunker oil. "Bunker fuel" is the generic term applied to the heavy oils burned in ship power plants. Bunker fuels consist mostly of a residual fuel oil, which is what remains after light fractions have been removed from a crude oil in the refining process. Neat residual fuel oils are highly viscous, and must be "cut" with a lighter fuel, typically diesel, in order to be pumped. The *Cosco Busan* carried IFO380, which is a residual oil cut with roughly 3% marine gasoil (equivalent to No. 2 diesel) to produce a viscosity of 380 centistokes. The specific gravity of residual oils varies from slightly less to greater than 1.0, and depending on water density and state of weathering, may float or sink. The diesel-cutting agent weathers more quickly, leaving behind the heavier residual oil. Because only a very small percentage of the oil is subject to evaporative weathering, IFO380 has the tendency to form tar balls that can become widely distributed.

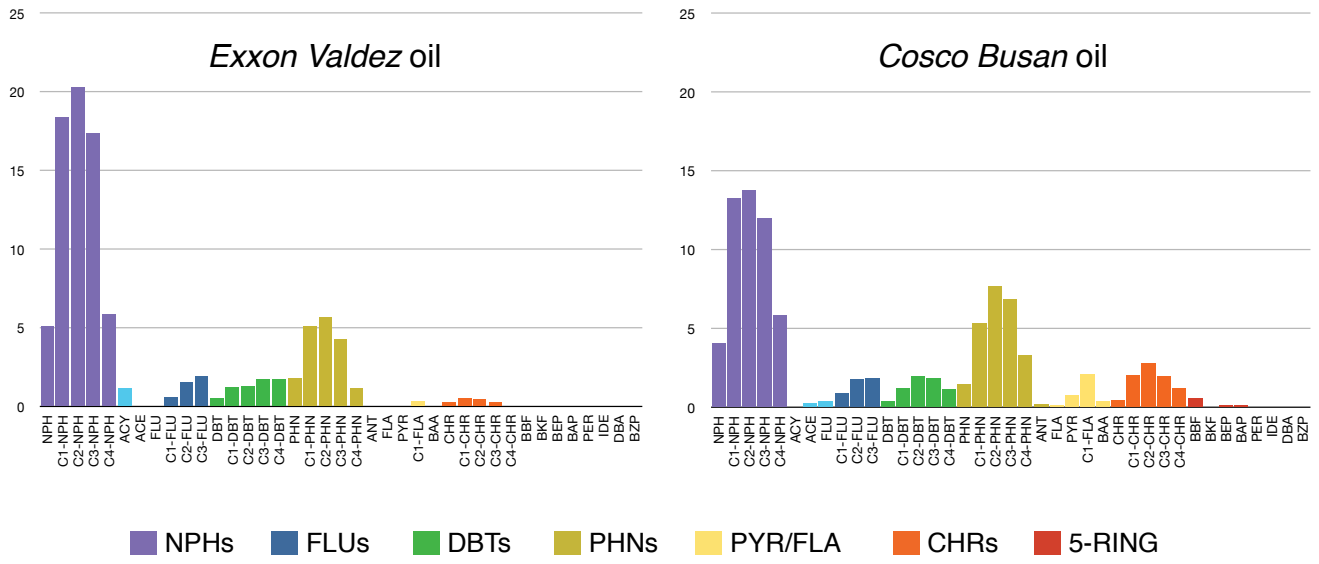

Figure 1-2: PAH composition of *Exxon Valdez* hold oil compared to *Cosco Busan* bunker oil. The x-axis is percentage of total PAHs. PAH subclasses are color-coded and degree of alkylation increases to the right (C1-, C2-, etc.). NPHs, naphthalenes; FLUs, fluorenes; DBTs, dibenzothiophenes; PHNs, phenanthrenes; PYR, pyrene; FLA, fluoranthene, CHR, chrysenes; 5-ring indicates benzo[a]pyrene, etc. EV data from NOAA Auke Bay Lab, CB data from NOAA NWFSC.

Many chemical and elemental components of crude oil are much more highly concentrated in residual oils (Table 1-1) [1, 2]. Residual oils and its mixed products such as IFO380 have a higher percentage of aromatic compounds, a higher total mass of PAHs, and importantly, fractions of uncharacterized polar

compounds, a higher total mass of PAHs, and importantly, fractions of uncharacterized polar compounds or "unresolved complex mixture" that can approach 30% of the mass [1]. In addition, residual oils are enriched with a higher content of metals such as nickel and vanadium [1]. Compared to Alaska North Slope crude oil (ANSCO) carried on the *Exxon Valdez*, *Cosco Busan* oil has three times the PAH mass (Table 1-1) and a higher percentage of the PAH classes that are toxic to fish early life history stages (Figure 1-2, detailed below in Section 1.6; note also that the chemical profile of "ANSCO" varies slightly depending on the exact oil field source). These compositional differences between bunker and crude oil are important in terms of predicting the potential toxicity of *Cosco Busan* oil. Relative to the size of the *Exxon Valdez* oil spill, the volume of the *Cosco Busan* spill was relatively small. However, strictly on the basis of normalized PAH toxicity, the *Cosco Busan* spill could be viewed as equivalent to approximately 150,000 gallons of *Exxon Valdez* oil. Moreover, although bunker fuels have not been studied nearly as intensively as ANSCO, the available studies generally indicate that residual oils are more toxic than predicted based on PAH content alone, consistent with their larger fraction of uncharacterized compounds (detailed in Section 1.6).

1.3 Pacific herring biology and the natural history of herring in San Francisco Bay. Estuaries provide essential habitats for Pacific herring reproduction, and are therefore an integral part of the herring life cycle. Reciprocally, herring are forage fish, and the adults, eggs, and larvae are important components of estuarine food webs. For this reason, herring are a keystone species. As such, they play a complex role in the dynamics and productivity of many predator populations, including other fish, birds, and marine mammals. They are also economically important to an international fishery that targets reproductive animals for the purposes of collecting ovaries (Kazunoku) and spawned eggs attached to kelp (Kazunoku Kombu).

General life history patterns for Pacific herring are alike throughout their range, which extends from Japan to the Arctic to California. Spawning, embryonic development, larval growth, and early juvenile life occur within estuaries, where lowered salinity and protected waters offer conditions conducive to success for early life stages [3]. San Francisco Bay supports the southern-most reproductive stock of Pacific herring in the Eastern Pacific Ocean. The San Francisco Bay stock is the youngest at first reproduction, and possesses the earliest and longest annual spawning seasons. Minimum age for reproduction is 2 years in the California stocks, 2-3 (in some years up to age 5) years in British Columbia fish and 4-5 years in Alaskan stocks [3-6]. The spawning season for the San Francisco Bay stock in most years extends from December through March with the peak of spawning occurring during January and February, although it has begun as early as October [5, 7-9].

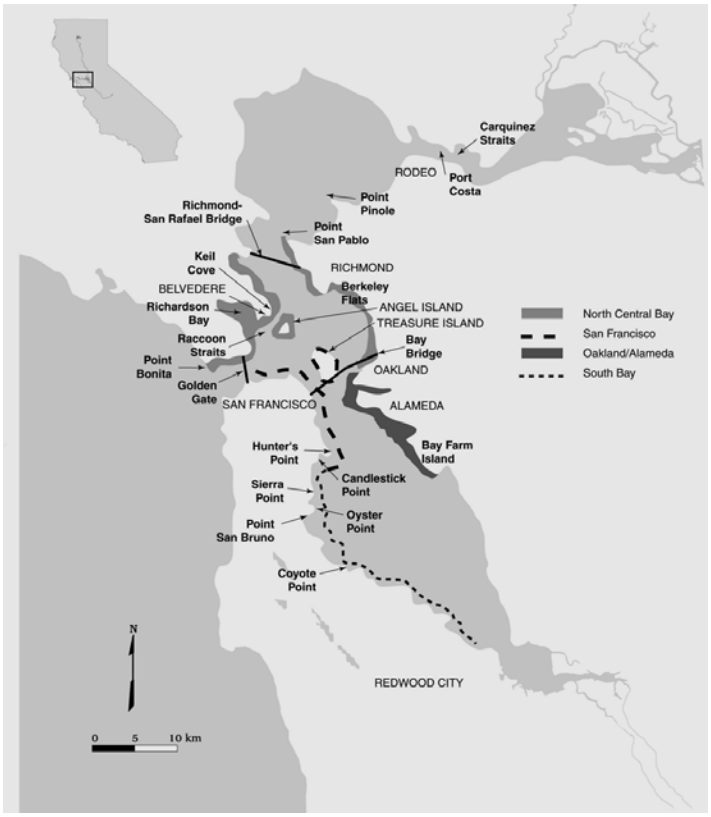

Figure 1-3: Historical Pacific herring spawning regions within San Francisco Bay. (From reference 11)

San Francisco Bay is a large, complex body of water that consists of at least three sub-bays.

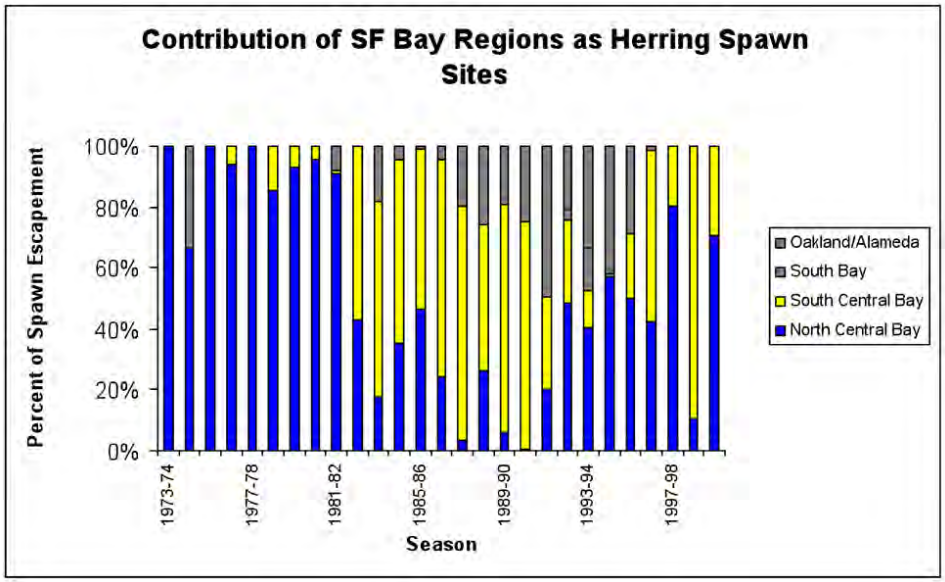

Figure 1-4: Percent of Pacific herring spawning adult biomass (i.e., escapement) by region for each season in San Francisco Bay, 1973–2000. Average percent biomass for each region was 54.9% North Central bay, 34.2% South Central Bay, 9.8% Oakland–Alameda, and 1.1% South Bay. (From reference 11; San Francisco spawn data is listed as South Central Bay).

These sub-bays, although physically connected, have different biological communities and fish assemblages. They include the North Bay, Central Bay, and South Bay. Use of the larger bay by herring as spawning and nursery sites varies within and between years. In terms of historical spawning patterns, the San Francisco shoreline can be divided into four regions [10]; the North Central Bay, San Francisco, Oakland/ Alameda, and the South Bay (Figure 1-3). The North Central Bay encompasses the Marin County shoreline from Point Bonita through Richardson Bay to Point San Quentin. From 1974-2000 the North Central Bay was used for spawning in every year but one, and was the predominant spawn region in 13 out of the 26 years (Figure 1-4; [10].

During spawning, females deposit the adhesive eggs onto substrates such as marine vegetation, gravel, and rocks while males continue to release sperm in close proximity [11]. In San Francisco Bay, there have been declines in the percent cover of the eelgrass *Zostera*, a preferred substrate for spawning, and marine algal species (e.g. *Gracilaria* sp. and *Laminaria* sp.). Non-biological substrates, both natural and man-made (rocks, sand, pilings, boats) have been increasingly used as substrates for spawn [5, 10]. Herring avoid mud or silt-laden habitats. In 1979 divers sampled 15 sites in Richardson Bay and found *Zostera* and *Gracilaria* to be the only two significant marine vegetative species, with *Zostera* occupying only patches of subtidal habitat [5]. Density of vegetative coverage was variable throughout Richardson Bay, ranging from 0.003 kg of vegetation per square meter (northeast of Strawberry Point) to 0.164 kg/ m^2 (off Belvedere, near the mouth of Richardson Bay).

Surveys of herring spawn locations were conducted from 1973-74 through 1979-80 in San Francisco Bay, focusing on the North Central Bay [5]. In addition to intertidal and shoreline spawning from just inside the Golden Gate Bridge to Paradise Cove, major subtidal spawning areas were discovered in Richardson Bay and in the flats off Richmond and Oakland. Spawning during this period was also documented to occur off of Coyote Point in the South Bay, but was not surveyed for size [5]. During the period of these studies, estimates of spawning biomass for the Bay per season varied from 3,682 tons (1977-78) to 46,439 tons (1979-80). Similar wide fluctuations have been reported for Pacific herring spawning biomass in other regions (e.g. Alaska, British Columbia, and Washington). Spawning of a school of herring may take place over several hours or days depending on the size of the school. Typically several separate schools enter San Francisco Bay to spawn every two to three weeks over the course of a season. These spawning "waves" are typically separated temporally, but may overlap geographically. It is also not

uncommon for one or two of these waves of spawners to contribute the majority of the spawn for the season [5, 9].

Herring eggs are monospermic in that normal fertilization requires that only one sperm fuse with and enter an egg. Embryonic development in *C. pallasi* is typical for teleosts [12-15]. Temperature and salinity correlate with changes in embryonic development times [15, 16], and the timing and landmark stages of Pacific herring embryonic development have been detailed for the San Francisco Bay stock [12]. These stages (periods) are generally: cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. Early cleavages are confined to the animal pole of the egg with the first cleavage occurring about 3 hrs post-fertilization. Subsequent cleavages continue through the next 12 hrs and result in the formation of a cap of blastomeres (cells) termed the blastodisc, that migrate at a cell sheet (epiboly) to encase the vegetal regions of the embryo, producing the gastrula stage at about 20-21 hrs post-fertilization. By this stage the embryo has a definite bilateral symmetry with anterior/posterior, dorsal/ventral, and right/left axes evident. The next landmark stage, segmentation, becomes apparent with the development of somites by 42-48 hrs, and the pharyngula period is reached by day 5 of development. Hatching of swimming larvae in San Francisco Bay begins at 10.5 days at 10.5 ^{o}C [7]. In the laboratory, larval hatching occurs over a protracted period of 2-3 days, 8-10 days post-fertilization at 12^{o}C [12]. At hatching, herring larvae are transparent, retain a yolk-sac, and measure approximately 6-9 mm in length [13, 16].

1.4 Natural and anthropogenic causes of morbidity and mortality during herring egg stages. Herring spawning sites in San Francisco Bay are susceptible to several natural threats. Other threats originate from various human activities (past and present) in this heavily urbanized and industrialized estuary. Mortality during embryonic development in relatively pristine areas varies with location and year; it can range from 56-99% in British Columbia. In a two-year study conducted in Barkley Sound and the Strait of Georgia (1988-90), spawn sites were sampled to determine total biomass remaining as embryonic development proceeded. Predation is the primary cause of mortality, with average daily loss at 6-8% producing an overall loss of 50-70% by hatching [17]. Two additional potential natural causes of mortality involve embryos being dislodged from substrata and presumptive hypoxia when eggs are deposited in multiple layers of greater than eight eggs thick at spawning [3]. Both field observations and lab studies have shown that herring embryos can be significantly delayed or suffer high rates of mortality in the deeper layers at very high densities [18-22]. There is only one report of egg layers approaching or exceeding eight eggs in field-collected samples from San Francisco Bay [23].

The morphological effects of hypoxia on herring embryos have not been described in detail. However, some predictions can be made based on studies in other fish species. The embryos of a range of teleosts are generally resistant to lethal hypoxia at early developmental stages, and become more sensitive closer to hatching. In fish with relatively small eggs such as herring, this may be due to the very large surface-to-volume ratio [21]. In several species, hypoxia was shown to be a mild teratogen. At moderate levels of hypoxia, the most common effect is developmental delay with no overall changes in gross morphology. In zebrafish, an increase in body axis defects was observed with severe hypoxia (0.8 mg/L O_2), but only at much later stages of development (after hatching), and then in only about 20% of the animals [24]. Subtle somite defects have been observed in the embryos of several pelagic marine species, leading to vertebral abnormalities in juveniles and adults [25-27]. Hypoxia is not associated with cardiac arrhythmia and has not been found to induce edema in any species. Zebrafish embryos respond to hypoxia with an accelerated heart rate [24].

Temperature and salinity also influence herring development. Higher temperatures decrease embryonic development times, but result in larvae that are smaller than those developing at lower

temperatures [16]. Eggs deposited in the intertidal are vulnerable to exposure and temperature shock. In Oregon the estimate for mortality in intertidal zones was dependent on weather, with higher mortality rates in warm, dry weather and lower mortality in cool, moist weather [28]. Hatching success declines with increasing water depth. Only 10-12% of embryos developing at 18 meters hatched compared to those that develop near the surface [19]. Herring embryos from stocks in the White Sea (Russia) arrested at early cleavage in salinity at or below 1 ppt [29]. At a slightly higher salinity (3 ppt), abnormal development occurs. This salinity is the lowest at which herring embryos have been reported to hatch. Hatching of White Sea herring occurred over a wider range (5-34 ppt) than that reported for Pacific herring from San Francisco Bay [12]. Consistencies between California herring and White Sea herring include higher numbers of malformed embryos and even larvae at both high and low salinity, incidences of partial hatching at low salinity, and delayed hatching at high salinity [12, 29].

Suspended sediments pose another potential threat. Theoretically, coating of eggs with fine suspended sediments could result in hypoxia. These effects might be expected to mimic those of hypoxia induced experimentally using water with low dissolved oxygen. Also, sediment-induced hypoxia might be similar to the effects of heavy spawn density. However, several studies using either Pacific or Atlantic herring embryos failed to find any significant effects of suspended sediments on embryos [30-33]. The potential threats associated with sedimentation have been a recurring issue for San Francisco Bay herring spawning grounds. This is due to the periodic need for dredging associated with the widespread maintenance of channels and harbors. However, a recent assessment found there to be little risk for impacts of suspended sediments on herring spawn in San Francisco Bay [34].

Inputs of effluent or overflows from sewage treatment plants are common in urbanized waterways such as San Francisco Bay. During the period of January-February 2008, there was a leakage of 2.7 million gallons of partially treated sewage into Richardson Bay (January 31) and a 1500 gallon spill of raw sewage from the San Quentin prison (February 14). Although the primary effects of sewage effluent are related to endocrine disruption by xenoestrogens, impacts of sewage on early development in fish has not been studied in detail. A single study tested the effects of sewage sludge on Atlantic herring development [35]. Concentrations of suspended sludge $\geq$ 0.1% caused premature hatching but no mortality in embryos. There were otherwise no significant effects at concentrations $\leq$ 0.2%. Given that concentrated sewage in the form of sludge is likely to be more toxic than partially treated effluent, it is highly unlikely that sewage spills to San Francisco Bay would produce acute morphological defects in herring embryos.

1.5 Timeline and pattern of spill in relation to herring spawning. The spill occurred in early November, two months before the average peak of herring spawning. Based on the last 10 years of surveys by California Department of Fish and Game, the most likely sites for spawning in 2007-2008 were in the North-Central portion of San Francisco Bay from Golden Gate to Point San Quentin. Major sections of this shoreline that had visible oil included areas near the Golden Gate, the Sausalito waterfront, and the southern part of the Tiburon peninsula (Figure 1-5). In the 2007-2008 season, spawning occurred much later than typical. Schools of herring began to enter the Bay intermittently in January 2008, but sampling showed low percentages of fish with ripe gametes. Small spawning events occurred intermittently through February, and major spawning occurred on the San Francisco waterfront for the first time since this area was oiled by the *Cape Mohican* spill in 1996. Ripe fish caught near Richardson Bay provided gametes for the outplant portion of this study (see Section 1.7) starting the second week of February. Spawning along the North-Central shoreline, including oiled sites, occurred fairly widely but at relatively low densities starting February 17, a full 14 weeks after the spill.

1.6 Impacts of petroleum hydrocarbons on herring and other fish embryos. The body of scientific research that followed the 1989 *Exxon Valdez* oil spill in Prince William Sound was a major advance in terms of understanding the toxicological impacts of crude oil on early life history stages of fish. Our current understanding of how petroleum hydrocarbon exposures impact the normal development of fish embryos and larvae has been largely determined by research and monitoring in the years since *Exxon Valdez*. Much of this work was published after 1996 and was hence unavailable to inform the response to and damage assessment for the last major oil spill in San Francisco Bay (*Cape Mohican*). The *Exxon Valdez* spill contaminated spawning grounds for Pacific herring and pink salmon. In subsequent years, a large number of field and laboratory studies revealed that the embryos of both species are highly sensitive to polycyclic aromatic hydrocarbons (PAHs) in petroleum products. In both herring and pink salmon, PAHs from weathered oil caused a common syndrome of developmental defects [36-39]. Lower frequencies of essentially identical defects were previously described in earlier studies focusing on higher concentrations of fresh oil [40-43]. Gross malformations included pericardial and yolk sac edema, small jaws, and spinal curvature, accompanied by heart rate reduction (bradycardia) and cardiac arrhythmia. These effects of petroleum-derived PAH mixtures were subsequently documented in a variety of other teleost species [44-46] as well as in herring embryos exposed to PAH-rich creosote [47]. Overall, these toxicological effects occur at relatively low (ppb) total aqueous PAH concentrations, and do not require direct contact with oil droplets or particulate oil [48].

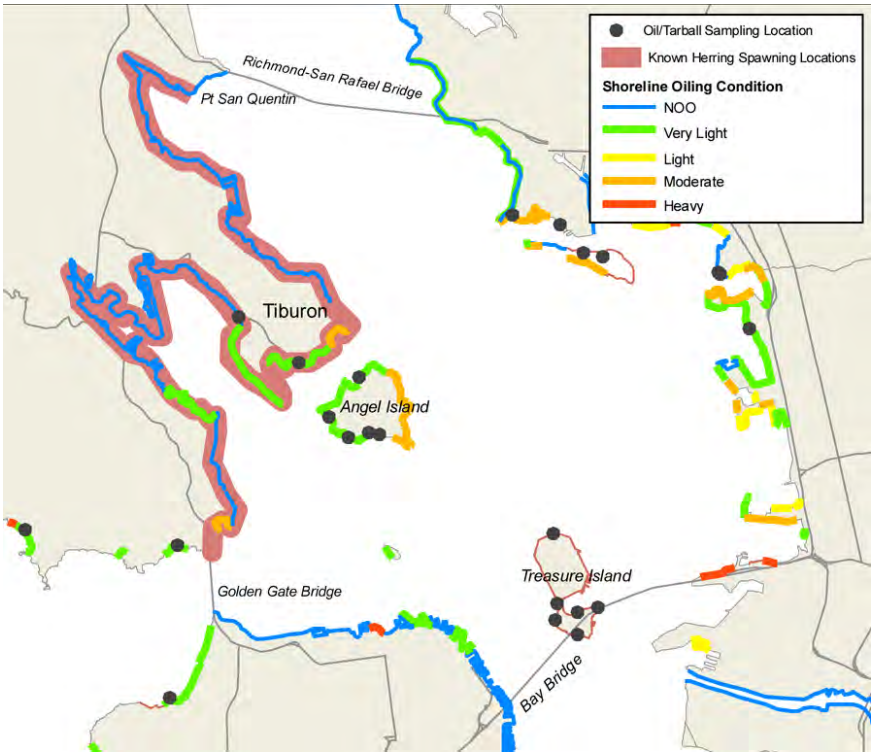

Figure 1-5: Relationship of shoreline oiling to recent herring spawning grounds in the Central Bay.

Unrefined crude oils generally contain PAH fractions that consist of roughly 50-60% naphthalenes, 40-50% tricyclic compounds (fluorenes, dibenzothiophenes, and phenanthrenes), and 1-3% chrysenes [2]. Higher molecular weight PAHs such as benzo(a)pyrene usually constitute < 1% of the total PAHs in crude oils. During the weathering of oiled substrates (e.g. beach gravel) by water, PAHs (and other constituents) move into water from the substrate over time. This timed release is in essence the definition of weathering, described by first-order loss-rate kinetics [49], and results in a 'water-washed' pattern of dissolved PAHs. Lower molecular weight compounds with fewer alkyl substitutions are dissolved most readily, and dissolution rates are proportional to hydrophobicity. Effluent from substrates with relatively fresh oil is initially dominated by the relative proportions of naphthalenes. Over time, the concentrations of tricyclic PAHs and alkylated isomers become proportionately greater. As the pattern of dissolved PAHs shifts to these tricyclic compounds, both mortality and defects such as pericardial edema occur at much lower total

PAH concentrations [38, 39]. Thus, oil toxicity to fish embryos is predominantly associated with fluorenes, dibenzothiophenes, and phenanthrenes.

Considerable progress has been made over the past five years in terms of elucidating the different toxicological pathways by which crude oil and these individual PAH compounds disrupt fish development. Several lines of evidence from studies using zebrafish and other experimental models have identified the developing heart as a primary target for PAHs enriched in crude oil. These studies demonstrated that the now-familiar morphological defects associated with oil exposure are (1) attributable to the tricyclic PAH fraction, (2) secondary to direct impacts on cardiac function, and (3) independent of the aryl hydrocarbon receptor/cytochrome P4501A (AHR/CYP1A) pathway traditionally associated with toxicity of high molecular weight PAHs [46, 50-53]. Importantly, these studies have made key distinctions between the effects of crude oil and its most abundant low molecular weight PAHs, and the effects of other aromatic compounds that are widely distributed in San Francisco Bay. These include the higher molecular weight pyrogenic PAHs such as pyrene, benz[*a*]anthracene, and benzo[*a*]pyrene, the co-planar PCBs, and dioxins. Most of these compounds disrupt teleost heart development in a manner similar to dioxins through activation of the AHR. However, cardiac rhythm disturbances are not the primary response associated with exposure to potent AHR ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, co-planar PCBs, or benz[*a*]anthracene, and all of these compounds produce cardiac malformations at later developmental stages than the tricyclic PAHs [52, 54-56].

In zebrafish embryos, exposure to non-alkylated tricyclic PAHs through the pharyngula stage (36-48 hours post-fertilization, hpf) produces a dose-dependent reduction of heart rate (bradycardia), followed by more complex arrhythmias consistent with atrioventricular conduction block [50, 51]. Somewhat more complex effects, including reduced contractility, were observed in zebrafish embryos exposed to weathering oil that produced total tricyclic alkyl-PAH aqueous concentrations in the range of 20-30 ppb [50]. Comparison to the phenotypes of known zebrafish cardiac mutants suggests several potential myocardial targets for oil toxicity, including cardiac potassium channels [57-59], sarcoplasmic or plasma membrane calcium channels [60] or gap junctions [61]. These findings recently were extended to Pacific herring embryos (Figure 1-6), thereby confirming that early cardiac dysfunction (i.e. arrhythmia) is the primary and earliest toxicological response to unrefined crude oil exposure in herring, occurring at the same developmental stage as in zebrafish [62]. Therefore, the best available science indicated that an assessment of *in vivo* cardiac defects and their sequelae (e.g. edema) would likely be the most sensitive indicator of toxicity in herring embryos exposed to *Cosco Busan* oil.

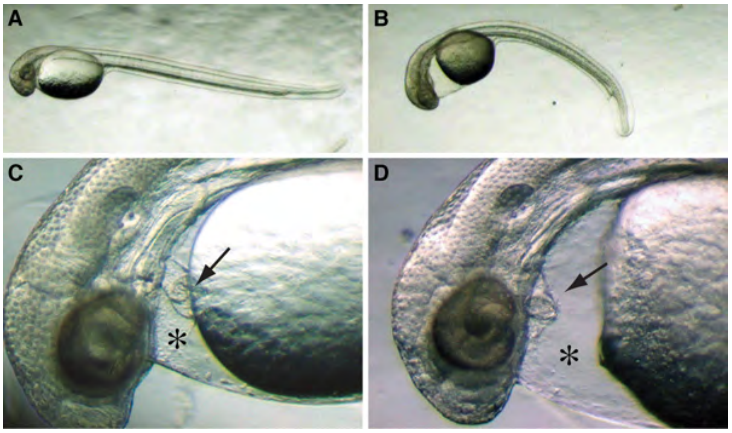

Figure 1-6: Cardiogenic edema in Pacific herring embryos exposed to ANSCO. (A, B) Gross morphology of embryos at 7 days post-fertilization exposed to clean (A) or oiled (B) gravel effluent. (C, D) Higher magnifications showing the heart (arrows) and pericardial space (asterisks) in embryos exposed to clean (C) or oiled (D) gravel effluent. From reference 60.

Despite these recent advances in our understanding of PAH and the toxicity of unrefined crude oil, there are still significant data gaps concerning the toxicity of heavier residual oil products that comprise "bunker" fuels. The heavier distillates of crude oil have not been studied nearly as intensively as crude oils, particularly Alaskan crude oils. However, studies on a variety of

invertebrates and fish generally have shown that crude oil distillates typically have comparably higher toxicity than unrefined petroleum [63-65]. Moreover, the toxicity of heavier refined products often cannot be attributed to just the PAH fraction. This is because the observed toxicity of refined oil is higher than predicted by the aqueous concentrations of PAHs [66-68]. Some studies suggest that exposure of fish embryos to heavy residual oils may not produce the canonical syndrome associated with Alaska North Slope crude oil. A field study following a spill of bunker fuel in a freshwater lake found no association of edema with oil exposure in lake whitefish (*Coregonus clupeaformis*), but increased incidence of body axis defects was highly correlated with incubation near oiled sites [69]. Similarly, a laboratory study using spotted halibut (*Verasper variegates*) embryos described novel defects in spinal neural development caused by heavy oil exposure, apparently with the absence of edema [70]. Finally, very small spills of bunker fuel have been associated with high rates of mortalities in other marine vertebrates [71].

1.7 General goals and approach of the fish injury assessment. The overall aims of this assessment were to monitor the *in situ* exposure of herring embryos to *Cosco Busan* oil at sites with varying histories of visible oiling, and to assess the toxicological response of herring embryos over the same range of oil exposures in the field. A simple approach to estimating herring spawn exposure to *Cosco Busan* oil would be to compare the distribution of visible oil (or tar balls) along the shoreline of San Francisco Bay with specific spawning locations for the 2007/2008 season as determined from California Department of Fish and Game field surveys. However, the presence or absence of visible oil in the days immediately after the spill may be a poor indicator of the spatial distribution of dissolved-phase PAHs or other oil compounds months later during the herring spawning season. Instead, a tiered approach was developed. The aim of the first tier (Tier 1) was to determine the extent of bunker oil exposure by analyzing PAH profiles in 1) the tissues and eggs of pre-spawning adult females, 2) eggs spawned naturally at locations within and external to the visible *Cosco Busan* oil spill zone, 3) eggs fertilized and outplanted at locations within and external to the visible spill zone, and 4) passive sampling devices deployed in tandem with the outplanted herring embryos. A related Tier 1 aim was to assess the early development, viability, and larval performance of naturally spawned and outplanted herring embryos for evidence of early life stage toxicity that might be attributable to exposure to residual *Cosco Busan* bunker oil. The goal of Tier 2 was to determine whether *Cosco Busan* oil could be detected in intertidal and subtidal sediments adjacent to locations where natural spawn and outplanted eggs incubated, respectively. Collections for the Tier 3 analysis were intended to qualitatively assess and quantify the induction of CYP1A (a biomarker for PAH exposure; *51*, *53*) in both naturally spawned and outplanted embryos. This report describes the results of the Tier 1 studies.

To determine whether there were biological impacts to herring spawn from *Cosco Busan* oil, the basic approach was to look for the morphological and functional defects associated with (crude) oil exposure (described in Section 1.6) in embryos collected from spawning locations with different degrees of *Cosco Busan* oiling (based on SCAT maps). The same observations were made for embryos collected from non-oiled urban reference sites. Since it was unknown in advance where herring would actually spawn, laboratory-fertilized embryos were outplanted in moored cages at sites selected by recent history of spawning and degree of oiling. Four sites were chosen in the Central Bay/Marin area that had different degrees of oiling based on SCAT surveys, but also different degrees of cleanup activity, and two non-oiled reference sites were chosen further northeast on the same shoreline. There was generally delayed and reduced spawning in the Central Bay in early 2008, and only three of the oiled sites and one reference site were assessed for impacts to naturally spawned embryos. Natural spawn and caged artificial spawn also differed in their incubation by depth and distance from shore: all natural spawning occurred in the intertidal zone, while caged embryos were incubated in the shallow subtidal zone.

To characterize oil exposure to herring embryos, PAH levels were analyzed in composite samples from natural spawn and caged artificial spawn. PAHs were also analyzed in the bodies and ovaries of adult animals to determine whether there could be maternal contribution to any exposure In addition, polyethylene membrane devices (PEMDs) were deployed to passively sample PAHs over the normal duration of herring egg incubation at the cage deployment sites. PEMDs bind dissolved-phase PAHs, eliminate the potential for PAH metabolism associated with fish tissues, and, unlike eggs, are less susceptible fouling by sediments and artifactual measurements of sediment-bound PAHs. Sediment samples were also collected for potential Tier 2 PAH analysis from the same transects in the intertidal zone where natural spawn was sampled, as well as the subtidal locations for caged embryos. Samples of embryos were also retained in order to qualitatively and quantitatively assess induction of CYP1A if necessary (Tier 3).

Section 2: Methods and Implementation

2.1 Selection of natural spawn sampling sites and collection of natural spawn. Selection of natural spawn sampling sites was opportunistic. The 2007-2008 spawning season was atypical, with ripe fish appearing in large numbers relatively late in the season. Significant spawning events did not occur along the Central Bay waterfront until late February. Spawning occurred at only four of the six study sites chosen for deployment of caged embryos (see below). Over the period from 2/26/08 through 2/29/08 natural spawn samples were collected at San Rafael Bay (MRU01), Sausalito (MRQ10/P01), Peninsula Point (MRQ01), and Keil Cove (MRR20). No natural spawning occurred over the course of the study at the Horseshoe Cove site (MRP04), and although natural spawning was observed at the Point San Quentin site (MRT04), the spawning density there was very light. It also occurred concurrently with a sewage spill from the San Quentin prison near this site which prohibited access to the water, and at the same time as a much more dense natural spawning event at the nearby reference site, San Rafael Bay. It was not possible to process natural spawn samples from two sites in the same day, and it was decided not to hold field-collected natural spawn samples in the lab after arrival from the field prior to the beginning of laboratory processing. Accordingly, natural spawn samples were not collected from Point San Quentin. At subtransects within the Sausalito (N5) and Keil Cove (N6) sites, it was necessary to combine two adjacent subtransect collections (20-m total distance at each) together to be able to collect enough sample quantity to provide the required laboratory subsamples (see Table 2-1).

Table 2-1: Summary of Natural Spawn Sampling Sites

Site	N1	N2	N3	N4	N5	N6	N7	N8
San Rafael Bay (MRU01)	37°56.690N x 122° 28.841W	37°56.693 x 122° 28.849	37°56.696 x 122° 28.854	37°56.698 x 122° 28.859	37°56.702 x 122° 28.864	37°56.707 x 122° 28.873	37°56.711 x 122° 28.877	37°56.717 x 122° 28.887
Date/Time begun	2/26/08 10:30 am	2/26/08 10:40 am	2/26/08 10:48 am	2/26/08 10:57 am	2/26/08 11:04 am	2/26/08 11:12 am	2/26/08 11:24 am	2/26/08 11:40 am
Sausalito (N1-5, MRQ10 N6-8, P01)	37°51.688 x 122° 29.174	37°51.691 x 122° 29.181	37°51.693 x 122° 29.185	37°51.696 x 122° 29.191	37°51.697 x 122° 29.199 & 37°51.698 x 122° 29.205	37°51.482 x 122° 28.719	37°51.487 x 122° 28.718	37°51.493 x 122° 28.716
Date/Time begun	2/27/08 10:11 am	2/27/08 10:23 am	2/27/08 10:36 am	2/27/08 10:49 am	2/27/08 11:05 am	Feb 27, 2008	Feb 27, 2008	Feb 27, 2008
Keil Cove (MRR20)	37°52.826 x 122° 26.413	37°52.824 x 122° 26.407	37°52.821 x 122° 26.402	37°52.816 x 122° 26.391	37°52.814 x 122° 26.387	37°52.811 x 122° 26.382 & 37°52.809 x 122° 26.376	37°52.806 x 122° 26.371	37°52.803 x 122° 26.365
Date/Time begun	2/28/08 11:53 am	2/28/08 11:58 am	Feb 28, 2008	Feb 28, 2008	Feb 28, 2008	Feb 28, 2008	Feb 28, 2008	Feb 28, 2008
Peninsula Pt. (MRQ01)	37°52.056 x 122° 27.994	37°52.052 x 122° 27.989	37°52.048 x 122° 27.988	37°52.042 x 122° 27.983	37°52.039 x 122° 27.978	37°52.036 x 122° 27.975	37°52.032 x 122° 27.969	37°52.030 x 122° 27.963
Date/Time begun	Feb 29, 2008 1:	Feb 29, 2008	Feb 29, 2008	Feb 29, 2008	Feb 29, 2008	Feb 29, 2008	Feb 29, 2008	Feb 29, 2008

Generally, the spawning occurred on substrates in the intertidal and shallow subtidal zones. Abundant spawn was not found at depths adjacent to moorings for cages (see below). Collection of naturally spawned herring eggs was conducted in the intertidal zone, at positions shoreward of all of the subtidal cage/mooring deployment sites where natural spawning events also occurred. At all sites, samples were collected seven days after the natural spawning event had originally been detected by rake or shore-based surveys, or based on field examination of the embryo developmental stage at each site by visual inspection after fixation in Stockard's solution. At each location, attempts were made to collect marine vegetation with spawned herring eggs attached from the middle to lower intertidal zone. These efforts were successful in most situations. At some sites, marine vegetation was largely absent in the lower intertidal, and the herring had primarily spawned in the upper intertidal zone. In these situations samples were collected as low in the intertidal zone as possible, and in no cases were samples collected above the waterline.

The protocol for collection of natural spawn was performed uniformly at all sampling sites. At each site, samples were collected along a 100-m transect at positions shoreward of the cage deployment positions (A1 through A5) and parallel to the shore within the intertidal zone. Each 100-m transect was divided into ten distinct 10-m subtransects from which vegetation samples with attached spawn were pooled into eight distinct samples (N1-N8); two subtransects within the 100-m transect were randomly skipped at each site. GPS coordinates were recorded at the midpoint of each subtransect. Samples were collected from the shoreline by personnel with chest waders and/or by snorkeling. Algal holdfasts were cut with a knife and the entire sample placed in heavy duty ziplock bags containing ambient seawater. When a sufficient sample size for processing the required laboratory subsamples had been collected at each subtransect, the ziplock bag was filled with ambient water at the same subtransect, sealed, and placed in a large cooler lined with freshly frozen blue ice. Individual samples from the same site (e.g. N1-N8 from San Rafael Bay) were separated from one another by frozen blue ice blocks, to maintain an ambient, or lower than ambient, water temperature during transport back to the laboratory at the Bodega Bay for sample processing and imaging. In all cases, processing of the natural spawn samples in the lab was begun within six hours after the last natural spawn subtransect site was collected in the field.

Figure 2-1: Collection of natural spawn

2.2 Mooring design and deployment. The design of moorings for caged embryo outplants and PEMDs was the same as that described in the SOP manual, to address the potential for cages or PEMDs to contact bottom sediments at low tides. Briefly, the anchor-buoy units consisted of a large primary and small secondary float attached to either end of a braided polypropylene line that was passed through a stainless steel O-ring attached to the middle of a pair of concrete blocks weighing 60-lb. Cages and passive samplers were attached with two heavy duty Zipties through the braided line just beneath the small secondary float, which line was in turn attached by heavy duty Zipties to the same and opposite

Figure 2-2: Construction of moorings

line leading to the primary float, which maintained the cages vertical at a preset depth (~1 foot from the bottom, the depth of the cinder blocks plus a few inches of line between the cinder block and the attached cage) no matter the level of the tide (Figure 2-2). Anchor-buoy units were installed 1-2 days prior to embryo cage deployment to allow any disturbed bottom sediments to clear.

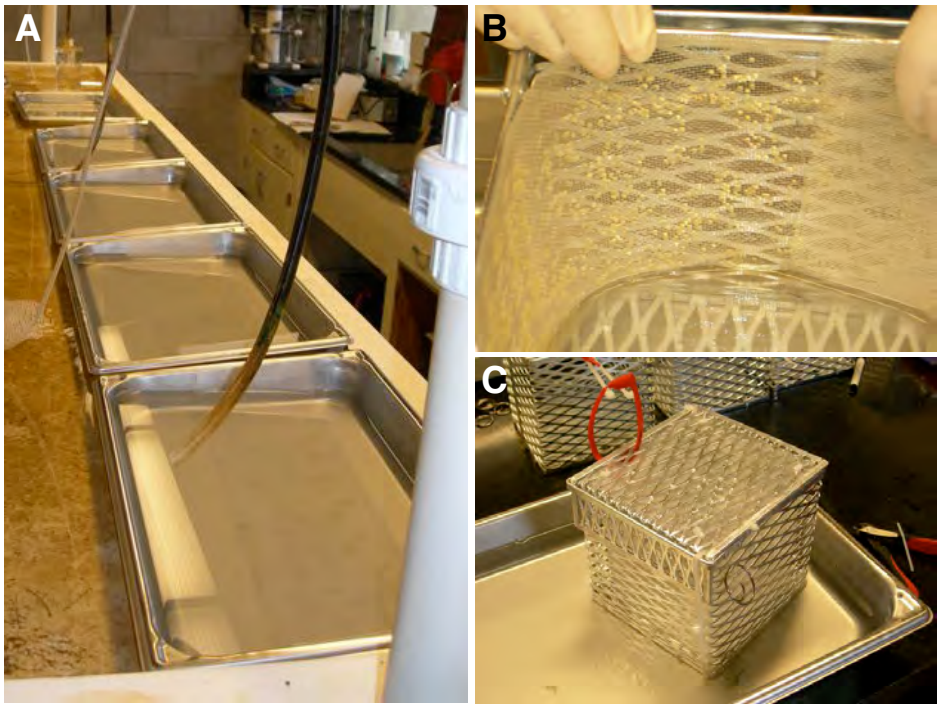

Figure 2-3: Distribution of fertilized eggs onto nitex sheets and cage assembly. (A) Five replicate sheets with monolayers of eggs incubating in milt. (B) Insertion of nitex sheet with fertilized eggs into cage. (C) Fully assembled cage with numbered security tag.

2.3 Collection of adults for analyses of background PAHs and persistent organic pollutants (POPs) in whole body and ovary samples. These steps were carried out as described in the SOP manual.

2.4 Preparation of caged embryos, cage deployment and retrieval. These steps were carried out as described in the SOP manual. Cage assembly and the process of deployment are shown in Figures 2-3 and 2-4, respectively.

2.5 PEMD deployment and retrieval. PEMDs were deployed and retrieved as detailed in the SOP manual, and following procedures developed at the NOAA Alaska Fisheries Science Center, Auke Bay Lab (Juneau, AK). Additional details and field observations are described here. Three PEMDs were deployed at each cage deployment site, as follows: one PEMD was attached to the mooring line just above the cage at each Mooring #1 (A1), Mooring #3 (A3), and Mooring #5 (A5), as part of the cage deployment process. At each deployment, previously prepared PEMDs (double-wrapped in aluminum foil and placed in a sealed ziplock bag) were opened while under water by personnel wearing fresh nitrile gloves, removing the outer bag and both layers of aluminum foil. While still underwater, the PEMD was then attached to the mooring line leading to the secondary, smaller mooring buoy (which served as flotation for the PEMD and cage) by two heavy-duty plastic zip-ties, at a position 6" to 1' above the cage. The primary mooring line bearing the larger orange primary marker buoy was then pulled taut by shipboard personnel, so that the attached cage became adjacent to and just above the stainless steel ring in the center of the double-cinder block anchor resting on the bottom. A snorkeler then attached the two lengths of the mooring line with two heavy duty zip ties, thereby ensuring the cage and PEMD were maintained above the center of the mooring anchor and out of contact with the sediment. At the time of deployment and without boat engines running, a PEMD "air blank" was deployed by unwrapping (while wearing fresh nitrile gloves) the PEMD and exposing it to ambient air for a 60 seconds, and then re-wrapping it in a double layer of aluminum foil. It was then labeled, double-bagged it in ziplock bags, placed it on ice in a cooler reserved only for PEMDs. Following transport to the BML, PEMDs were stored in a locked freezer. PEMD air blanks at deployment were routinely conducted at the mooring #3 (A3) at the cage deployment sites.

Figure 2-4: Deployment of cages: Cages were transported to the field in large ziplock bags with half-strength seawater on ice, lowered into the water in a closed bag, removed from the bag underwater and handed to a free diver to be clipped onto the mooring. Retrieval was a reverse of this process.

At retrieval, the PEMDs were collected in a reverse process of the deployment procedure. After the snorkeler had severed the zip ties connecting the two sides of the mooring line, the secondary buoy was brought to the surface and handed to shipboard personnel at the waterline. At that point the cage containing herring embryos was collected, double-bagged underwater in a heavy-duty ziplock bag filled with ambient water and placed on ice in a cooler. Next, while keeping the PEMD underwater, shipboard personnel hanging over the side of the boat double-wrapped the PEMD in aluminum foil, placed the wrapped PEMD in an appropriately labeled ziplock bag, then drained the excess water from the foil-wrapped PEMD and placed the PEMD and inner ziplock bag into another larger, labeled ziplock bag. The PEMD was then placed on ice in a cooler reserved only for PEMDs, transported back to the BML and placed in a locked freezer. Following the same methods described above for the PEMD deployment process, at retrieval of cages and submerged PEMDs, a PEMD "air blank" sample was also collected at the #3 mooring (A3) at each of the cage sites.

2.6 Laboratory processing of embryos and imaging. These steps were carried out as described in the SOP manual.

2.7 Laboratory assays of hatching and larval swimming behavior.

2.7.1 Hatching rates. Upon arrival of natural spawn samples in the laboratory, several strands of vegetation with attached embryos were placed into 11- x 21-mm rectangular glass dishes containing 600-700 ml half-strength, 0.45 µm-filtered seawater (½ FSW) and incubated in a 12°C incubator. The initial methodology for quantifying hatching success was to incubate embryos on natural substrate. However, following overnight incubation of the first spawn samples (San Rafael Bay), it was subsequently determined that visualization of embryos on the substrate presented several logistical problems, including opacity of vegetation obscuring the developing embryos and contamination of the incubation media with vegetation-associated organisms. Therefore, up to 100 embryos were carefully removed from the vegetation into 6-well culture plates (20-30 embryos/well) and incubated at 12°C with daily water changes. 48 hrs post retrieval, embryos, larvae, and empty chorions (egg shells) were enumerated as follows: eyed non-hatched embryos, dead or unfertilized embryos, number of empty chorions, and normal and abnormal larvae. Due to adherence of sediments or other suspended particles to caged embryos, it was difficult to determine incidence of non-fertilized versus embryos with arrested development, thus these embryos were counted as dead/unfertilized. Partially hatched larvae (embryos/larvae that had partially exited the

chorion but were non-viable, see Figure 3-6) were counted as non-hatched embryos. Larvae were defined as normal if they had straight body axes, lack of pericardial or yolk sac edema, regularly beating hearts, and ability to swim and respond to stimuli (touch). Normal larvae (up to 50) were transferred to dishes containing ½ FSW for larval survival (see below). Abnormal larvae exhibiting scoliosis, yolk and/or pericardial edema, or opacity were removed, and any live abnormal larvae were euthanized in an overdose of MS-222. Subsequent daily counts enumerated only eyed non-hatched embryos, partially hatched embryos, abnormal larvae that were removed and euthanized, and normal larvae transferred to glass culture dishes for larval survival or behavior studies.

For caged embryos, upon arrival at the lab, a section of mesh containing up to 200 embryos (assessed macroscopically) was removed from the larger mesh and placed into 250 ml glass culture dishes containing 200 ml ½ FSW and incubated in a 12˚C incubator. Most of the sites showed evidence of hatching (empty chorions) prior to retrieval, so initial numbers of embryos for monitoring purposes were reduced. Daily water changes of ½ FSW were performed for the duration of incubation and counts were performed daily for 2-6 days, based on the variability in days to hatching observed between sites.

Normal hatching was defined as the number of normal larvae per total number of hatched and unhatched embryos combined.

2.7.2 Larval Survival. Normal larvae from caged embryos (N = 6 sites with 4-5 cages/site) or natural spawns (N = 8 transects/site for San Rafael Bay, and 4 transects/site for Keil Cove) were incubated in ½ FSW with 50% daily water changes. Hatching success for naturally spawned herring embryos was significantly reduced at Peninsula Point (no normal larvae), Sausalito (only 1 normal larva), and Keil Cove (only 4 transects with normal larvae, and no normal larvae from the other 4 transects). Thus, monitoring for larval survival was only performed for San Rafael Bay (transects N1-N8) and Keil Cove (transects N1-N4 only). Larvae were observed daily, and abnormal or dead larvae were removed and/or euthanized in MS-222. Types of abnormalities (body axis defects, edema, opacity) were recorded. Typically larvae that appeared moribund were incubated for an additional day. Observations were carried out for 4-6 days. In cases where few larvae were available (Keil Cove), larvae were assessed for survival, and then utilized for larval behavioral assessments. All surviving larvae were euthanized in MS-222 and larval lengths recorded. Percent survival was defined as the number of normal larvae surviving for 4-6 days per number of initial larvae.

2.7.3 Statistical Analysis. All data were arcsin transformed and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD Test for all pairwise multiple comparisons, or Kruskal-Wallis one Way Analysis of Variance on Ranks (larval survival for caged embryos). Results were considered to be significant at $p < 0.05$.

2.7.4 Swimming Behavior. *Test Chamber*: The test system consisted of a rectangular chamber, recirculating temperature control water supply, illumination, black and white CCD camera and a video recorder. A black plastic flow chamber was used for the swimming behavior tests. The overall chamber measured 284 mm long, 22 mm wide and 14 mm deep. Since video recordings were made for each test a smaller recording chamber was established (58 mm long) in the center of the main chamber. The bottom of this chamber had a clear plastic insert so that it could be illuminated from below. A fine-meshed grid was used at each end of the smaller chamber to separate it from the larger system. Flat black plastic covers were placed over the top of the mesh dividers to prevent light being "piped" up the mesh and interfering with digitizing the image. The input and outlet tubes (5 mm ID) were placed at each end of the larger chamber. Tubing from each chamber end was passed through a Cole-Parmer 1-100 rpm peristaltic pump connected to a Masterflex speed controller. The pump output side was connected to 7 foot coiled stainless steel tubing placed in a Neslab RTE 221 water bath and then attached to the input side of the chamber.

Temperature was controlled at 12°C± 1°C within the imaging chamber. Temperature measurements were made in the test chamber with a stainless steel digital thermometer (traceable to NTSF standards). There was some slight temperature increase depending upon the length of the test caused by heat from the lighting system.

Lighting System. Because the herring larvae are nearly transparent is was necessary to produce dark field images that could be detected by the CCD camera and later digitized for further analyses. An adjustable intensity fiber-light (Dolan-Jenner) was used with a fiber optic ring light (140-mm diameter, Edmunds Scientific). This lighting unit was placed 70 mm under the test chamber (clear bottom section) and covered with an IR glass filter with a 720 Hz transmission band. The use of the IR illumination was done to reduce any behavioral changes in the larvae when exposed to light from below (not a natural situation) A flat black ring (115 mm diameter) with a 63 mm hole was centered 10 mm directly below the test chamber. A Sanyo B&W CCD camera with a Fuijinon TV 1:14/25 lens was located 240 mm above the bottom of the clear test chamber. The system components, camera, black disc and ring light were carefully centered to produce near dark field. The CCD images were monitored (Ikegami Monitor) and also recorded on a Sony VHS recorder.

The characteristics of the velocity profile in the chamber are important in the consideration of swimming speed. It is difficult to maintain laminar flow at higher velocities in the chamber design used for the experiments. However, the use of fine-meshed grids produced a rectilinear front of fairly uniform turbulence. Based on dye releases in the test chamber it would appear that sufficient turbulence persists to eliminate most advantageous wall effect developing at the downstream end of the chamber.

Larvae. Larvae from cages and natural spawn samples were transferred to the Motion Analysis laboratory for swimming behavior analyses. Each set of larvae, from a single sample area (i.e., cages) was transferred and the behavioral test completed before the next set was exchanged. Samples moved to the Motion Analysis laboratory were placed in an ECHO-term chilling incubator and keep at 12°C until tested. The number of larvae used for each test set was limited to five or fewer. Four factors determined the maximum number used in each test and the number of tests that could be conducted for each sample. The first was that all larvae had to exhibit healthy behavior. Second, five larvae were the maximum that could be observed in the test and still trace the fatigue time for each larva. Third was the length of time to conduct each test and still keep up with incoming samples and lastly, in some samples there were very limited larvae available for testing. Larval behavioral testing was conducted for most samples 5-7 days after the samples were removed from the field. If larvae had significant differing body lengths, the critical velocity achieved by each larvae is adjusted to the equivalent maximum velocity of the larvae of the mean body length by means of the formula: $U_{crit} = \sqrt{}$ mean U_{crit}^2/L for the purpose of standardization. In reviewing the measured body lengths for each sample (conducted for the survival analysis) and applying the standardization formula the correction factor for a length difference of 0.2 mm would be >0.01 mm/sec. Based on this analysis, it was determined if the SD was less than 0.5 around the mean, the lengths would not be adjusted for this first analysis of swimming speed. The larvae for each behavioral test were preserved and can be measured in the future, if necessary.

Incremental Velocity Test Protocol. There are two experimental procedures to quantify swimming performance of fish, the fixed velocity (or fatigue) test and the incremental velocity test [72, 73]. Tests on juvenile fish have used widely variable test periods (minutes to hours) and flow velocities. More recent studies of larval swimming speeds for coral reef fishes used much shorter velocity increase steps (e.g. 2 minutes) [74]. During the incremental velocity tests, herring larvae were forced to swim in an increasing current field. The current velocity, and thus the swimming speed, were not increased gradually, but rather

in steps, each speed being maintained for a certain period of time until exhaustion occurs (fatigue or threshold speed).

The fatigue speed or critical speed (U_{crit}) for increased velocity tests is calculated as:

$$U_{crit} = Vp + \left(\frac{t_f}{t_i} \times Vi \right)$$

Vi = velocity increment (cm/sec)
Vp = penultimate velocity as which the larvae swam before fatigue
t_f = elapsed time from the velocity increase to fatigue
t_i = time between the velocity increments

A peristaltic pump was used to provide a recirculating water flow, which could be increased in five steps. The step velocities are as follows:

Step 2 = 0.29 cm/sec
Step 4 = 0.57 cm/sec
Step 6 = 1.14 cm/sec
Step 8 = 1.67 cm/sec
Step 10 = 1.93 cm/sec

Because of the limitations of the pump controller the differences between the steps were not evenly spaced. Step 2-4 increased by 0.28 cm/sec, step 4-6 increased by 0.57 cm/sec, step 6-8 increased by 0.53 cm/sec and step 8-10 increased by 0.26 cm/sec. While it would have been more uniform to have even velocity increases, these differences were taken into consideration by the formula used to calculate U_{crit}.

Based upon recent larval swimming reports [74, 75] and preliminary tests run on herring in our laboratory, three-minute time intervals were selected and used between velocity increments (Vi) for this project. This time interval worked well for the first series of samples when many larvae became fatigued before reaching the last velocity increment. In later samples it was found that some larvae could continue swimming, without fatigue, for over 15 minutes at the highest velocity level. Over half (53%) of the 124 individual larvae tested did not demonstrate fatigue at the highest velocity after 3 minutes of exposure. Wide ranges for U_{crit} values are frequently observed [72, 76, 77]. These prolonged times to fatigue values at the highest exposure velocity presented potential problems with analysis. If 3 minutes was scored for those fish that swam beyond the time limit, the U_{crit} might be under estimated. Extending the time to fatigue period during the last velocity step to cover those larvae that were able to swim beyond the original 3-minute time period would make the velocity increments uneven. Thus, it would not be possible to determine if larvae would fatigue at a lower velocity during a longer exposure period rather than moving to the next higher increment. Based upon these facts it was decided to conduct two types of analyses. First, 3-minute increments were used for each velocity change for a total of 12 minutes, and U_{crit} was calculated. To investigate the longer fatigue times the data were adjusted using the last increment for the 12-minute period where most of the larvae reached fatigue. Thus, all increments except the last level (1.93 cm/s) were treated as 3-minute increments.

Only larvae that could actively swim and had no visible morphological abnormalities were used for testing. Larvae were required to swim for the acclimation period, for three minutes at step 2 before the test was started, and after the completion of the tests (recovery analysis). The larvae were acclimated to the test

chamber for 20 minutes before the test was started at step 2 (0.29 cm/sec), run for three minutes, then moved to step 4 and continued to be exposed to increasing flow velocities until fatigue occurred or the time for the penultimate increment had elapsed. If a larva could not extend one body length off the downstream barrier for 1 minute it was considered fatigued**.**

Light was supplied through the bottom of the test chamber as IR (790 Hz) so that the larvae were not affected by light coming from a direction not encountered in nature. Room lights were dimmed during the test period. Recirculating water was checked in the video chamber for temperature increase after each velocity increment change. Water was pumped from the system after each full test (five velocity increments) and exchanged with 12 ˚C oxygenated water before the next test series was started.

2.8 Selection of sediment collection sites and sediment collection.

2.8.1 Subtidal sediment collection at cage deployment sites and moorings. Subtidal sediment collection at cage deployment sites and moorings. Subtidal sediments were collected at cage deployment sites as detailed in the SOP. Briefly, sediments were collected from the BML vessel *Cape Horn* or *Klamath* with a small Ponar grab deployed from the boat. At each mooring (five per site), three replicate grabs were taken adjacent to the mooring anchor. The top 2 cm of the grab contents of each of the three grabs at each mooring were then combined into an isopropyl alcohol-rinsed stainless steel bowl and thoroughly mixed with a isopropyl alcohol-rinsed stainless steel spoon. The contents of these three replicate grabs were then placed into two separate pre-labeled, rinsed ICHEM glass jars (one for analysis of PAHs, and another for sediment grain size). Therefore, at each cage deployment site, five sediment samples were collected for PAH analysis (representing a composite of three grabs at each mooring) and sediment grain size analysis. All samples were placed on ice in the field and transported on ice to the BML. The jars for PAH analysis and sediment grain size analysis, respectively, were transferred to the locked freezer and refrigerator at the BML.

2.8.2 Intertidal sediment collection at natural spawn sites. Intertidal sediments were collected at the natural spawn sites as detailed in the SOP manual, with the exception that "cookie cutter" devices were not used to collect sediments. Instead, an isopropyl alcohol-rinsed large stainless steel spoon was used to hand-collect all intertidal sediments by wading Sampling positions were chosen to coincide with locations where marine vegetation (or substrate) with attached herring eggs at the natural spawn sites. At each natural spawn site, we conducted five separate ~10-m transects perpendicular to the shoreline, moving from high water to low water within the intertidal zone, which were conducted at the 10-m, 30-m, 50-m, 70-m, and 90-m marks of the original 100-m transect laid out for collection of natural spawn samples (parallel to the shoreline). The individual transects perpendicular to the shoreline were intended to encompass the range of suitable herring spawning habitat, from the high intertidal to low intertidal. Depending on the shoreline grade, the length of each transect covered from approximately 5-m to 10-m. In all cases, sediments were collected within the intertidal area where natural spawn samples had been collected. The only exception to this transect pattern was at the Sausalito site, where the five transects were conducted at every 10-m mark along the original transect where the natural spawn samples were collected at the Sausalito Bay site (corresponding to natural spawn samples N1 through N5). This change was necessary because the appropriate sediments were not available within the Spinnaker Cover portion of the Sausalito site. As with the subtidal locations, samples from each transect were combined into a stainless steel bowl, mixed thoroughly and divided into two separate 4 oz. ICHEM jars (one for analysis of PAHs, and another for sediment grain size). All samples were placed on ice in the field and transported on ice to the BML. Samples for PAH analysis and sediment grain size analysis, respectively, were transferred to a locked freezer or refrigerator for storage at the BML.

2.9 Analytical chemistry. Analyses of the whole body and ovary samples of adult female herring for PAHs, POPs and lipid content, as well as PAH and lipid analyses of natural spawn and cage-deployed eggs, were conducted as described in the SOP manual. For the PAH analyses of the PEMDs, the following modification was done. An additional cleanup step using size-exclusion high-performance liquid chromatography was conducted for each PEMD extract to remove any additional compounds that were found to interfere with PAH determinations (as determined from PEMD test samples).

2.10 Data analysis and statistics. For the biological responses, statistical treatments are described independently for each section or figure. For analytical chemistry, concentrations of sum LAHs, sum HAHs and sum PAHs, as well as the various sum values of POPs (i.e., sum PCBs, sum DDTs) were $\log_{10}$-transformed and the percent lipid values were arcsine transformed to increase the homogeneity of variances. One-way analysis of variance (ANOVA) and the Tukey-Kramer HSD Test were used to determine if mean concentrations of PAHs, POPs, percent lipid or dry weight values varied among collection sites. The Tukey-Kramer HSD Test is one of a number of post-hoc methods recommended to use to test differences between pairs of means among groups that contain unequal sample sizes. The correlations between percent lipid and contaminant concentrations, as well as dry weight and contaminant levels, between paired whole body and ovary samples were assessed by simple correlation analyses. One-way ANOVA and simple t-Test were used to compare mean concentrations of contaminants, percent lipid and dry weight values between whole body and ovary samples of adult female herring. If the sum contaminant value was reported as less than the lower limit of quantification (< LOQ) in a sample, a value of zero was substituted for this value prior to calculating the mean and standard error values and conducting statistical analyses. All statistical analyses were completed using JMP Statistical Software (SAS Institute, Inc., Cary, NC). The level of significance used for all statistical tests was $\alpha \leq 0.05$.

Section 3: Results

3.1 Overview of study sites. Sites were selected based on likelihood of proximity to oiled substrates as determined by SCAT surveys and other observations of oiling. The sites (Table 3-1) differed in the degree of oiling and cleanup, so the actual amount of residual oiling at each location at the time of the assessment is unknown. Accessibility, safety, and stability of moorings were also considered. Reference sites were chosen to most closely match the habitat, temperature, and salinity conditions at sites within the (visible) spill zone. Satellite images showing locations of natural spawn subtransects and caged embryo/ PEMD placement are shown in Figure 3-1. Green pins show caged embryo moorings, with black diamonds indicating moorings with PEMDs. Blue pins indicate natural spawn sample locations. Natural spawn of sufficient density was only available at four of the six sites where caged embryos were deployed. These were Sausalito, Peninsula Point, Keil Cove, and San Rafael Bay. All natural spawn occurred in the intertidal zone, while caged embryos were all incubated in the shallow subtidal (-3 to -6 ft mean low water).

Table 3-1: Characteristics of study sites

site	NRDA designation	SCAT rating	cleanup	land use and other features related to PAH inputs
Horseshoe Cove (HC)	MRP04	moderate-light	extensive wiping of rip-rap	marina, adjacent to US101
Sausalito (SA)	MRQ10/P01	very light-light	some wiping	marina, commercial, residential
Peninsula Point (PP)	MRQ01	light	some wiping	residential
Keil Cove (KC)	MRR20	heavy-light	extensive wiping, removal of rock	residential, undeveloped
Point San Quentin (PSQ)	MRT04	no oil	NA	residential, industrial, adjacent to I580
San Rafael Bay (SRB)	MRU01	no oil	NA	commercial, adjacent to I580

Figure 3-1A: Horseshoe Cove

Figure 3-1B: Sausalito

Figure 3-1C: Peninsula Point

Figure 3-1D: Keil Cove

Figure 3-1E: Point San Quentin

Figure 3-1F: San Rafael Bay

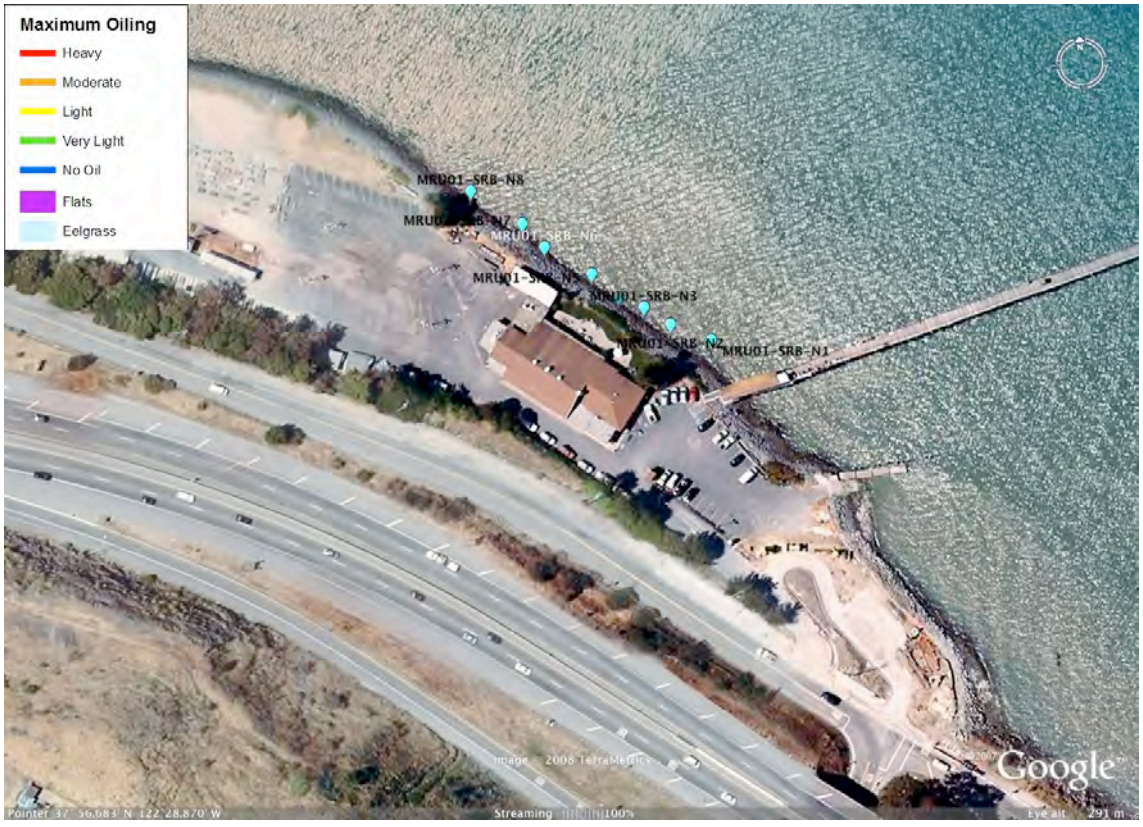

Figure 3-1G: Close-up of San Rafael Bay natural spawn grabs

3.2 High rates of body axis defects, neural tissue necrosis, and cardiac arrhythmia in natural spawn samples from oiled sites. General descriptions of natural spawn grab samples are provided in Table 3-2. The predominant substrates were brown and red bladed algae such as *Fucus*, *Cryptopleura*, and *Chondrocanthus*, filamentous red algae (e.g. *Gracilaria, Microcladia*, or *Odonthalia*), and some green algae (*Ulva*). The highest density spawn was at San Rafael Bay adjacent to the Marin Rod and Gun Club, where the samples were collected almost exclusively on *Fucus*. *Fucus* also predominated at Sausalito, but samples were much more variably mixed at Peninsula Point and Keil Cove, the former predominated by filamentous red forms. Typical samples are shown in Figure 3-2. The spawn at San Rafael Bay was medium density, approaching 4 layers of embryos (Figure 3-2E). Spawning at the three oiled sites was less dense, ranging from very light "salt-and-pepper" density (Figure 3-2C) to light density with contiguous patches of a single layer (Figure 3-2D).

Table 3-2: Characteristics of natural spawn samples

site	dates of deposition/date sampled	spawn density	predominant substrate
SA	20-22 Feb/27 Feb	light	*Fucus*
PP	20-22 Feb/29 Feb	very light-light	mixed, *Gracilaria, Fucus*, *Cryptopleura*, *Chondrocanthus*, *Ulva*
KC	20-22 Feb/28 Feb	very light-light	mixed, *Fucus*, *Cryptopleura*, *Chondrocanthus*, *Gracilaria, Ulva*
SRB	17-19 Feb/26 Feb	medium	*Fucus*

Because of the requirement to document cardiac function with digital video, only embryos with obvious heart beats were selected for imaging. This was a challenge in most natural spawn samples from the three oiled sites. Viable embryos were scored for gross abnormalities including body axis defects, tissue opacity (indicative of necrosis), and pericardial or yolk sac edema. Cardiac abnormalities were scored in video clips as arrhythmia based on the presence of atrioventricular conduction block, silent ventricle, severe bradycardia, minimal overall contractility, or complete absence of heart beat. Representative images of dechorionated embryos from natural spawn grabs by site are shown in Figure 3-3, and scores for abnormalities summarized in Table 3-3. The most striking features of embryos obtained from Sausalito, Peninsula Point, and Keil Cove were high rates of body axis defects (Figure 3-3) and tissue opacity (Figure 3-4). These abnormalities were consistently observed at all three oiled sites, but were entirely absent in samples from San Rafael Bay (Figure 3-4A). At Sausalito, body axis defects were observed in 7/8 grab samples, and occurrence ranged from 25-82% (mean 60%, $p < 0.05$). At Peninsula Point, body axis defects were observed in 8/8 grabs, and occurrence ranged from 85-100% (mean 98%, $p < 0.05$). At Keil Cove, body axis defects were observed in 8/8 grabs, and occurrence ranged from 60-100%

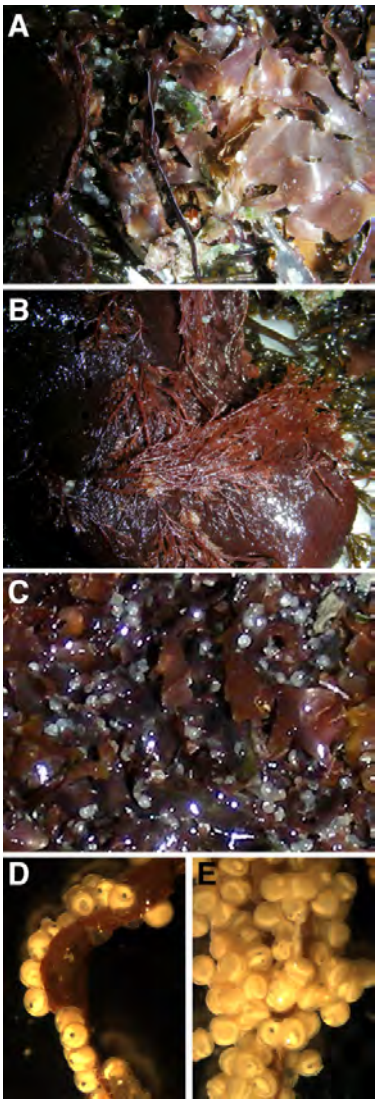

Figure 3-2: Representative natural spawn samples. (A) and (B) Mixed algae typical of Peninsula Point and Keil Cove. (C) Close-up of fresh natural spawn sample from Peninsula Point with "salt-and-pepper" density. (D) Light single-layer spawn sample from Keil Cove fixed in Stockard's. (E) Medium density spawn from San Rafael Bay (Stockard's fixed), up to four embryos deep at points.

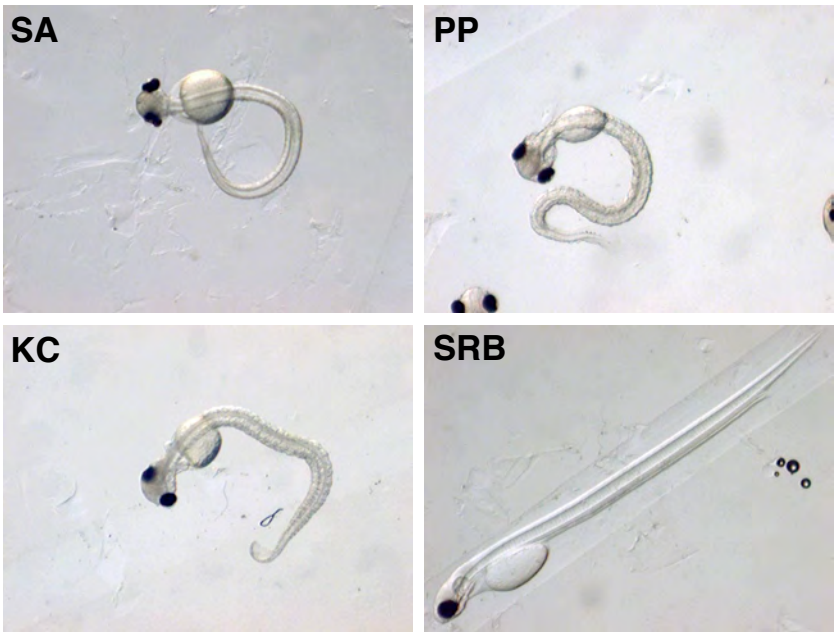

Figure 3-3: Body axis defects in natural spawn samples from oiled sites compared to typical normal morphology from San Rafael Bay samples

(mean 90%, $p < 0.05$). The body axis defects did not appear to be a malformation *per se*, but rather a failure to straighten after dechorionation (movie files available) due to loss of neuromuscular capacity.

Notably, the primary tissue opacity observed was in the developing central nervous system, starting anteriorly in the brain (Figure 3-4). CNS opacity appeared to progress from a pair of bilateral structures in the diencephalon and ventral midbrain in milder cases (Figure 3-4D - F), to the entire brain and anterior spinal neural tube in the most severe cases (Figure 3-4B, C). These defects appeared identical in embryos from Sausalito, Peninsula Point, and Keil Cove (Figure 3-4D - F). At Sausalito, tissue opacity was observed in 7/8 grabs, with the same grab (#7) showing an absence of both body axis defects and tissue opacity. Opacity could not be quantified in two grabs due to inconsistent lighting in the images. However, in the five remaining grabs, the occurrence ranged from 55-71% (mean 60 %, $p < 0.05$). At Peninsula Point, tissue opacity was observed in 8/8 grabs, and occurrence ranged from 85-100% (mean 96%, $p < 0.05$). At Keil Cove, tissue opacity was observed in 8/8 grabs, and occurrence ranged from 55-100% (mean 86%, $p < 0.05$).

Due to the failure of most embryos to straighten after dechorionation, images of the appropriate lateral view often could not be obtained for samples from Sausalito, Peninsula Point, and Keil Cove. This precluded the planned quantitative measure of pericardial edema in individuals (as detailed in the SOP). Instead a binary score was obtained for the presence of absence of edema. Edema was defined as increased ratio of pericardial space to apparent heart volume in addition to flattening of the yolk profile from its typical radial curvature (Figure 3-5), or the presence of fluid between the yolk and lateral portion of the yolk sac. At Sausalito, edema was observed in 8/8 grabs, and occurrence ranged from 5-76% (mean 33%, $p < 0.05$). At Peninsula Point, edema was

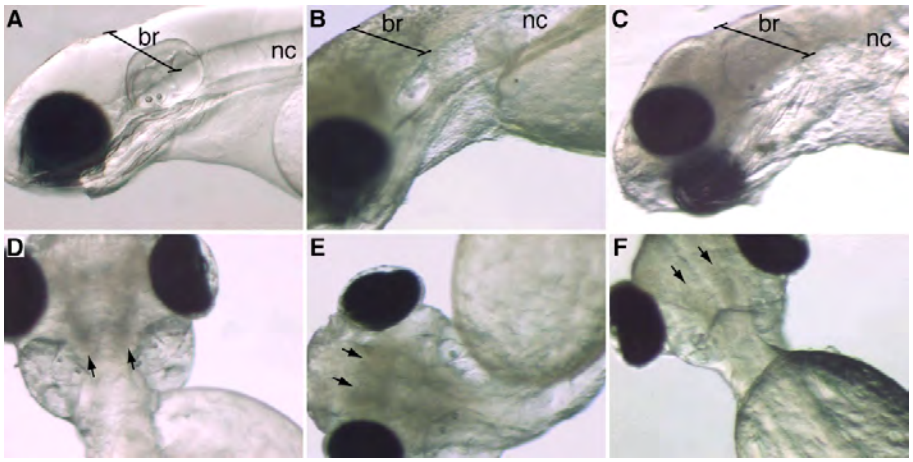

Figure 3-4: CNS opacity in natural spawn samples from oiled sites. (A-C) lateral views of the head. (A) Typical translucent embryo from San Rafael Bay. The line indicates the span of brain tissue (*br*), which lies above anterior portion of the notochord (*nc*). (B) Embryo from Peninsula Point. (C) Embryo from Keil Cove. (D-F) Dorsal or ventral views, arrows indicate bilateral structures in the base of the diencephalon/midbrain. Embryos are from (D) Keil Cove, (E) Peninsula Point, and (F) Sausalito.

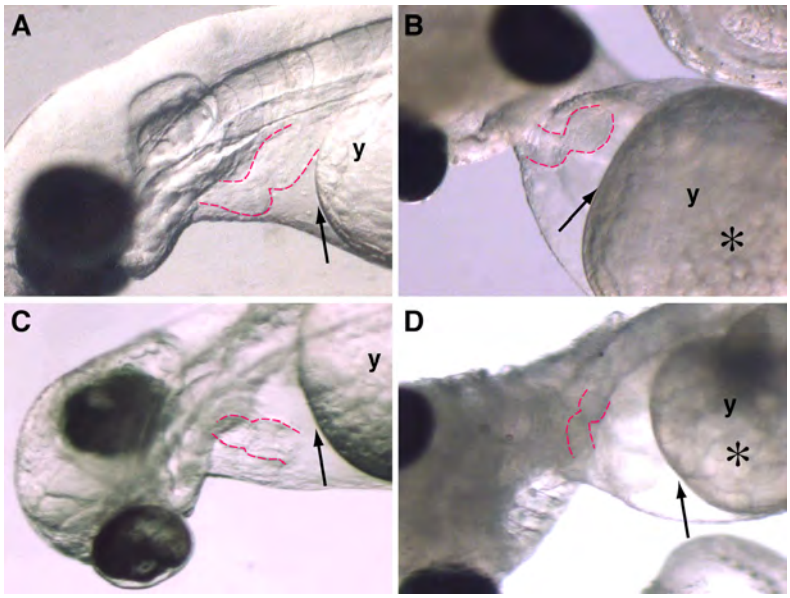

Figure 3-5: Pericardial edema in natural spawn samples from oiled sites. (A) San Rafael Bay, (B) Sausalito, (C) Peninsula Point, (D) Keil Cove. Red dashed lines indicate the outline of the heart. In the embryos from SRB (A), the heart takes up most of the pericardial space, and the anterior end of the yolk (*y*) is smoothly arced (arrow). The yolks in embryos from oiled sites (B-D) are flattened anteriorly (arrows), and in many cases yolk platelets had an abnormal appearance (asterisks).

observed in 6/8 grabs, and occurrence ranged from 5-26% (mean 11%, $p > 0.05$). At Keil Cove, edema was observed in 8/8 grabs, and occurrence ranged from 5-20% (mean 11%, $p > 0.05$). At San Rafael Bay, a single edematous embryo was observed in each of two grabs for a mean of 1%.

Finally, cardiac function was assessed in video clips for each individual. Abnormal heart rhythms were observed in 8/8 grabs from Sausalito, with occurrence ranging from 15-76% (mean 48%, $p < 0.05$). At Peninsula Point, arrhythmia was observed in 8/8 grabs, with occurrence ranging from 50-100% (mean 91%, $p < 0.05$). At Keil Cove, arrhythmia was observed in 8/8 grabs, with occurrence ranging from 45-95% (mean 70%, $p < 0.05$). No abnormalities in cardiac function were observed in samples from San Rafael Bay.

Table 3-3: Overall scores for abnormalities in natural spawn samples

site	measure / subtransect	N1	N2	N3	N4	N5	N6	N7	N8	mean %
SA	total	22	21	20	19	20	20	20	20	
	body axis defect	18	16	13	16	14	16	0	5	***60 ± 11***
	tissue opacity	14	15	9	nd	nd	11	0	2	***41 ± 10***
	edema	12	13	3	5	10	5	6	1	***33 ± 7***
	arrhythmia	15	16	10	9	4	11	10	3	***48 ± 7***
PP	total	20	20	20	20	20	19	20	20	
	body axis defect	19	17	20	20	20	19	20	20	***98 ± 2***
	tissue opacity	19	17	19	20	20	18	20	20	***96 ± 2***
	edema	1	4	3	4	0	5	1	0	11 ± 4
	arrhythmia	19	12	10	10	19	18	17	20	***91 ± 11***
KC	total	20	20	19	20	20	19	20	22	
	body axis defect	12	19	18	15	20	19	19	22	***90 ± 5***
	tissue opacity	11	19	16	11	20	19	19	22	***86 ± 7***
	edema	4	3	2	3	1	3	1	0	11 ± 2
	arrhythmia	9	10	17	12	15	18	13	16	***70 ± 6***
SRB	total	20	21	20	20	20	20	20	20	
	body axis defect	0	0	0	0	0	0	0	0	0
	tissue opacity	0	0	0	0	0	0	0	0	0
	edema	1	0	0	1	0	0	0	0	1 ± 0.6
	arrhythmia	0	0	0	0	0	0	0	0	0

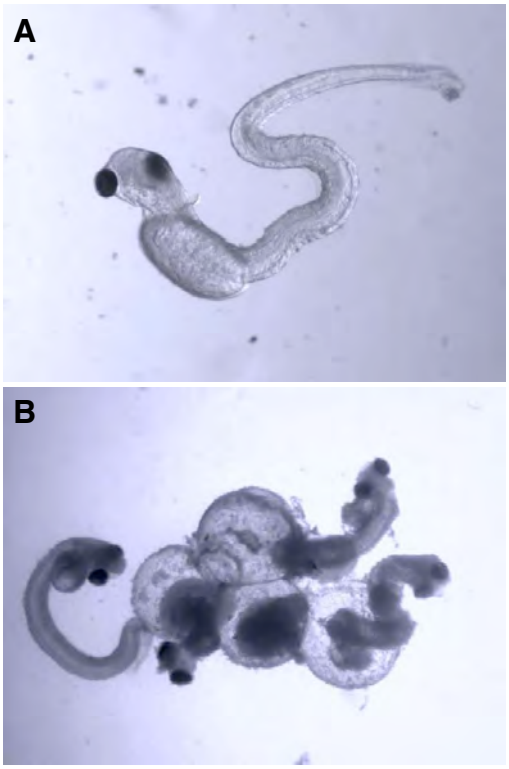

Figure 3-6: (A) Hatched abnormal larva with typical body axis defects; (B) Partially hatched larvae

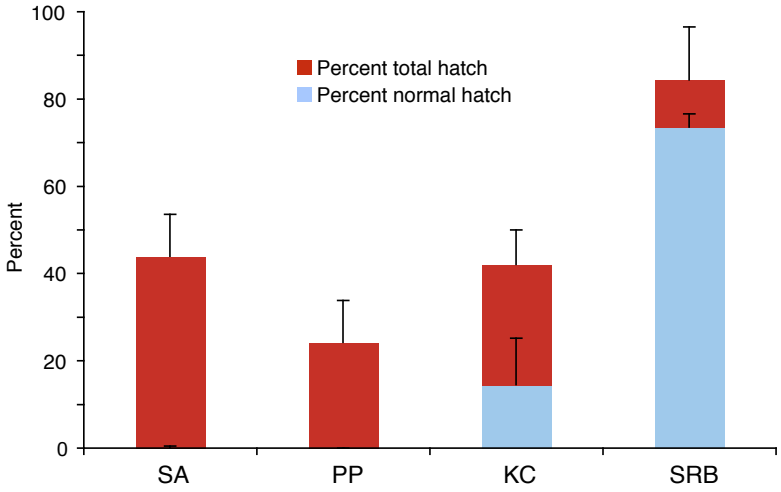

Figure 3-7: Laboratory hatching in samples of natural spawn. Red bars, percent total larvae hatched; blue bars, percent hatched larvae with normal morphology.

Because only viable embryos with obvious heartbeats were imaged, these data probably underestimate the true frequency of abnormalities. The total values presented in Table 3-3 do not reflect the number of embryos that were not assayed.

3.3 Reduced hatching success and high rates of abnormal morphology in larvae from natural spawn at oiled sites. Data for hatching rates and occurrence of abnormal morphology (Figure 3-6A) in hatched larvae are summarized in Table 3-4 and Figure 3-7. For samples from San Rafael Bay, 84 ± 11 % of the embryos incubated in the laboratory hatched, and 74 ± 3% showed normal morphology (range 48 – 94%). For samples from Sausalito, 44 ± 10% of embryos incubated successfully hatched, with only 0.1 ± 0.1% showing normal morphology. For samples from Peninsula Point, 24 ± 10% of embryos hatched, with none

Table 3-4: Numbers of natural spawn embryos assayed for hatching and percentage hatched with normal morphology

site	subtransect	N1	N2	N3	N4	N5	N6	N7	N8	mean (± SE)
SA	embryo N	83	81	81	77	91	85	84	88	83
	percent normal hatch	0	0	0	0	1	0	0	0	0.1 ± 0.1
PP	embryo N	70	69	72	61	79	53	70	55	66
	percent normal hatch	0	0	0	0	0	0	0	0	0
KC	embryo N	52	47	39	72	53	55	49	58	53
	percent normal hatch	67	6	23	19	0	0	0	0	15 ± 11
SRB	embryo N	93	71	49	81	58	53	50	63	64
	percent normal hatch	72	90	94	88	64	70	48	63	74 ± 3

showing normal morphology. Finally, for samples from Keil Cove, 42 ± 8% of embryos hatched, and 14 ±11 % had normal morphology (range 0 – 67%). Only four subtransects from Keil Cove produced larvae with normal morphology. Keil Cove grab N1 was the only sample with relatively high rates of normal larvae, and this same grab had the lowest number of body axis defects detected in dechorionated embryos (Table 3-3). Significantly higher numbers partially hatched embryos (Figure 3-6B) were observed at Sausalito and Peninsula Point. The total hatch as San Rafael Bay was significantly different from all three oiled sites ($p < 0.001$). The percentage of normal larvae hatching from San Rafael Bay samples was also significantly different from all three oiled sites ($p < 0.05$ for Sausalito and Peninsula Point, $p = 0.052$ for Keil Cove).

3.4 Reduced survival of larvae from natural spawn at oiled sites. The only oiled site with surviving larvae was Keil Cove. An average of 77 ± 4% (67-85%) from 4 grabs survived an average of 5.25 days post-hatching. In contrast, a significantly greater average of 88 ± 3% (75-100%) that hatched from 8 San Rafael Bay grabs survived an average of 5.9 days post-hatching (Figure 3-8).

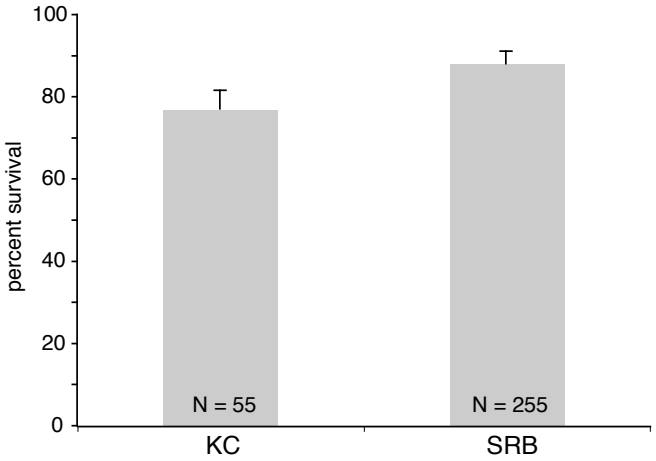

Figure 3-8: Percent survival of hatched larvae from naturally spawned embryos. Dead or abnormal larvae (abnormalities included body axis defects, edema, or opacity) were removed each day. Percent survival was determined at the end of the 5 day incubation period. (KC is significantly different from SRB; $p<0.05$; ANOVA with Tukey-Kramer HSD test for all pairwise multiple comparisons).

3.5 Behavior in larvae from natural spawn. Critical swimming speeds were determined for larvae hatched from natural spawn. However, there were only larvae available to develop U_{crit} values for two sites, Keil Cove and San Rafael Bay. There were no statistically significant differences between the mean U_{crit} for larvae from the two sites (Figure 3-9).

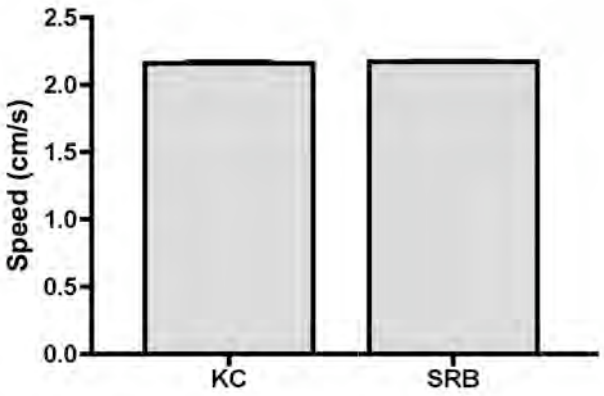

Figure 3-9: Critical swimming speed (U_{crit}) in larvae hatched from natural spawn samples. Keil Cove provided the only surviving oiled-site larvae that satisfied the requirements for swimming performance (see Section 2.7.4). There is no statistical difference between the two sites for Ucrit based on a Mann-Whitney Rank Sum Test (p= 0.469).

3.6 Increased occurrence of pericardial edema in caged embryos incubated adjacent to oiled sites. Higher magnification lateral views centered on the heart and pericardial area provided the basis for measurements (Figure 3-9). Images were screened for quality and excluded if the embryos were dorsally or ventrally rotated. In embryos exposed to either individual petrogenic tricyclic PAHs or unrefined crude oil (e.g. ANSCO), cardiogenic edema results in expansion of the pericardial space and alteration of the position of the heart tube relative to the ventral surface of the pericardium [50, 51, 62]. To quantify these effects, two measurements were made from lateral views, overall pericardial area and the distance between the atrioventricular junction and the ventral surface of the pericardium (i.e. surface epithelium; atrioventricular-pericardial [AVPC] distance).

For pericardial area measurements, only two cages fell into a significantly different group, A4 and A5 from Horseshoe Cove (Figure 3-10). For AVPC distance, nine cages grouped significantly different from the others (Figure 3-11), two from Horseshoe Cove (A1 and A2), all five

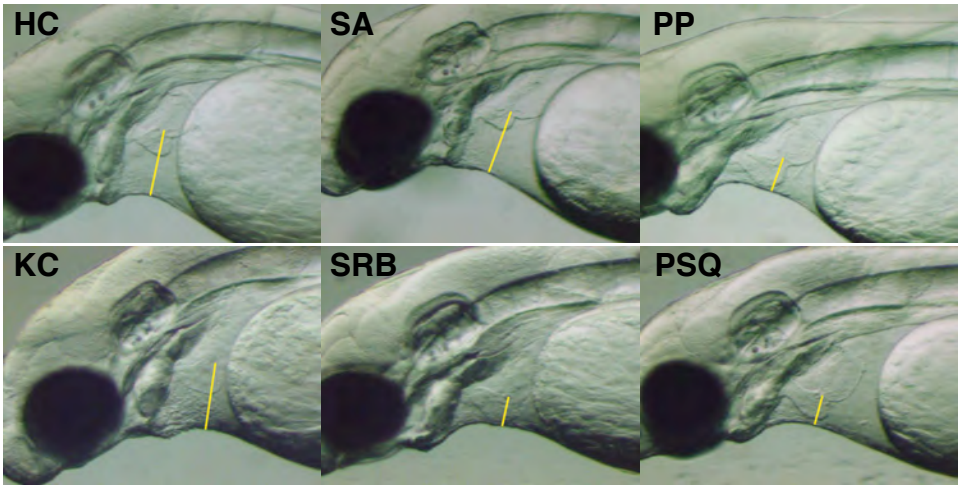

Figure 3-9: Representative images from each site used for pericardial measurements. The yellow line indicates the AVPC distance measurement.

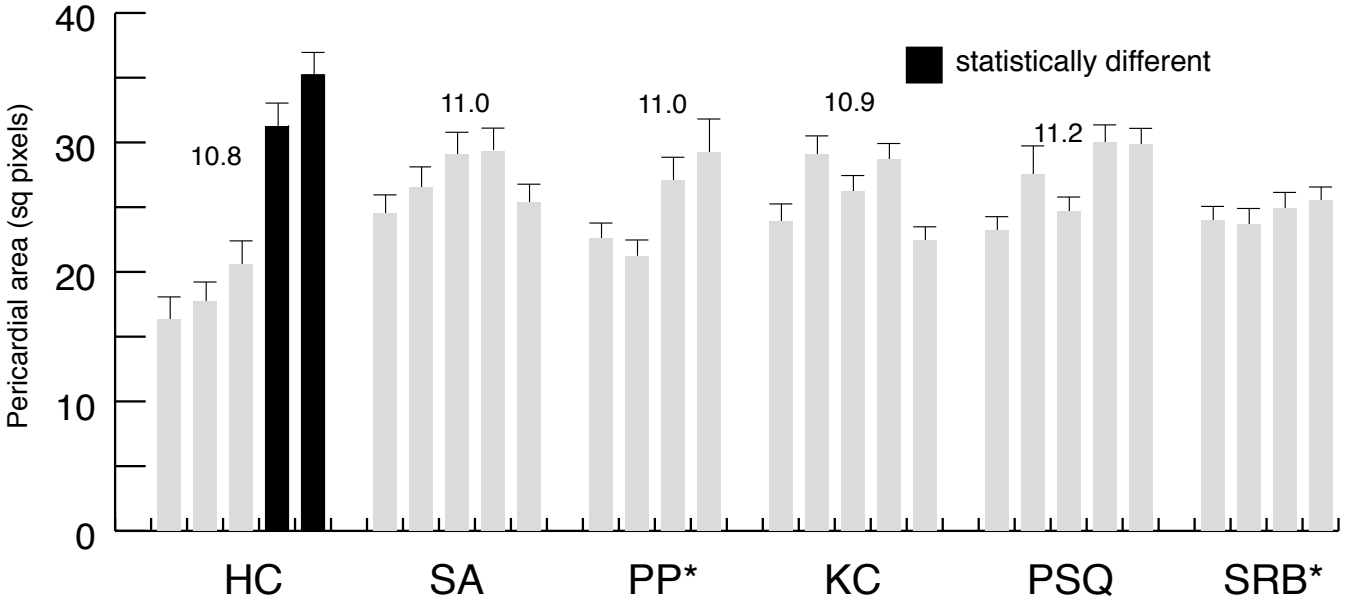

Figure 3-10: Pericardial area by cage.*N = 5 cages for all sites except Peninsula Point, where one cage contained no eggs when retrieved, and San Rafael Bay, where one cage was lost due to current during retrieval. Individual means are derived from N = 15-30 after excluding images that failed QC. Black bars indicate statistically different cages determined by One-way ANOVA followed by Tukey's HSD test (α = 0.05). Mean temperature logged during incubation is shown above each set. Logger failed for SRB.

Sausalito cages, and two cages from Keil Cove (A1 and A2). Based on the mean AVPC distance calculated for each site, two sites stood out as significantly different (Sausalito and Keil Cove; Figure

3-12). Horseshoe Cove, Peninsula Point, Point San Quentin, and San Rafael Bay all grouped together statistically. Between both measurements, all individual cages that were significantly different came from oiled sites.

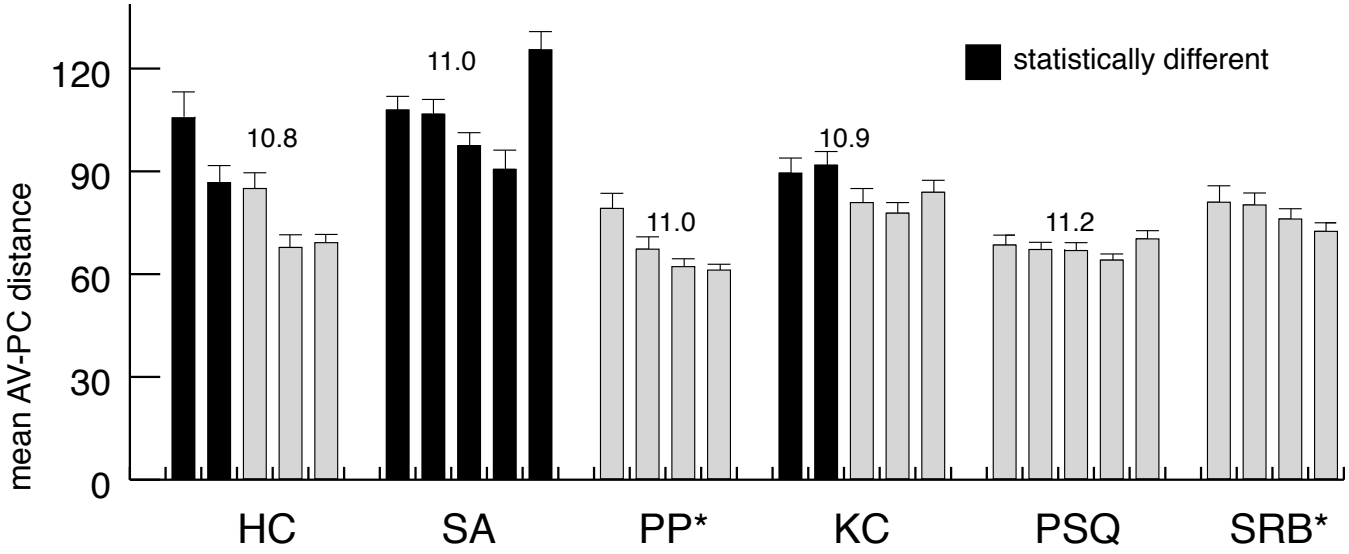

Figure 3-11: Atrioventricular canal - pericardial distance by cage. *N = 5 cages for all sites except Peninsula Point, where one cage contained no eggs when retrieved, and San Rafael Bay, where one cage was lost due to current during retrieval. Individual means are derived from N = 15-30 after excluding images that failed QC. Black bars indicate statistically different cages determined by One-way ANOVA followed by Tukey's HSD test (α = 0.05). Numbers above each set of bars is the mean temperature logged during incubation. Logger failed for SRB.

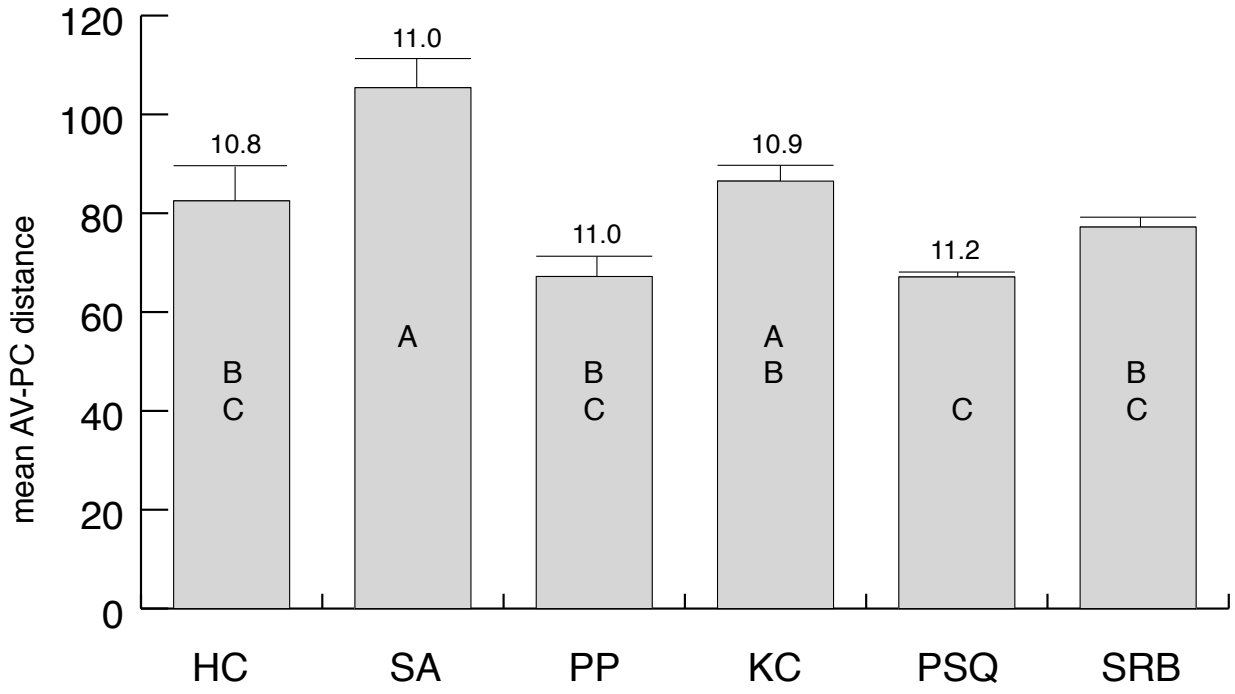

Figure 3-12: Atrioventricular canal - pericardial distance by site. Site means were derived from 5 cages at Horseshoe Cove, Sausalito, Keil Cove, and Point San Quentin, and from 4 cages at Peninsula Point and San Rafael Bay. Letters indicate statistically similar groupings determined by One-way ANOVA followed by Tukey's HSD test (α = 0.05). Numbers above each set of bars is the mean temperature logged during incubation. Logger failed for SRB.

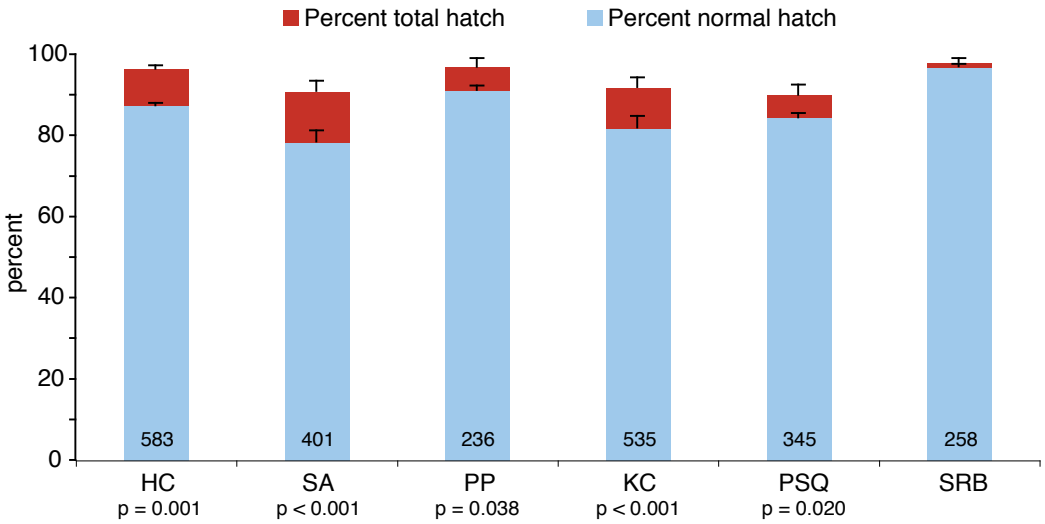

Figure 3-13: Mean hatching success of embryos incubated in deployed cages. Total hatched larvae as percent of embryos are indicated by red bars, percent of total with normal morphology indicated by blue bars. Total number of embryos indicated at the bar base. P-value in comparison to SRB indicated below other site names.

3.7 Hatching success and morphology of larvae from caged embryos. Average total hatching for caged embryos incubated in the laboratory ranged from 79 ±2 % at Sausalito to 97 ± 1% at San Rafael Bay (Figure 3-12), differences that were not statistically significant. In contrast, of embryos that did hatch, significantly fewer numbers with normal morphology were observed at all four oiled sites, but also at Point San Quentin. In concordance with observations AVPC distance (Figure 3-11), Sausalito and Keil Cove showed the lowest numbers of hatched larvae with normal morphology.

3.8 Larval survival from caged embryos. Average survival in laboratory incubations for larvae from caged embryos ranged from 78% at Point San Quentin to 92% at San Rafael Bay. No statistical differences in survival were observed between any of the sites ($p > 0.119$) (Figure 3-14).

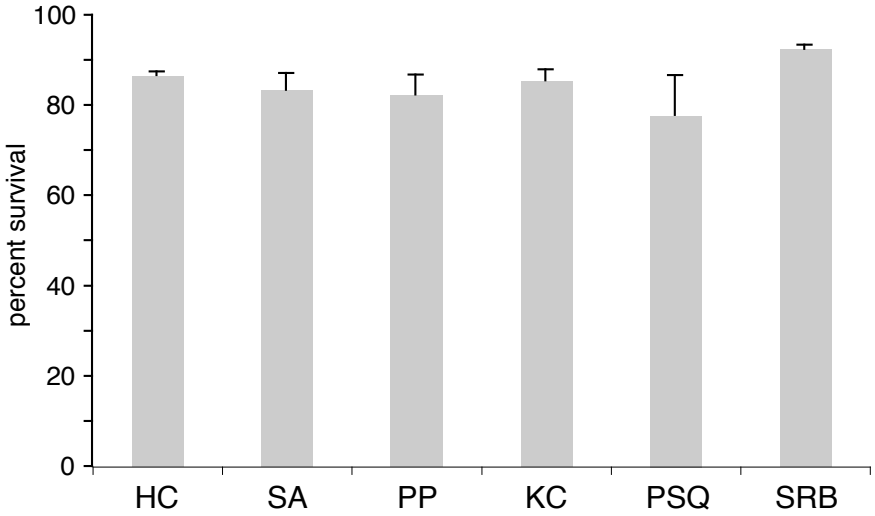

Figure 3-14: Percent survival of hatched larvae from naturally spawned embryos. Dead or abnormal larvae (abnormalities included body axis defects, edema, or opacity) were removed each day. Percent survival was determined at the end of the 5 day incubation period. (KC is significantly different from SRB; $p<0.05$; ANOVA with Tukey-Kramer HSD test for all pairwise multiple comparisons).

3.9 Lower critical swimming speed in larvae hatched from cages incubated at oiled sites. Results of the critical swimming speed analysis were combined for each set of cages at each station. For the first analysis, the 3-minute increment steps were used for a total of 12 minutes and the U_{crit} calculated. Based on this analysis,

station PP and KC are statistically different from the other locations (Figure 3-15). A Kruskal-Wallis one-way ANOVA of Variance on Ranks was applied to determine differences between stations (P= <0.001). A second analysis was conducted by adjusting the last increment to a 12-minute exposure rather than the 3-minute used in the first analysis. This was done to compensate for the fact that over half of the larvae tested did not reach fatigue within 3 minutes at the highest speed (1.93 cm/s). There were no statistically significant differences between the two different approaches to determining the U_{crit} values. To determine within-site variability, the six stations were analyzed individually by comparing the results for each cage within the station (Figure 3-16). Sample sizes were small so that statistical analyses were limited. One-way ANOVAs were conducted on the U_{crit} values for all stations except SRB where a Kruskal-Wallis ANOVA on Ranks was applied. These results indicate no statistical differences between cages at the HC site while three of the cages were statistically significant at the SA site. All other sites had one cage at each station that was statistically significant with site PP having two cages that were significantly different.

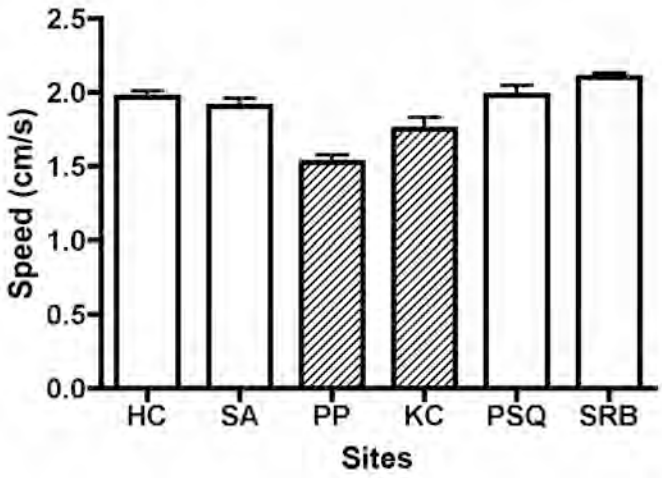

Figure 3-15: Mean critical swimming speed in larvae from caged embryos by site. Sites PP and KC are statistically different from the remaining locations. The individual cage results were combined for each station. A Kruskal-Wallis One Way ANOVA of Variance on Ranks was applied to determine differences between stations (P = <0.001).

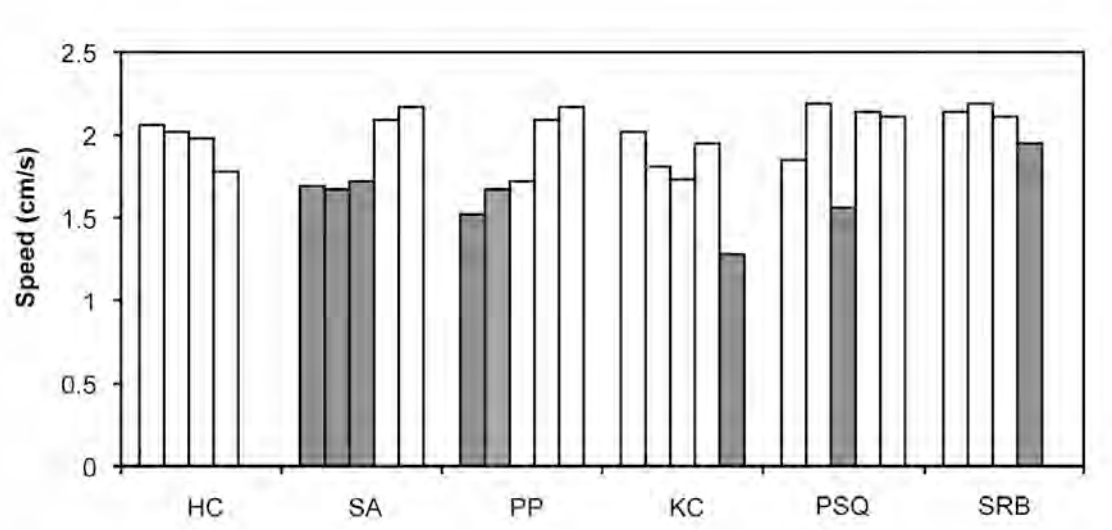

Figure 3-16: Mean critical swimming speed in larvae for each cage. Dark bars indicate a statistically significant difference between cages within the station. A one way analysis of variance was conducted on all the stations groups except for station SRB where a Kruskal-Wallis ANOVA on Ranks was used.

3.10 Background levels of PAHs and POPs in reproductively mature Pacific herring from San Francisco Bay. Very low levels of PAHs were detected in herring entering San Francisco Bay during the 2007-2008 spawning season. In samples of fish captured at Richardson Bay and South of the Bay Bridge in February 2008 (Table 3-5), concentrations of sum PAHs ranged from 23 – 52 ng/g in whole bodies and 8.6 – 13

Table 3-5: PAHs and POPs in maternal tissues of herring in San Francisco Bay and Puget Sound

				Percent	Mean (± SE), ng/g, wet weight			Mean (± SE), ng/g, wet weight		
Tissue	Region	Site	Year	lipid	Sum LPAHs	Sum HPAHs	Sum PAHs	HCB	Sum DDTs	Sum PCBs
Whole body	SF	Richardson Bay (n=6)	2008	3.4 ± 0.24	44 ± 1.7	3.2 ± 0.3	47 ± 11	0.95 ± 0.10	33 ± 4.0	12 ± 0.61
	PS	Port Orchard (n=56)	1999-2004*	6.4	NA	NA	NA	1.6	19	160
	PS	Quartermaster Harbor (n=10)	1999-2004*	8.1	NA	NA	NA	1.8	19	120
	PS	Cherry Point (n=20)	1999-2004*	3.3	NA	NA	NA	0.44	11	41
Ovary	SF	Richardson Bay (n=6)	2008	0.40 ± 0.10	11 ± 0.7	0.5 ± 0.1	12 ± 0.6	0.28 ± 0.03	2.8 ± 0.32	1.6 ± 0.40
	PS	Port Orchard (n=2)	2001†	3.1 ± 0.14	23 ± 0.0	2.2 ± 0.1	25 ± 0.1	1.3 ± 0.07	5.5 ± 0.57	51 ± 7.1
	PS	Quartermaster Harbor (n=1)	2001†	2.9 ± 0.0	24	1.1	25.1	1.1 ± 0.0	4 ± 0.29	44 ± 0.0
	PS	Cherry Point (n=2)	2001†	2.8 ± 0.1	23 ± 0.7	1.6 ± 0.7	24 ± 0.0	1.1 ± 0.07	12 ± 0.07	70 ± 18

*Data from West et al., 2008, Sci. Total Environ. 394:369-378. Standard error values not reported.
NA - not analyzed for PAHs
†Data from O'Neill and West, WDFW PSAMP

ng/g. Low molecular weight PAHs (LAHs) comprised more than 90% of the sum PAHs measured in both whole body and ovary samples, and naphthalenes represented roughly 90% of the sum LAHs (Table). For the Richardson Bay fish, mean sum PAH levels (based on wet weight) in the whole bodies and ovaries were significantly different ($p < 0.0001$), with whole body concentrations being 3-6 times higher as those measured in ovaries. Similarly, significant differences ($p < 0.0001$) in mean sum LAHs and HAHs values were also found between these tissues. Percent lipid values were significantly correlated with the ovary sum LAHs ($r^2 = 0.9177$; $p = 0.0017$) and sum PAHs ($r^2 = 0.7931$; $p = 0.0109$) concentrations whereas the percent dry weight values of the ovaries were not significantly correlated with these sum LAH, HAH or PAH values at the $p < 0.05$ level. For herring whole body samples, lipid concentrations were not significantly correlated with sum LAHs ($p = 0.1258$), HAHs ($p = 0.2279$) or sum PAHs ($p = 0.1275$). Dry weight percentage values were also not correlated with herring body sum LAH ($p = 0.3415$), HAH ($p = 0.6218$) or PAH ($p = 0.3912$) levels.

Persistent organic pollutants (POPs) were also measured in whole body and ovary samples of the San Francisco Bay herring, with DDTs and PCBs being the most abundant classes of POPs (Table V). Other classes of POPs (e.g., chlordanes, polybrominated diphenyl ethers, hexachlorocyclohexanes) were also detected in the herring whole body samples but were less than the limit of quantification (< LOQ) in the ovaries. Sum DDTs and PCBs (based on wet weight), as well as percent lipid values, were significantly different ($p < 0.0001$) between the tissues of the Richardson Bay fish, with whole body levels being 4–18 times higher than the ovary values. Percent lipid values were not strongly correlated with wet weight concentrations of sum DDTs and sum PCBs measured in whole body ($p = 0.1551$ and $p = 0.9190$, respectively) or ovary ($p = 0.1815$ and $p = 0.1461$, respectively) samples. Similarly, no significant relationships were found between percent dry weight values and sum PCBs or sum DDTs in herring whole bodies ($p = 0.3796$ and $p = 0.7229$, respectively) or ovaries ($p = 0.1146$ and $p = 0.6872$, respectively).

The contaminant levels determined in the California herring tissues were compared to those measured in the same tissues of herring captured from various sites in Puget Sound, WA. In general, the mean PAH levels measured in ovaries of the California herring were lower than those determined in ovaries of Puget Sound herring captured in 2001 (O'Neill and West pers. commun.) (Table X). In the whole body samples, mean sum DDT concentrations were higher in the California herring whereas the mean sum PCBs were higher in the Puget Sound fish. It should be noted that PAH concentrations have yet to be determined in whole body samples of herring captured in Puget Sound, WA so these comparisons could not be made.

3.11 PAHs detected in embryos from natural spawn samples and deployed cages. The mean PAH concentrations measured in embryos compared to background levels in ovaries from adult females are shown in Figure 3-17. The mean sum PAH levels from natural spawn samples ranged from 13 ng/g to 53 ng/g, wet weight. The highest levels were detected in embryos from Sausalito (53 ± 34 ng/g), followed by Keil Cove (40 ± 20 ng/g), with similar levels detected in embryos from Peninsula Point (14 ± 6 ng/g) and San Rafael Bay (13 ± 2 ng/g). Mean sum LAHs contributed 57 – 77% to sum PAHs in embryos from Keil Cove, Peninsula Point and San Rafael but comprised only 47% of mean sum PAHs in embryos collected from Sausalito. In cage-deployed embryos levels of sum PAHs in these samples ranged from 9 ng/g to 70 ng/g. The highest levels were detected in embryos incubated at Horseshoe Cove (70 ± 12 ng/g), followed by Sausalito (41 ± 7 ng/g), Keil Cove (16 ± 3 ng/g), Point San Quentin (16 ± 2 ng/g), Peninsula Point (11 ± 1 ng/g), with the lowest levels at San Rafael Bay (9 ± 3 ng/g). Embryos incubated at both Horseshoe Cove and Sausalito had approximately equal percentages of LAHs and HAHs (~ 50% each) contributing to the sum PAH values whereas at the other four sites, the deployed embryos contained primarily LAHs

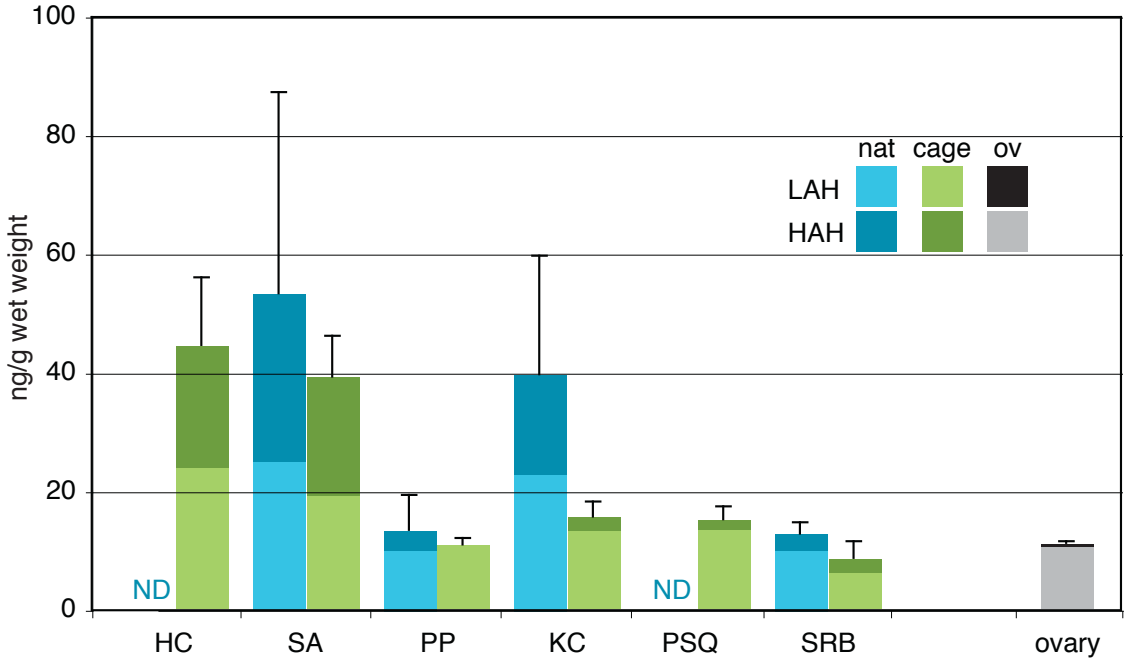

Figure 3-17: Mean sums of PAHs in embryos from natural spawn samples, caged embryos, and prespawn ovaries from SF Bay adults. Blue bars are natural spawn embryos, green bars are caged embryos; lighter shades represent sum of low molecular weight PAHs, darker shades sum of high molecular weight PAHs. ND, not determined.

(comprising > 70% of sum PAHs). Background ovary mean sum PAH level was 11 ± 0.3 ng/g and predominantly LAH naphthalenes.

We found no differences (p = 0.1468) in percent lipid values of embryos collected among the four natural spawn sites. However, significant differences (p < 0.0001) in egg percent lipid values were found among the deployment stations, with embryos from San Rafael and Sausalito containing lower lipid content than those measured in embryos deployed at Point San Quentin (Table Z). Embryos from Horseshoe Cove also had higher percent lipid values than those determined in embryos deployed at San Rafael Bay. In embryos from natural spawn samples, there was a weak correlation between percent lipid and sum LAHs (r^2 = 0.1227; p = 0.0279) whereas no significant relationships were found for lipid values and sum HAHs (p = 0.6704) or sum PAHs (p = 0.2451). Similarly for caged embryos, there was significant but weak relationship (r^2 = 0.1223; p = 0.0414) between percent lipid and sum LAHs whereas no significant correlations were found between lipids and sum HAHs (p = 0.6362) or sum PAHs (p = 0.3972

Table 3-6: Lipid content of cage-deployed embryos

Collection site	Date deployed	Date retrieved	Percent lipid
HC (n = 4)	Feb 10, 2008	Feb 17, 2008	0.39 ± 0.08*a,b*
SA (n = 5)	Feb 12, 2008	Feb 19, 2008	0.29 ± 0.04*b,c*
PP (n = 4)	Feb 18, 2008	Feb 25, 2008	0.48 ± 0.07*a,b*
KC (n = 5)	Feb 13, 2008	Feb 20, 2008	0.36 ± 0.05*a,b*
PSQ (n = 5)	Feb 18, 2008	Feb 25, 2008	0.65 ± 0.1*a*
SRB (n = 4)	Feb 15, 2008	Feb 22, 2008	0.13 ± 0.06*c*

Unlike italic letters after values in Percent lipid column indicate significant differences using Tukey-Kramer honestly significant difference (HSD) test ($p < 0.0001$).

Differences were apparent in the mean sum alkyl-phenanthrenes detected in embryos (Figure 3-18). These are the most abundant of the toxicologically relevant tricyclic PAHs in Cosco Busan bunker oil (Figure 1-2). The natural spawn samples with the highest total alkyl-phenanthrenes were Sausalito (3.4 ± 2.5 ng/g) and Keil Cove (2.3 ± 0.9 ng/g), while Peninsula Point (1.2 ± 1 ng/g) was higher than San Rafael

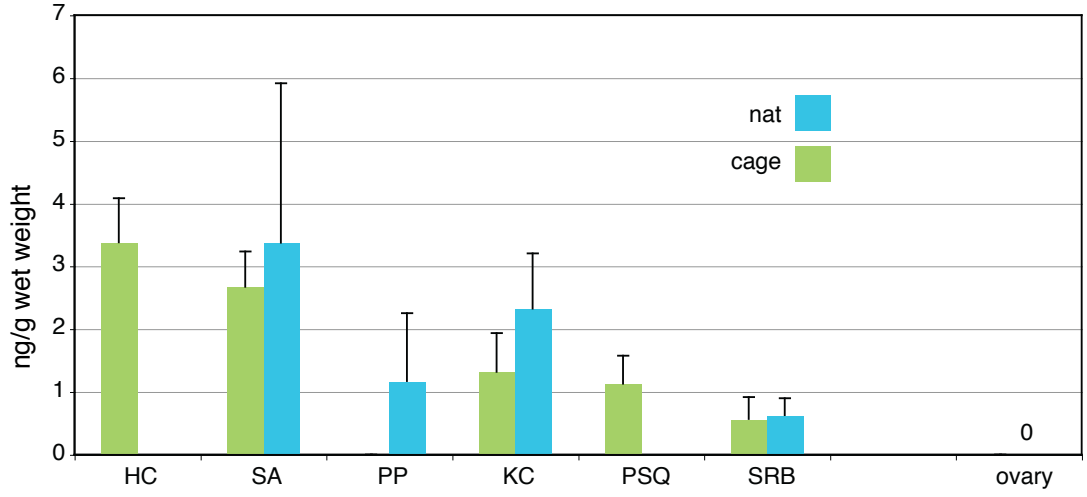

Figure 3-18: Sum alkyl-phenanthrenes (C1- through C4-PHN) in embryos from natural spawn samples, cages, and prespawn ovaries. Values are mean and standard error. Alkyl-phenanthrenes were below detection limits in ovaries.

Bay (0.6 ± 0.3). Thus, while Peninsula Point and San Rafael Bay have similar total PAH levels (14 ± 6 ng/g vs. 13 ± 2 ng/g, respectively), the levels of alkyl-phenanthrenes were 2-fold higher at Peninsula Point. A similar ranking was observed for caged embryos, where the highest alkyl-phenanthrene levels were observed at Horseshoe Cove (3.4 ± 0.7 ng/g), followed by Sausalito (2.7 ± 0.6 ng/g), Keil Cove (1.3 ±0.6 ng/g) and San Rafael Bay (1.1 ± 0.4 ng/g). Caged embryos incubated at Peninsula Point had alkyl-phenanthrene levels that were below detection limits. In both cases, Keil Cove stands out as having high alkyl-phenanthrene levels with an absence of obvious PAH inputs from land-based or other maritime sources.

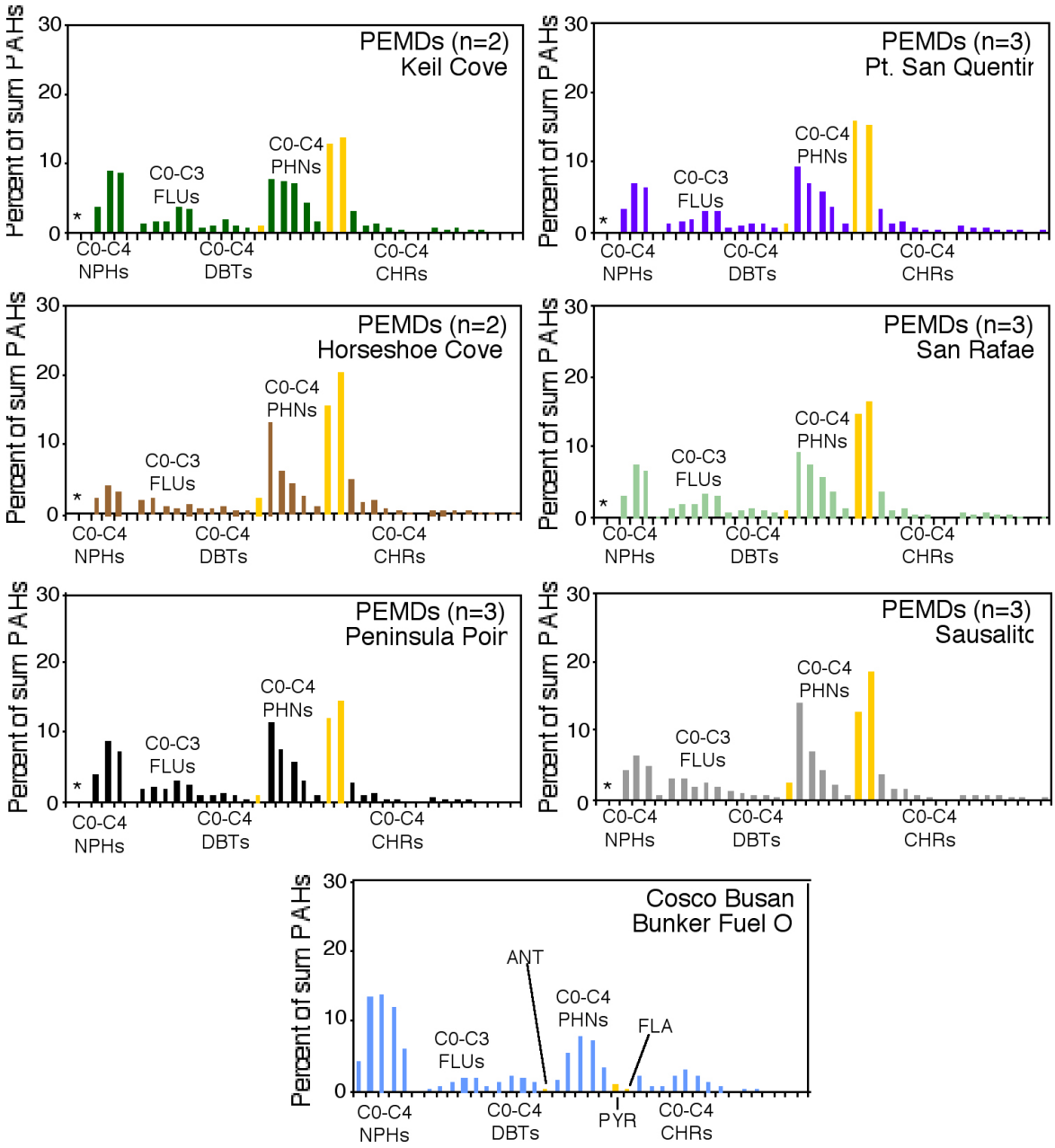

Figure 3-19: PAH patterns in PEMDs. Profiles of individual PAHs (shown as percent of sum PAHs) measured in Cosco Busan Bunker fuel oil, as well as PEMDs deployed at six sites in San Francisco Bay. Pyrogenic PAHs are shown in yellow. Because the recovery of one of the surrogate standards (naphthalene-d8) for several of the PEMDs (23 out of 30) was below our quality assurance criteria of 60%, NPH and C1-NPHs were not included in this figure. PAH abbreviations: NPHs = naphthalenes; FLUs = fluorenes; DBTs = dibenzothiophenes; PHNs = phenanthrenes; CHRs = chrysenes; ANT = anthracene; PYR = pyrene; FLA = fluoranthene.

3.12 PAHs detected in PEMDs. PAHs were measured in the PEMDs deployed at all six sites in San Francisco Bay, with sum LAHs contributing 56 – 73% to the sum PAH values. The PEMD field blanks from each site also had quantifiable levels of PAHs; however, the sum LAHs contributed more than 88% to the sum PAHs in these samples. It should be noted that the recoveries of the surrogate standard naphthalene-d8 did the not meet our minimum surrogate recovery criterion (≥ 60%) in more than 75% of the PEMD field samples and all PEMD spiked blanks. As a result, the concentrations of naphthalene and C1 naphthalenes determined for all PEMD field samples analyzed were not used for any mean calculations or PAH pattern analyses. In addition, the surrogate standard benzo[a]pyrene-d12 did the not meet our minimum surrogate recovery criterion (≥ 60%) in 2 PEMD field samples from Horseshoe Cove and Keil Cove. Because these two samples failed recovery guidelines for two out of three surrogate compounds, all concentration data from these two samples were disregarded for mean calculations and PAH pattern analyses.

We examined the PAH patterns in the field PEMDs by plotting the percent contribution of each PAH to the sum PAH values (Figure 3-19) and compared the patterns to those of the *Cosco Busan* bunker fuel oil. The most abundant PAHs measured in the samples were fluoranthene (FLA), pyrene and phenanthrene, which are PAHs that are routinely measured in environmental samples from urban marine areas. In addition, alkyl-phenanthrenes, as well as the alkylated naphthalenes, were measured in the PEMDs from the six deployment sites, as well as the bunker fuel oil sample. From these data, it appears that the PEMDs deployed in the field were exposed to both pyrogenic and petrogenic sources of PAHs.

The data for mean total alkyl-phenanthrenes are compared for each site in Figure 3-20. Similar to what was observed in embryo samples, the highest levels of alkyl-phenanthrenes were found in PEMDs deployed at Horseshoe Cove (220 ± 67 ng/g) and Sausalito (111 ± 17 ng/g), with the next highest levels at Peninsula Point (82 ± 3 ng/g) and Keil Cove (82 ± 6 ng/g), with the lowest at Point San Quentin (68 ± 5 ng/g) and San Rafael Bay (75 ± 2 ng/g).

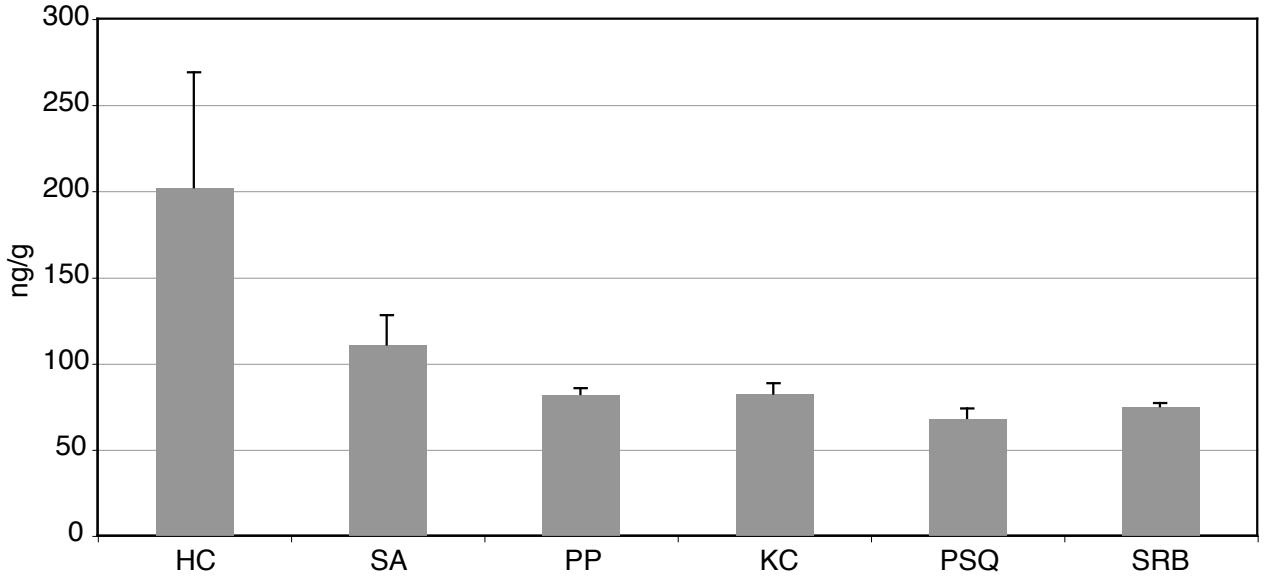

Figure 3-18: Sum alkyl-phenanthrenes (C1- through C4-PHN) in PEMDs. Values are mean and standard error.

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Appendix 1: Summary of samples collected

1. Caged embryo ouplant samples representing 6 sites, Horseshoe Cove (HC), Sausalito (SA), Peninsula Point (PP), Keil Cove (KC), San Rafael Bay (SRB), and Point San Quentin (PSQ).

   A. Image and video datasets for 30 embryos each from 5 cages at HC, SA, KC, and PSQ. For PP and SRB, 4 datasets were collected due to loss of eggs from one cage (PP) and loss of a cage at retrieval (SRB). (28 datasets total, 150 embryos per site)
   B. Fixed embryos from all imaged specimens for all sites (for CYP1A immunofluorescence).
   C. Frozen pools of 100 embryos from each of the 28 cages (for RNA isolation).
   D. Frozen 3 g samples of embryos for PAH quantification from 27 cages. A single cage from HC did not have sufficient embryos for chemical analysis (but enough for images analysis and RNA sample).
   E. Continuous temperature and salinity recordings for 5 sites; logger at SRB failed to collect data.

2. Natural spawn samples representing 4 sites, SA, PP, KC, and SRB. From each site 8 grab samples were collected along a transect.

   F. Image and video datasets for 20 embryos each for 8 grabs from all 4 sites (32 datasets total, 160 embryos per site.
   G. Fixed embryos from all imaged specimens for all sites (for CYP1A immunofluorescence).
   H. Frozen pools of 100 embryos from each of the 32 grabs (for RNA isolation).
   I. Frozen 3 g samples of embryos for PAH quantification from 32 grabs.

3. Passive samplers (PEMDs) deployed at the embryo outplant sites. 5 PEMDs for each of the 6 sites: 3 PEMDs deployed for the duration of egg incubation anchored with 3 of the 5 cages; 1 PEMD air blank exposed at deployment, 1 PEMD air blank exposed at retrieval.

4. Sediment samples for analytical chemistry. 5 samples each of subtidal sediments taken from the 6 outplant sites. 5 samples each of intertidal samples taken from 3 sites, KC, SA, and SRB.

5. Samples of adult male and female herring caught in SF Bay for analytical chemistry (addressing potential maternal transfer of PAHs/POPs to eggs). 94 fish resulting in 11 composite samples for analysis of PAH metabolites in bile, 7 composite samples for PAH/POP analysis of ovaries and carcasses.

The 2007 *Cosco Busan* oil spill: assessing toxic injury to Pacific herring embryos and larvae in the San Francisco estuary

Draft Proposal
November 30th, 2007

NOAA Fisheries
Northwest Fisheries Science Center
Environmental Conservation Division
Ecotoxicology and Environmental Assessment Programs

Introduction:

On the morning of November 7, 2007, the cargo container ship *Cosco Busan* left the Port of Oakland bound for South Korea. Soon thereafter, the vessel struck one of the support pilings for the Bay Bridge. The accident tore open the bow of the ship and spilled approximately 58,000 gallons of bunker oil into San Francisco Bay. The spill quickly broke into several small slicks. Over the next several days, the shorelines of the Central Bay (as well as areas outside of the Bay) were visibly contaminated with *Cosco Busan* oil. The degree of oiling was variable and discontinuous along the beaches, coves, and headlands of the Central Bay.

In terms of injury to natural resources in and around San Francisco Bay, the *Cosco Busan* oil spill poses a near and long term threat to a wide variety of species. These include marine mammals, birds, finfish, shellfish, epifauna, macrophytes, zooplankton, and phytoplankton. In the days immediately following the spill, the distributed oil slicks were a major concern for marine mammals and seabirds. To date, thousands of dead and oiled birds have been reported in the affected area. The subsequent oiling of the shoreline poses an ongoing risk to the many organisms that inhabit intertidal and shallow subtidal habitats.

Among finfish, the potential for injury to Pacific herring (*Clupea pallasi*) is of particular concern. As forage fish, herring are a cornerstone of the pelagic food web. They therefore play an influential role in the ecology of the estuary. Herring also constitute the only commercial fishery in San Francisco Bay, and the shoreline of the Central Bay serves as one of the largest spawning locations for herring in the state of California. Visible oiling of herring spawning habitat (as indicated by usage in recent years) has ranged from non-detectable to heavy, with the most oil observed between Keil Cove and Horseshoe Cove near the base of the Golden Gate Bridge. The season for herring spawning typically spans November to March, with peak spawning in December and January. Thus, in the weeks ahead, herring are likely to spawn on eelgrass, seawalls, riprap, and other surfaces that have been contaminated to various degrees with *Cosco Busan* oil.

Early life stages of herring are likely to be disproportionately impacted by the *Cosco Busan* spill. This is due in part to the fact that adults are expected to enter the Bay soon after the spill and begin depositing eggs in proximity to oiled habitats. Also, numerous studies have shown that herring embryos are highly sensitive to the toxicological effects of oil. This toxicity can arise from direct contact with particulate oil (e.g., droplets) or exposure to dissolved-phase oil in surrounding seawater. This raises the possibility of developmental defects and embryo mortality in locations that did not have visible shoreline oiling after the spill.

This injury assessment will characterize the toxicological responses of herring embryos to *Cosco Busan* oil under both natural and laboratory exposure conditions. The objective is to provide a scientific basis for estimating the oil-induced loss of individual herring larvae from the 2008 year-class. In preliminary discussions with the Trustees (Nov. 14th, 2007), this was identified as the highest priority in terms of assessing injury to fish. However, impacts to herring may be representative of toxicological effects on other forage fish that spawn in the nearshore. These include, for example, the California grunion (*Leuresthes tenuis*). Grunion spawners have been observed in San Francisco Bay in recent years, albeit later than the herring run (typically

beginning in March). Eggs remain on the beach in the sand for approximately two weeks and therefore may be at risk for residual oil exposure. Other forage fish that spawn in the Central Bay nearshore include northern anchovy and jacksmelt.

The proposal does not address oil exposure and potential injury to other species of fish in the San Francisco Bay. These include, for example, salmonids, leopard shark, white sturgeon, striped bass, midshipmen, rockfish, staghorn and prickly sculpin, threespine stickleback, white croaker, shiner perch, bay goby, California halibut, and starry flounder. In addition, the proposed assessment will not provide a basis for monitoring longer-term exposures to oil or recovery from injury over time for species other than herring. Certain species, such as white croaker and starry flounder, have been monitored at various times since the 1980s as sentinels for hydrocarbon exposure in San Francisco Bay (e.g., as part of the National Benthic Surveillance Program) and may therefore be useful in terms of assessing any lingering impacts of *Cosco Busan* on fish in the estuary.

This draft proposal was prepared by the Northwest Fisheries Science Center, with input from staff from both the Ecotoxicology and Environmental Fish Health Program and the Environmental Assessment Program. The proposal is intended to provide scientific and technical support for the Trustees as part of an overall resource injury assessment for the *Cosco Busan* spill. The approach proposed in this initial draft version (Nov. 30th, 2007) is likely to be modified pending further discussion with and feedback from the Trustees. Certain key elements, such as the number and specific location of sampling sites for herring eggs, have yet to be determined. Moreover, many of the logistics for the field and laboratory components of this assessment (e.g., location of an uncontaminated wet lab for processing herring spawn) are still in the planning phase.

Background:

Our current understanding of how oil exposures impact the normal development of fish embryos and larvae has been largely determined by research and monitoring in the years since the 1989 *Exxon Valdez* oil spill. Much of this work was published after 1996 and was hence unavailable to inform the response to and damage assessment for the last major oil spill in San Francisco Bay (SS Cape Mohican). The *Exxon Valdez* spill contaminated spawning grounds for Pacific herring and pink salmon. In subsequent years, a large number of field and laboratory studies revealed that the embryos of both species are highly sensitive to polycyclic aromatic hydrocarbons (PAHs) in petroleum products. In both herring and pink salmon, PAHs from weathered oil caused a common syndrome of developmental defects [1-4]. Gross malformations included pericardial and yolk sac edema, small jaws, and spinal curvature, accompanied by heart rate reduction (bradycardia) and cardiac arrhythmia. These effects of petroleum-derived PAH mixtures were subsequently documented in a variety of other teleost species [5-7] as well as in herring embryos exposed to PAH-rich creosote [8]. Overall, these biological effects occurred at total PAH concentrations that were relatively low (low ppb range) with a severity that was proportional to the percentage of tricyclic PAHs in the mixture [3,4,9].

Considerable progress has been made over the past five years in terms of elucidating the different toxicological pathways by which crude oil and individual PAH compounds disrupt fish

development. Several lines of evidence from studies using zebrafish and other experimental models have identified the developing heart as a primary target for PAHs enriched in crude oil. These studies demonstrated that the now-familiar morphological defects associated with oil exposure are (1) attributable to the tricyclic PAH fraction, (2) secondary to direct impacts on cardiac function, and (3) independent of the aryl hydrocarbon receptor/cytochrome P4501A (AHR/CYP1A) pathway traditionally associated with toxicity of high molecular weight PAHs [7,10-13]. These findings have recently been extended to Pacific herring embryos, thereby confirming that early cardiac dysfunction is the primary and earliest toxicological response to oil exposure in herring [14]. Therefore, the best available science at this time indicates that an assessment of in vivo cardiac function will be the most sensitive indicator of toxicity in herring embryos exposed to *Cosco Busan* oil.

Although oil toxicity to developing fish occurs primarily through AHR/CYP1A-independent pathways, induction of CYP1A remains a very sensitive indicator of PAH exposure. Moreover, CYP1A induction is predictive of early life stage toxicity in salmonid embryos. In pink salmon embryos exposed to dissolved PAHs in oiled gravel effluent, the lowest effects concentration for PAH toxicity (< 0.94 µg/L TPAH) was lower than the lowest concentration (< 3.7 µg/L TPAH) associated with immunologically detectable CYP1A induction [15]. In juvenile Atlantic cod exposed to dispersed oil, hepatic CYP1A induction was detected at exposure levels of 0.3 µg/L TPAH [16].

This is important because San Francisco Bay receives PAH inputs from urban, industrial, and other sources unrelated to the *Cosco Busan* oil spill. However, it is unlikely that herring embryos in the Central Bay (those not deposited on creosote-treated pilings) will have significant levels of CYP1A induction in response to these background exposures. The Central Bay locations contaminated with *Cosco Busan* oil have a much lower PAH background than the more heavily industrial and urbanized South Bay (SFEI RMP Database, http://www.sfei.org/RMP/report). Water column monitoring in recent years (2000-2006) found that dissolved TPAH concentrations for 36 parent and alkyl-PAHs in the Central Bay averaged 0.011 µg/L. The average for particulate TPAH was 0.035 µg/L. These ambient PAH levels in the Central Bay are therefore approximately 10-fold lower than the lowest TPAH concentrations reported to induce detectable CYP1A in laboratory studies. Other CYP1A inducers are similarly unlikely to cause significant induction. The average dissolved total PCB concentration in the Central Bay for 2000-2006 was 112 pg/L. This is well below the threshold for CYP1A induction by potent PCB congeners such as PCB126 [17,18].

Most of the PAHs in the San Francisco Bay are associated with bottom sediments or particulates, with a background pattern enriched in high molecular weight pyrogenic PAHs [19-21]. Pyrogenic PAHs induce a tissue-specific pattern of CYP1A induction in fish embryos that is distinct from the pattern induced by oil exposure [10,12,14]. This is due to the higher bioavailability of chrysenes in oil compared to soot-bound pyrogenic PAHs in the sediments of urbanized embayments. Therefore, the tissue-specific pattern of CYP1A induction in herring embryos exposed to petrogenic PAHs, as well as the levels of induction, should be distinguishable from CYP1A induction (if any) in herring embryos from reference locations (e.g. Tomales Bay) and non-oiled Central Bay sites. A tissue-specific and quantitative assessment of

CYP1A induction in herring embryos should therefore be diagnostic of in situ exposure to *Cosco Busan* oil.

Specific Aims:

We propose the following Specific Aims:

1. Monitor the in situ exposure of herring embryos to PAHs at sites with varying degrees of oiling.

2. Assess the toxicological response of herring embryos over a range of PAH exposures in the field.

3. Characterize the toxicity of *Cosco Busan* oil to herring embryos under controlled laboratory exposure conditions.

4. Estimate the loss of individual herring from the San Francisco Bay population due to both lethal and sublethal impacts of oil.

Injury Assessment Study Design:

1. *Monitor the in situ exposure of herring embryos to PAHs at sites with varying degrees of oiling.*

Rationale:

A simple approach to estimating herring spawn exposure to *Cosco Busan* oil would be to compare the distribution of visible oil (or tar balls) along the shoreline of San Francisco Bay with specific spawning locations for the 2007/2008 season as determined from California Department of Fish and Game field surveys. However, the presence or absence of visible oil in the days immediately after the spill may be a poor indicator of the spatial distribution of dissolved-phase PAHs weeks or months later when herring enter the Bay to spawn. To accurately characterize oil exposure to herring embryos, we will collect composite samples of herring eggs from spawning locations with different degrees of *Cosco Busan* oiling (based on SCAT maps) and compare these levels with tissue concentrations in eggs collected from urban (non-oiled) and non-urban reference locations. Cages containing artificially spawned herring will also be deployed at these sites, along with polyethylene membrane devices (PEMDs) to passively sample PAHs over the normal duration of herring egg incubation. Sediment samples will be collected along a transect in the intertidal zone as well as the subtidal location for egg collection or caging. PAHs will be analyzed in herring eggs, PEMDs, and sediments, and herring embryos from the different locations will be assessed for qualitative and quantitative patterns of CYP1A induction.

Approach:

1.1 Field collection of naturally spawned herring eggs.

Herring embryos will be collected from multiple sites along a potential pollution gradient for analyses of both oil exposure (this Aim) and oil-induced developmental toxicity (Aim 2). Eggs will be collected with a vegetation rake where herring have spawned on eelgrass or other vegetation. In areas with hard substrates, such as gravel, cobble, or riprap, eggs will be collected by SCUBA. In either case, samples will be placed in bags prior to breaking the surface of the water and will remain in bags while in transport to the laboratory to prevent contamination from boat engine exhaust and other sources of PAHs. Basic water quality conditions (e.g., temperature, dissolved oxygen) will also be maintained during transport. Eggs will then be scraped into PAH-cleaned glass jars using pre-cleaned stainless steel spatulas.

For chemical analyses, a minimum of three grams of embryos (wet weight) will be collected for each individual composite sample. For a subset of samples, additional tissue (9 g) will be collected to allow triplicate analyses for the purpose of QA.

Approximately 10 composite samples will be collected at each location in San Francisco and Tomales Bays and stored at -80 ^{o}C. Of these, three will be analyzed via GC/MS (see below). Statistical power will be computed based on the variance associated with these three initial samples. If needed, more power can be achieved with the iterative analysis of additional samples. This approach is intended to balance the tradeoff between a need for statistical power and practical need to control costs associated with analytical chemistry.

1.2 In situ placement and retrieval of artificially spawned embryos in cassettes.

Overlapping herring spawns can produce closely associated aggregations of eggs that differ in age. To reduce this source of exposure variability, as well as variability associated with differences in egg location (i.e., relative to substrate and tidal depth) between sites, adult prespawn herring will be captured and gametes fertilized in the laboratory. Ripe herring will be obtained by gillnet in Tomales Bay and transported in coolers to a wet lab. Ovaries will be dissected and eggs distributed by swirling clumps taken from ovaries with a dry spatula over 12-cm x 12-cm swatches of nitex mesh laid out in glass dishes containing filtered seawater. Testes will be dissected, minced with a scalpel, and macerated in 2 ml seawater with a spatula in a plastic weigh boat. Swatches coated with unfertilized eggs will be transferred to a beaker of filtered seawater on a stir plate. Milt will then be decanted into the beaker and allowed to stir for 5 min before removing and rinsing the eggs with fresh filtered seawater. Eggs will be incubated in constant flow filtered seawater at ambient SF Bay temperature until all fertilizations are complete, then inspected for fertilization rate and developmental stage.

Replicate swatches with newly fertilized embryos will be inserted into individual cassettes that will exclude predators but will allow exposure to dissolved-phase PAHs [22]. Cassettes will consist of 15 cm lengths of 10.2 cm diameter aluminum pipe capped with 0.32-cm nylon mesh. These will be attached to anchor-float lines at field sites by SCUBA at locations that will minimize differences in abiotic habitat factors between sites. Anchor-float lines will be installed at selected study sites prior to the deployment of cassettes. To avoid contamination during deployment and retrieval, cassettes will be bagged before boat transport and crossing the sea

surface. Entire cassettes may be retrieved as needed, or individual swatch replicates subsampled and cassettes returned for further incubation. Herring eggs retrieved in this manner will either be fixed or maintained live in coolers with filtered seawater as appropriate for subsequent laboratory processing and observation (see below).

1.3 In situ deployment of passive sampling devices

Because herring embryos may be exposed intermittently to relatively low levels of total PAHs, passive sampling devices offer a reliable and cost-effective approach for measuring cumulative PAH exposure at herring spawning sites in San Francisco Bay. The traditional approach has typically made use of semi-permeable membrane devices (SPMDs), such as low-density polyethylene tubing filled with a lipid solution (e.g. triolein) to mimic a biological matrix. However, a simpler, less expensive, and equally effective approach is the polyethylene membrane device (PEMD), which is essentially the same type of plastic tubing without the lipid contents. PEMDs reliably sample lipophilic hydrocarbons from aquatic environments at low and fluctuating concentrations. Results provided by PEMDs under these conditions are comparable to those for SPMDs [23,24]. PEMDs are a single strip of low-density polyethylene measuring 2.6 cm × 50 cm × 88 cm serpentined around aluminum nails in an aluminum pipe (11 cm outside diameter × 6 cm depth) with perforated aluminum end caps that allowed seawater to flow freely past the strips. Hydrophobic contaminants such as PAHs are sequestered in the polyethylene matrix inside the perforated canisters. Following retrieval, membranes are removed from the canisters in the laboratory using hydrocarbon-free forceps. The membranes are stored in hydrocarbon-free glass jars with aluminum foil or teflon-lined lids and placed in a -20ºC freezer until extraction.

Passive sampling devices will be deployed at sites along the shoreline that are selected to capture the gradient of visible oiling in herring spawning habitats in the Bay Area, from heavily or moderately oiled locations in the Central Bay to non-oiled reference locations in Tomales Bay. The devices will be co-located in space and time with herring embryos that are naturally spawned (and then collected) as well as herring embryos that are artificially spawned and placed in cages. As with eggs, care will be taken during placement, retrieval, and transport of PEMDs to avoid PAH contamination.

1.4 Collection of sediments

Surface sediments at the herring spawning sites and egg caging sites will be collected for detailed analysis of PAHs. Collections will occur within the intertidal zone upslope of where the subtidal spawning sites are located, and in the immediate vicinity of the area of subtidal egg collection (or retrieval). Samples will be collected along a 30 m transect perpendicular to the beach in the intertidal zone and by SCUBA at the subtidal location for each site. Surface sediment samples (0-2 cm in depth) will be collected along the intertidal transect, using an isopropyl alcohol-rinsed stainless steel cookie cutter ~ 4 cm in diameter x 2cm in depth. A similarly rinsed stainless steel spatula will be thrust underneath the corer to retain the sediment sample, which will then be placed into a prerinsed and prelabelled 250 ml jar. PAH values derived from these will represent the average extent of intertidal oiling present at each sampling site. For subtidal sampling, collection jars will be sealed at depth to prevent contamination.

Prior to analysis, all samples will be thoroughly homogenized. Locations of all sediment samples will be documented by GPS.

Should herring spawning sites occur at depths below 20 m, sediments will be collected from boats using a Van Veen grab sampler ($0.1m^2$), or a smaller Ponar-type sediment grab, with three grab events occurring at this depth. The top 2 cm of sediment will be collected from the surface of the grab into a pre-rinsed stainless steel bowl, using a pre-rinsed stainless steel spoon, the contents homogenized for 1 min, and then a portion of this total placed into a single pre-rinsed and pre-labelled 250 ml glass jar (as in an IChem jar). All sediment samples will be frozen within 3 hrs. of collection for storage until analysis.

1.5 Analyses of PAHs in herring eggs, PEMDs, and sediments.

Herring egg samples and sediments will be extracted and analyzed for PAHs using the method of Sloan *et al.* [25]. This method involves: (1) extraction of tissues or sediments using dichloromethane in an accelerated solvent extraction procedure, (2) clean-up of the dichloromethane extract on a single stacked silica gel/alumina column, (3) separation of PAHs from lipid or other biogenic material by high-performance size exclusion liquid chromatography, and (4) analysis on a low resolution quadrupole GC/MS system equipped with a 60-meter DB-5 GC capillary column. The instrument will be calibrated using sets of up to ten multi-level calibration standards of known concentrations.

For passive sampling devices, the low-density polyethylene strips will be extracted and analyzed for PAHs. An internal standard solution will be applied to each sample, and a spiking solution of analytes will be added to the spiked samples. The samples will then be extracted in 1:1 dichloromethane:pentane by sonication. The sample extracts will be eluted through silica columns using 1:1 dichloromethane:pentane to remove any interfering biogenic compounds. The cleaned up extracts will be concentrated to 100 µL using a stream of nitrogen and analyzed for PAHs by GC/MS in a series which includes six concentration levels of calibration standards ranging from approximately 0.001 to 1 ng/µL for each analyte. The concentrations of analytes (ng per sample) will be calculated using internal standards and point-to-point calibration. The percent recoveries of the internal standards will be calculated for each sample using the GC internal standard. The analyte percent recoveries for the spiked lab blank strips and spiked solvent method blanks will be calculated relative to the analysis of the spiking solution.

Sum "low molecular weight PAHs" (LMWAHs) will include summing the concentrations of naphthalene, C1- through C4-naphthalenes, biphenyl, acenaphthylene, acenaphthene, fluorene, C1- through C3-fluorenes, phenanthrene, C1- through C4-phenanthrenes, dibenzothiophene, C1-through C3-dibenzothiophenes and anthracene. Sum "high molecular weight PAHs" (HMWAHs) will include adding the levels of fluoranthene, pyrene, C1-fluoranthenes/pyrenes, benz[a]anthracene, chrysene/ triphenylene, C1- through C4-chrysenes/ benz[*a*]anthracenes, benzo[*b*]fluoranthene, benzo[*j*]fluoranthenes/ benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, idenopyrene, dibenz[*a,h*+*a,c*]anthracene, benzo[*ghi*]perylene. Total PAHs will be calculated by summing the concentrations of LMWAHs and HMWAHs.

The herring egg samples will also be analyzed for lipid classes (i.e., sterol esters/wax esters, triglycerides, free fatty acids, cholesterol, polar lipids) and percent lipid by thin-layer chromatography/flame ionization detection (TLC/FID) using an Iatroscan Mark 5 as described [26]. Prior to sample cleanup on the alumina/silica column, a 1-mL aliquot of sample extract will be transferred to a clean vial. The volume of each 1-mL egg extract will be reduced to ~100µL using nitrogen gas to ensure that each lipid class will be in the linear range of the FID. Each lipid sample extract will be spotted on a Type SIII Chromarod and developed in a chromatography tank containing 60:10:0.02 hexane:diethyl ether:formic acid (v/v/v). The percent lipid values will be calculated by summing the concentrations of five lipid classes for each sample. The dry weight determinations for the egg and sediment samples will be determined as described [27].

1.6 Analyses of CYP1A induction in herring embryos.

For assessment of CYP1A induction pattern, embryos collected from field sites and cassettes will be fixed in the field in 4% buffered paraformaldehyde with overnight incubation at room temperature. After washing in phosphate-buffered saline (PBS), embryos will be dehydrated through a graded methanol/water series for long-term storage in 100% methanol. Fixed embryos will be processed for CYP1A immunofluorescence and analyzed by laser scanning confocal microscopy as described previously [10,12]. CYP1A induction will be quantified by measuring *cyp1a* mRNA levels using real time RT-PCR. Collected embryos will be flash-frozen in liquid nitrogen after transport from the field and stored at -80˚C. Embryos will be disrupted using handheld Pellet Pestle Motor and RNase free pestles and tubes (Kontes Glass Company, Vineland, NJ), homogenized with QIAshredders (QIAGEN, Valencia, CA) and total RNA isolated using the RNeasy Protect Mini Kit (QIAGEN) according to the manufacturer's instructions. RNA will be quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and quality verified by an optical density (OD) absorption ratio OD 260 nm/ OD 280 nm > 1.9 and agarose electrophoresis of 1 µg RNA. A TaqMan real-time quantitative RT-PCR will be developed using the Pacific herring *cyp1a* sequence [18] based on an assay previously established in zebrafish [12].

1.7 Measurement of maternal contributions of PAHs and other CYP1A-inducing compounds in eggs.

Because adult fish readily metabolize and thus do not bioaccumulate PAHs, it is unlikely that PAHs will be maternally transferred to naturally spawned embryos. Some maternal transfer of low molecular weight PAHs predominated by naphthalenes did occur in female herring exposed to high levels of dissolved PAH (~ 60 ppb) [28], but these compounds are not strong CYP1A inducers. However, persistant organic pollutants (POPs), including DDTs and PCBs, could be transferred to eggs, thereby resulting in embryonic CYP1A induction. Due to the relatively short lifespan of herring and because fish that spawn in SF Bay are not resident there, accumulation of high levels of POPs and maternal transfer is unlikely. To evaluate maternal transfer, samples will be collected from adult prespawn herring and stored for later PAH and POPs analyses as necessary. Adult prespawn herring will be caught by gillnet in SF Bay. Bile will be collected for analysis of recent PAH exposure according to established procedures [29]. Dissected ovaries will

be extracted and spiked with internal standards for analysis of POPs as described for PAH analysis in section 1.5

2. *Assess the toxicological response of herring embryos over a range of PAH exposures in the field.*

Rationale:

Assessing the toxicity of oil to herring embryos is relatively simple and straightforward. Although somewhat thick, the chorion of herring eggs is translucent. This allows for the direct visualization of unhatched embryos, even with some degree of coating by diatoms and other microscopic debris. Due to the fairly large perivitelline space, herring embryos can be dechorionated manually with fine forceps, allowing more detailed microscopic examination of live embryos.

Approach:

2.1 Field collection of naturally spawned herring eggs.

Naturally spawned eggs will be collected and transported as described above in Aim 1.1. Studies of Atlantic herring [30] and Pacific herring [14] indicate that the first cardiac contractions begin in the range of 40-48 accumulated thermal units (days post fertilization x temp ˚C). Temperatures in the San Francisco Bay during Dec-Jan are in the range of 12˚C [http://sfbay.wr.usgs.gov/sediment/cont_monitoring/index.html], so cardiac contraction in naturally spawned embryos is anticipated to begin as early as three days post-fertilization. Spawning sites will be monitored daily for new spawn, and embryonic staging will be assessed in the field by observation of embryos fixed with Stockard's solution. Sites with cleavage-stage embryos will be resampled daily, and once embryos reach the organogenesis stages with a functional heart, subsamples will be transported daily through hatching stages (estimated 10 dpf) for laboratory assessment (see below).

2.2 In situ placement and retrieval of artificially spawned embryos in cages.

Methods for placing fertilized embryos in cages and deploying these at sites along a pollution gradient are described above in Aim 1.2.

2.3 Anatomical and physiological assessment for PAH-induced developmental toxicity.

Accumulation of fluid or edema of the yolk sac and pericardial space is the most common anatomical defect associated with oil exposure [2,3,4,10,14,31]. The presence of edema in herring embryos and larvae is readily documented in either live or fixed specimens [3,14,31]. Subsamples of embryos collected in the field will be fixed immediately in formalin to be analyzed for the presence of edema by visual inspection with a stereomicroscope. Embryos will be manually dechorionated if necessary. Live samples returned to the wet lab will be visually screened for defects while simultaneously analyzed for physiological effects of oil exposure. Analyses of cardiovascular function will be carried out the day of sampling by direct

microscopic observation of live animals. Live samples will be acclimated and held at constant temperature in the wet lab (based on current ambient temperatures in SF Bay). Initial observations will be made through the chorion with stereomicroscopes, and subsamples of eggs will be dechorionated as needed. Embryos will be held in flowing seawater at ambient temperatures for San Francisco bay. Temperature will be controlled on the stereomicroscope stage with a Peltier-cooled stage insert (Brooks Industries, Lake Villa, IL) designed to maintain the contents of a 100-mm petri dish as low as 5˚C. Still images and digital video will be acquired with an iFire400 digital video camera (Unibrain) connected to an Apple laptop computer using BTV Carbon Pro software.

Heart rates will be measured from 20-sec video segments collected from individual embryos. A quantitative assessment of cardiac arrhythmia will be obtained by determining the inter-beat variability from the same video segments (collected at 30 frames/sec). Briefly, the initiation of cardiac contraction is noted for each beat, and the corresponding video frame number recorded. Using Microsoft Excel, the number of frames between beat initiations is calculated and the mean and standard deviation obtained for each embryo. This standard deviation is a measure of heart rate irregularity. For example, a regular rhythm would have essentially the same number of frames between beats, and a low standard deviation. The standard deviations for individual embryos are then averaged to obtain a mean inter-beat variability for each exposure group. Generally, embryonic heart rates are consistent enough to allow detection of highly statistically significant differences among treatment groups with relatively small numbers of individuals (i.e., tens of animals) [11,14].

To confirm PAH exposure, analysis of CYP1A induction will be carried out as described under section 1.6. After initial stereomicroscopic assessment, subsamples of embryos will be (1) fixed in 4% buffered paraformaldehyde and archived for analysis of CYP1A induction by immunofluorescence and altered gene expression by in situ hybridization, or (2) flash frozen in liquid nitrogen for total RNA isolation for quantitative analyses of *cyp1a* mRNA levels.

3. *Characterize the toxicity of Cosco Busan oil to herring embryos under controlled laboratory exposure conditions.*

Rationale:

The presence of *Cosco Busan* oil in some herring spawning habitats raises the possibility of long-term toxicological effects from residual oil. The continuous dissolution of PAHs into the pore water surrounding shoreline gravel or cobble is the primary pathway by which demersal embryos are exposed to weathered oil. This aim will use a laboratory approach to determine the dose-response relationships between embryo toxicity and exposure to *Cosco Busan* oil with different PAH compositions that reflect weathering over time. The oiled gravel column approach provides a simple method for reproducing the different patterns and relative concentrations of PAHs found during the weathering of oil on intertidal/subtidal substrate [2-4,9,10,14,32]. Gravel is loaded with a measured mass of oil and then packed into columns. A flow of seawater is then passed through the columns and fertilized herring eggs are incubated in the column effluent. Dose-response relationships for oil toxicity are established both by loading different masses of oil into the columns and by extending the duration of seawater flow through

the column. Based on previous studies with other types of oil (e.g., Alaska North Slope crude), concentrations of dissolved total PAHs are expected to decline over time with a shift in composition to higher molecular weight compounds.

Approach:

3.1 Construction of oiled gravel columns.

Columns will be constructed according to previously published procedures [3,10,14]. Crushed rock (5/8-minus) will be obtained from a suitable local source, sieved to obtain a uniform distribution of 5 mm diameter gravel, and rinsed extensively in clean seawater. Gravel will be coated with a graded series of masses of *Cosco Busan* bunker oil by shaking in 1 gallon unlined stainless steel paint cans, followed by drying. To represent later stages of weathering, oil may be autoclaved to remove lower molecular weight compounds with higher volatility. 1-L beakers provide sufficient column bed volume for herring embryo studies. Gravel will be poured into the column around a glass tube that contacts the bottom of the column. Water can be fed by pump or gravity at a predetermined flow rate into the glass tube so that flow is from the bottom of the column, through the gravel, then over the top of the beaker. The columns will be placed in glass dishes that serve as a reservoir to hold a steady-state volume of effluent.

3.2 Exposure of herring embryos to oiled rock column effluent.

These laboratory studies may be carried out at any time during either the SF Bay/Tomales Bay spawning season, or if necessary during the Puget Sound spawning season. Prespawn fish will be caught by gillnet. Gametes will be processed as described under Section 1.2 except eggs will be distributed onto glass microscope slides instead of nitex swatches. Eggs will be fertilized using histology processing cassettes suspended in a beaker of filtered seawater. Replicates of individual slides with ~ 100 embryos attached will then be placed into the glass baking dishes holding the columns to allow incubation in the column effluent. Single slides will be subsampled daily and embryos examined in the same manner as described above under Section 2.3 (e.g., for cardiac dysfunction, edema, hatching rate, CYP1A induction). Studies may be carried out locally at the San Francisco Bay wet lab, the Bodega Marine Lab (Gary Cherr, personal communication) or at the NOAA/NWFSC Mukilteo Field Station on Puget Sound, where the herring spawning season is Jan-Apr.

3.3 Chemical analyses of PAHs in oiled rock column effluent.

Water samples (100 ml) will be collected at regular intervals and extracted with dichloromethane for PAH analysis by GC/MS. Samples of developing eggs (3 g) will be collected by scraping from the microscope slides, and stored at -80˚C in pre-washed I-Chem jars for subsequent tissue extraction and PAH analysis by GC/MS. See Aim 1.4 above for additional information on analytical methods.

4. *Estimate the loss of individual herring from the San Francisco Bay population due to both lethal and sublethal impacts of oil.*

The rationale and approach for Aim 4 will be defined following additional discussions with the Trustees. A proposed approach is not included here because this task is not as time-sensitive as the preceding Aims (particularly Aims 1 & 2).

Budget:

As of this date (Novermber 30th, 2007), there are several important details of the study design that have yet to be finalized in discussions with the Trustees. These include, for example, the overall number of spawning sites that will be assessed, the number of samples that will be collected at each site for the different matrices (i.e., tissue, sediment, and PEMDs), and the replicate design for the oiled gravel column exposures in the laboratory.

The most well defined costs at this point are those for analytical chemistry. These are provided on a per sample basis below:

PAHs, POPs, lipid and dry weight (egg or adult)	$874/sample
Subsistence PAHs, lipid + dry weight (egg)	$804/sample
Subsistence PAHs + dry weight (egg)	$781/sample
Subsistence PAHs + dry weight (sediment)	$776/sample
Subsistence PAHs (PEMD)	$738/sample

Thus, for a single hypothetical herring spawning site, if three naturally-spawned egg samples were analyzed (including lipids) together with three caged embryo samples, three PEMD samples, and three sediment samples, the approximate analytical costs would be $9.4K (not including additional samples for QA/QC). This does not include the cost of project planning, data analysis, and report writing, which will be estimated once the scope of the overall project is determined.

For the field exposure and effects assessments, we are assuming that eight NOAA staff will travel to the Bay Area for approximately three weeks of continuous investigative activity beginning in late December or early January. Estimated costs for this level of field effort are:

Airfare:	$2.4K
Housing (shared rooms):	$16.8K
Per diem:	$10.8K
Labor (not including overtime):	$43.4K
Transportation:	$3.0K
Supplies:	$5.0K
Materials:	$5.0K
Shipping:	$4.0K
	$91.4K

This estimate presumes that Center staff will work closely with the California Department of Fish and Game. Costs may be reduced if Center staff can partner with additional organizations that have the capacity to collect samples in San Francisco Bay. This includes, for example, the

field ecology research group at Bodega Marine Laboratory (Gary Cherr, personal communication).

Estimated costs for Aims 3 & 4 are not included in this draft version of the proposal, as many details still need to be discussed with the Trustees.

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STANDARD OPERATING PROCEDURES

for

2007 COSCO BUSAN oil spill: assessing toxic injury to Pacific herring embryos and larvae in the San Francisco Bay Estuary

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TABLE OF CONTENTS

1.0 GENERAL INFORMATION

Standard Operating Procedures are intended to provide detailed and explicit instructions for the research staff in the collection of study data, and should be fully reviewed by staff so that:

• They are versed on study objectives, methods, procedures, and details before sampling.
• Data are collected systematically and consistently throughout the study.
• Each staff member understands and adheres to the requirements.

Events may arise during this study that require revisions to the procedures documented. Revisions to procedures should be documented in writing, with a detailed explanation of why the revision was necessary. Revisions from the plan will be conducted only after the approval of the Principal Investigator and/or Co-Principal Investigators.

1.1 Documentation

1.11 Field Logbook

- All pertinent information on field activities and sampling efforts should be recorded in a bound logbook that is paginated.

- The field supervisor or data manager of the day should be responsible for ensuring that sufficient detail is recorded in the logbook.

- The logbook should enable someone else to completely reconstruct the field activity without relying on the memory of the field crew.

- All entries should be made in indelible ink, with each page signed and dated by the author, and a line drawn through the remainder of any page.

- All corrections should consist of permanent single line-out deletions, and corrected entries are dated and initialed.

At a minimum, entries in a logbook should include:

- Names of daily field supervisor/data manager and all other team members and responsibilities;
- Purpose of proposed sampling effort;
- Location of sampling site;
- Details of actual sampling effort, particularly deviations from standard operating procedures;
- Sampling activity start and end times;
- Field observations;
- Field measurements made (e.g., pH, temperature, flow).
- A summary of samples collected.

1.12 Field Datasheets

All information pertinent to the samples collected should be recorded on datasheets that to the extent possible are preformatted and pre-printed. The use of pre-formatted datasheets is a Quality Assurance measure designed to:

- ensure that all necessary and relevant information is recorded for each sample and each sampling activity;
- serve as checklists for staff so information is recorded consistently;
- to help ensure completeness of the data collection effort;
- assist the research staff by making data recording more efficient;
- minimize the problem of illegible or hard-to-follow notebook entries.

All study procedures and results will be documented on datasheets that should be placed in binders and retained for review. The researchers performing each procedure will be responsible for recording information on data. Data recorded on sheets should be error checked immediately upon completion of the sampling activity, and sheets should be signed by the reviewer.

1.13 Laboratory Logbooks and Datasheets

Laboratory Logbooks and Datasheets should be created and maintained similar to those for the Field. Specific guidance for each procedure can be found in the Chain of Custody Procedures and Standard Operating Procedures.

1.2 General consideration for samples collected for chemical analyses

The results from chemical analyses of fish tissue samples can be adversely affected by poor sampling techniques or poor sample quality. To ensure greater consistency, the guidelines for the collection and storage of tissue samples that will be analyzed for chemical contaminants follow. Sampling protocols describe the appropriate techniques in acquiring, storing, and transporting fish tissue samples for analyses for polycyclic aromatic hydrocarbons (PAHs) and persistent organic pollutants [e.g., PCBs, DDTs, polybrominated diphenyl ethers (PBDEs)].

General considerations. It is important to use standardized sampling procedures, so that, even when there are low levels of contaminants present, the differences in concentrations may be attributed to biological processes and contaminant exposure, rather than to variation in the collection and storage procedures. The following procedures are essential to prevent cross-contamination among animals and to assure proper preservation of the samples.

Avoid cross-contamination of samples. Careful cleaning of necropsy tools between fish from different sites is very important. Any contact with the fish should be with latex or

nitrile gloves. Equipment used to collected sediment should also be carefully cleaned between sites.

Rinse all tools and equipment with isopropyl alcohol before cutting tissue samples or collecting sediment. This procedure will minimize cross-contamination when sampling between fish. Isopropyl alcohol is the solvent of choice to remove lipid or other lipophilic substances. However, 95% ethanol can be used if isopropyl alcohol is not available.

Use pre-cleaned sample containers. Pre-rinsed vials or jars are used to store and ship fish tissue samples. Fish bile samples are collected and stored in 4-mL amber glass vials (standard GC-Vial) available from Sun Brokers. Other tissues collected for PAHs or POPs should be stored in I-Chem 300 or equivalent jars. Use of reliable containers of this type greatly reduces the possibility of external contamination.

Keep samples cold and freeze as soon as possible after collection. Some of the organic contaminants are volatile or are degraded by compounds released during cell death. Moreover, tissues can lose lipid and, thus, lose contaminants, if the temperature of the sample increases. To decrease changes in contaminant levels due to these processes, keep the samples on ice following collection and freeze as soon as possible. Storage at –20°C for periods of a few months is acceptable. For longer-term storage, the samples should be held at –80°C or lower.

1.3 Chain Of Custody Procedures

1.3.1 General COC Information

Chain of custody (COC) is defined as an unbroken trail of accountability that ensures the physical security of samples, data, and records. Due to legal aspects of Damage Assessment work, it is essential that the possession of samples be traceable from the time they are collected through analysis and introduction as evidence. Sample custody will be established through documentation during all stages of this study.

Samples are considered to be in "custody" if they are: 1) in the custodians' possession or view, 2) retained in a secured place (under lock) with restricted access, or 3) placed in a container and secured with an official seal(s) such that the sample cannot be reached without breaking the seal(s). The principle documents used to identify samples and to document possession are COC records, field notebooks, and field tracking forms. COC procedures will be used for all samples, no matter where in the analytical process, and all data and data documentation whether in hard copy or electronic format.

COC procedures are initiated during sample collection and maintained with field and laboratory logbooks. A COC record accompanies each sample [One COC form may be used to track a group of samples collected, processed and analyzed in the same manner at

the same time.] Each person who has custody of the samples signs the form and ensures that the samples are not left unattended unless properly secured.

The QA coordinators or designated scientists complete COC forms prior to transporting samples from the field or sampling vessel. Information on the sample labels is checked against sample log entries and sample tracking forms, and samples are recounted. All samples must be accompanied by COC forms that include sample numbers. The forms are signed at each point of transfer. **All COC forms must be filled out in indelible ink. If corrections are needed, draw a single ink line through the error, make the change, initial and date when the change was made and add a note why the change was needed**. Copies of all forms will be retained by NOAA.

An example of the COC Form is found in Appendix A.

Sample collection:

COC documentation begins with sample collection and initially includes:

- sample location, project name, unique sample number
- sample collection date and time
- Any special notations on sample characteristics or problems
- Initials of the person collecting the sample

The completed COC form is placed in a plastic zip-lock bag that is included in the container holding the listed samples (e.g. taped to the inside lid of an ice chest). The container is packed appropriately, (e.g. prevent breakage or ensure that samples stay cold or frozen as needed) completely wrapped with strong tape, and sealed with COC tape. Sealed containers may then be couriered to temporary laboratory space or shipped to the NWFSC laboratories in Seattle.

Considerations for storing sampling supplies

- Ensure that sampling supplies and equipment are housed in limited access or locked areas to avoid any tampering.
- Sample containers being used should be in boxes with original factory seals, or in boxes that have been resealed with custody seals and initialed. All seals should then be examined to ensure that they are intact prior to use.

Receipt of samples at the laboratory:

Upon receipt of samples at the laboratory, the shipping container COC seal is broken. The date of receipt and initials of the recipient is added to the COC form to document the transfer of custody. The condition of the samples is recorded by the recipient. COC records are included in the analytical report prepared by the laboratory, and are considered an integral part of that report. The QA coordinators at the laboratories ensure that the COC forms are properly signed and transferred upon receipt of the samples. They also note question or observations concerning sample integrity on the COC forms.

The laboratories will contact the QA Coordinator immediately if discrepancies between the COC forms and the sample shipment are discovered upon receipt. The laboratory QA coordinator will specifically note any containers that may contain compromised samples (e.g. not cold on arrival). The laboratory will not dispose of the samples until notified by the Project QA Coordinator.

COC documentation is needed to account for the custody of the samples and all times, including each time the sample changes hands and during periods of shipping or storage.

For instance if samples are processed in one laboratory, then sent to a different lab for analysis, the initials and dates of the people relinquishing custody must be noted, the container must be sealed with COC tape, and documentation must be included about shipping dates, (or dates the samples were stored in a locked freezer). The initials and dates when the next custodian receives custody must then be noted, along with comments on condition of the sample, noting if the COC seal is still intact.

Data must also be maintained in a secure manner, including being locked in a drawer or office when unoccupied, and maintained on password-protected computers. The documentation must be complete enough to legally document that no one could have tampered with the results at anytime between sample collection, analysis and data reporting. The documentation must show that the samples were either in the custody of the scientists, locked in a room or freezer where no one else has a key, or sealed with custody tape.

1.4 Description of COC procedures for Cosco Busan Oil Spill

A "quick guide" list of COC procedures is provided in Appendix B.

1.41 Designate a Data Manager

Previous experience shows that having a consistent, designated data manager throughout the day or for several days is a huge asset for maintaining good COC procedures. The designated data manager should check all logbooks and COC sheets to make sure all information has been filled out and matches the actual samples collected.

1.42 Field collection of herring eggs or sediment

Field Logbook: Note date, time, location, collector(s), how the eggs were collected and stored, sample numbers, condition of eggs, when samples are transferred, if they were security sealed, etc. Reference COC form #'s.

Chain of Custody forms: Start chain of custody forms, one form for each set of samples. Check to make sure that the samples listed on the forms actually match the samples in the box. Place COC forms in ziplock bags, inside sample boxes before sealing.

1.43 Caged herring eggs, deployment and sampling

Seal the cages in a tamper proof method: (e.g. cable ties)

Field logbook: Note date, time, sample ID number and location of where eggs were placed into cages. If deploying cages, note how and when they were sealed. If sampling cages, note if the seal was intact or broken. Note when the cages were resealed. Note condition of eggs, how they were collected and stored and when transported to the lab.

Custody forms: Begin custody forms as for other samples. Reference COC Form #'s in logbook

Seal sample containers if needed: If samples will be out of the custodian's sight (e.g. stored overnight), they must be sealed with security seals. COC forms are sealed inside the sample boxes.

1.44 Transfer of samples from the field to the on-site lab

Custody forms – transfer of custody: People receiving samples from the field must **c**heck the COC forms to make sure all the samples indicated on the form are actually now in the custody of Lab personnel. Sign and date forms to indicate exchange of custody. Note condition of samples. Reseal if the samples are to be stored or shipped.

Lab notebook: Note date, time location, names of personnel, how samples were transported, when custody is transferred and condition of samples. Describe processing procedures and include sample numbers. Note if eggs were repackaged for further processing, note storage and transporting details.

Custody Forms - if repackaging samples: After processing, if samples are repackaged for more than one analysis (e.g. chemistry vs. CYP1A) fill out a new chain of custody form, sign and date, **and reference the Form (tracking) # of the original COC form**. Put COC forms in a ziplock bag inside the sample box, secure the box with custody seals.

Custody forms - if samples are not repackaged: If samples were simply processed and returned to the same containers for storage or shipping with no change of custody, replace the custody forms in a ziplock bag, reseal the box, and note in the lab book any information on processing and storage. If there is a change of custody, then the COC forms need to be signed by both parties before sealing the box.

1.45 Adult herring, egg and tissue sample collection

Logbook: Note how adult herring were captured (e.g. BLM personnel, local fishermen), fishing gear, date, time, etc. Note how fish were transported to the laboratory, and who had custody of the fish during transport to the lab. Note when received at the lab, who now has custody of the fish, and what happened to the fish at the lab (e.g. held on ice

until necropsy, eggs spawned, etc.). Note what samples were collected and how they were processed, stored, etc.

Custody forms in the field: Prepare a custody form for the fish being transported to the lab.

COC during transport of fish: It is likely that the fish will be under the direct observation of the people preparing the original custody form and transporting them to the lab. However, if those fish will be left unattended for any length of time before they get to the laboratory, they should be secured in some manner, (e.g. placed in an ice chest with a security seal).

Custody forms in the lab: If custody is transferred to laboratory personnel, sign the fish custody forms. For tissue samples, new custody sheets will need to be started for each type of sample collected (e.g. bile vs. gonad samples), for each box. **Reference the form # of the original COC form.**

Seal samples before storing and transporting.

COC for spawned eggs: If eggs are artificially spawned and stored overnight before being deployed in cages, they should be placed in containers or tanks that can be secured with a seal, or kept in a locked, limited access facility. Document storage methods and security procedure in the logbook!

1.46 Laboratory Processing

Chain of Custody: When samples are first received in the lab, check to make sure the samples received are the same as those written on the COC forms. Note condition of samples. Sign and date forms. New custody forms will be started for sample analyses. Reference the form # of the original COC forms.

Logbook: Record when samples were received, how and where the samples are stored, when the samples were opened (date and time), what was done, etc, when samples were put back into storage.

Storing samples: Samples must continue to be under custody in the lab. They must either be under the direct observation of the "custodian", or placed in a locked laboratory or freezer, or sealed with custody tape.

Discarding samples: Sediment and tissue samples frozen or fixed for various analyses can not be discarded until released by the Trustees. For samples that have a finite lifetime (e.g., live fish embryos that have passed the appropriate developmental stage) keep records in the logbooks indicating when and why the samples were discarded. *[Need to check if this is correct]*

COC of data: Data must be maintained on a password-protected computer or hard copies locked in a file cabinet. Mark paperwork with "Attorney-Client Work Product."

Other considerations for maintaining COC during laboratory processing:

Each laboratory procedure will likely need different considerations for maintaining COC documentation.

Record keeping: When planning the workflow, provide sufficient paperwork to track the final data back to the original sample collection. This includes keeping track how data was collected (e.g. photographs, Excel datasheets, word documents), of file names, where they are stored, what was done to the data (e.g. what statistics were performed) etc. For instance, for microscopy examination of samples, a master database will contain summary COC information and link final image files and storage information. Backup electronic files of all imaging data will be maintained on duplicate external hard drives. Hardcopies of master database daily entries will be printed and entered into the laboratory notebook/logbook on a daily basis. Upon completion a final hardcopy will be printed and entered into the laboratory notebook/logbook.

Instrument calibration and monitoring: Paperwork is needed to verify that all laboratory instruments have been calibrated. If needed, equipment may need to be monitored (e.g. daily check of freezer temperature).

Storing samples: Samples must continue to be under custody in the lab. They must either be under the direct observation of the "custodian", or placed in a locked laboratory or freezer, or sealed with custody tape.

1.47 COC Procedures for Sample Shipment

All shipments must comply with Department of Transportation regulations (49 CFR, parts 172 and 173).

Frozen Shipments:
Ship all frozen samples via FEDEX. Ship frozen samples, using recommended procedures to prevent thawing. Ship frozen samples, early in the week (i.e. Monday or Tuesday), by FEDEX for OVERNIGHT NEXT BUSINESS DAY DELIVERY. Pack shipment with enough dry ice and ice packs if needed to ensure the samples remain frozen. Call the recipient of the samples the day the samples are shipped and provide them with the FEDEX Tracking number. By shipping early in the week, the possibility of the package arriving on a weekend because of shipping delays is greatly reduced.

Shipments with dry ice must be shipped by trained personnel. Dry Ice is considered "Dangerous Goods", but DOES NOT need a shippers declaration. Mark the FED EX airbill accordingly. The shipment must have the necessary placarding on the outside for

dry ice, i.e. diamond placard sticker, the UN number, amount of dry ice, shipper and consignee name and address.

Chain of Custody: An original Chain of Custody form should accompany the shipment. Shipper should ensure all seals are intact, and sign COC form to relinquish sample custody to the laboratory. When samples are first received in the lab, check to make sure the samples received are the same as those written on the COC forms. Note condition of samples. Sign and date forms. New custody forms will be started for sample analyses. Reference the form # of the original COC forms.

Custody forms:

- receiving personnel should confirm COC sample #'s match those recorded on COC form
-both parties (receiving and relinquishing) sign and date forms

Lab Notebook:

- Note everything (who, where, when, how) about transfer, storage, condition, processing, repackaging, etc.

Start new COC forms if repackaged:

-Indicate original COC form # so COC can be tracked to its origin.
-confirm COC sample #'s match those recorded on COC form
-sign and date

Seal sample containers

-if samples will be out of sight of "custodian" (placed in a freezer, stored overnight, shipped, etc.

2.0 STANDARD OPERATING PROCEDURES

2.1 List of Standard Operating Procedures

Field collection of naturally spawned herring eggs

Herring Embryo Collections by vegetation rake or SCUBA for Chemical Analyses

In situ placement and retrieval of artificially spawned embryos in cage devices

Field collection of pre-spawn adult male and female herring
Necropsy of adult male and female herring for collection of eggs and milt
Laboratory fertilization of eggs
Lab preparation of cage devices with fertilized eggs
Deployment, Monitoring and Retrieval of prepared cage devices
Setup, deployment and retrieval of dataloggers

In situ deployment of passive sampling devices

Lab preparation, Deployment, Monitoring and Retrieval of PEMDs

Subtidal and Intertidal Sediment Collection

Collection of sediments
(comment: this is to match text below)

Analyses of PAHs in herring eggs, PEMDs, and sediments

Detailed Analyses of PAHs in herring eggs and sediments
Detailed Analyses of PAHs in PEMDs

Measurement of maternal contributions of PAHs and other CYP1A-inducing compounds in eggs and tissue

Field collection of pre-spawn adult male and female herring
Collection of Tissues from Adult Herring
Methodology for sampling ovaries for PAHs and POPs analysis, extracting bile for bile FACs analysis, and creating composite samples for adult Pacific Herring
Analysis of herring bile for PAH metabolitesDetailed Analyses of PAHs and POPs in herring eggs and tissue

Morphological assessment of herring embryos
Hatching Success and Larval Survival
Larval Feeding Success
Analyses of CYP1A induction in herring embryos

2.2 FIELD AND LABORATORY SOPS

2.21 Field collection of naturally spawned herring eggs

Herring embryo collections by vegetation rake or SCUBA for chemical analyses

Objective: Herring embryos will be collected from multiple sites along a potential pollution gradient for analyses of both oil exposure and oil-induced developmental toxicity.

Procedure: Eggs will be collected with a vegetation rake where herring have spawned on eelgrass or other vegetation. Because the density of herring eggs may vary among sites, the number of rakes used to create each sample should be noted in the field logbook. In areas with hard substrates, such as gravel, cobble, or riprap, eggs will be collected by SCUBA. In either case, samples will be placed in bags prior to breaking the surface of the water and will remain in bags while in transport to the laboratory to prevent contamination from boat engine exhaust and other sources of PAHs. Basic water quality conditions (e.g., temperature, dissolved oxygen) will also be maintained during transport. Eggs will then be scraped into PAH-cleaned glass jars (I-Chem 300 series) using pre-cleaned stainless steel spatulas. Samples will then be frozen at – 20°C as soon as possible after collection then shipped to our laboratory in Seattle.

For chemical analyses, a minimum of three grams of embryos (wet weight) will be collected for each individual composite sample. For a subset of samples, a total of 9 g of embryos will be collected to allow for triplicate analyses for the purpose of quality assurance (QA). Approximately 10 composite samples will be collected at each location. Of these 10 composites, three samples will be randomly selected for GC/MS analyses. Statistical power will be computed based on the variance associated with these three initial samples. If needed, more power can be achieved with the iterative analysis of additional samples. This approach is intended to balance the tradeoff between a need for statistical power and practical need to control costs associated with analytical chemistry.

2.22 In situ placement and retrieval of artificially spawned embryos in cage devices

A. Deployment of anchor-buoy systems for placement of embryo cages, passive sampling devices, and data loggers

The anchor-buoy units consist of a large and small float attached to either end of a line that is passed through a D-ring attached to a pair of concrete blocks weighing 60-lb. Cages and passive samplers are clipped through the braided line toward the small float, which maintains the cages vertical at a preset depth (1 foot from the bottom) no matter

the level of the tide. Anchor-buoy units are installed 1-2 days prior to embryo cage deployment to allow any disturbed bottom sediments to clear.

1. Anchor-buoy units are constructed from clean, new materials including concrete blocks, zip ties, stainless steel rings, foam and rubber buoys, and 3/8-in polypropylene line
2. Vessel used for deployment is decontaminated before each cruise.
3. Units are transported to launch site in a carpeted minivan on a new plastic tarp.
4. Units are transferred to boat by hand and placed on the bow.
5. Units are stored on the bow of given vessel and remain there while underway.
6. Once the deployment site is located, boat approaches from downwind and is put in neutral, and unit moved to center of deck and lifted to gunwale.
7. The unit is lowered by hand to previously scouted position on the bottom.
8. All units placed between -3 and -6 feet mean tide.
9. Once on the bottom, the unit is carefully lifted slightly to ensure that it is level and secure on the bottom.
10. Once secured, GPS coordinates and exact depth of water are documented in the logbook.
11. After unit is deployed, the boat drifts in neutral away from anchor float.
12. Units are inspected from midwater by a diver, who does not handle the equipment nor touch bottom substrate.

B. Field collection of pre-spawn adult male and female herring

1. Ripe pre-spawn herring are captured by either hook-and-line, castnet, or mid-water trawl. Fish are not obtained from areas with obvious oil/fuel slicks and are brought in over the bow or centerline of the vessel.
2. Fish are immediately sexed and transferred to clean ziplock bags and stored in a clean cooler on ice. Fish may be placed in a decontaminated bucket prior to sexing if necessary.
3. Fish on ice are transported to the laboratory for dissection of gonads.

C. Necropsy of adult male and female herring for collection of eggs and milt

1. Field/boat personnel: Herring collected for gonad harvest should be ripe older fish as indicated by presence in spawner holding areas and presence of mature gonads. Whole fish should be stored on ice and delivered to the wet lab personnel within an hour or two post-mortem. (2-3 three large size female herring per cage should be enough for monolayer = 15 females + 3-4 males per site)
2. Necropsy/wet-lab personnel: Remove ovaries from mature to running ripe oozing females caught from common holding area in San Francisco Bay (Raccoon Strait). Remove ovaries using clean sharp scissors and immediately place in plastic Petri dishes. Place ovaries from approximately 20 females in individual dishes, label with individual fish numbers coordinated with COC forms). **Do not add any water.**
3. Seal entire edge of Petri dish and cover with parafilm to seal in moisture.
4. Wrap Petri dishes in 2-3 layers of moist paper towels and place in zip-lock bag. Place zip-lock bag in ice chest with ice.
5. Repeat steps 1-4 for adult male herring to obtain testes from 4-5 fish.

6. Record collection data including collection time, method, location and fork length on datasheet, create COC forms.
7. Harvested gonads may be retained in sealed Petri dishes at 4 degrees on ice and utilized for fertilization for up to 5 days post-harvest.

D. Laboratory fertilization of eggs

Herring eggs will be fertilized directly onto 5.5" x 22.5" Nitex mesh sheets according to methodology described in Griffin et al 1996 and Shannon Point Marine Center/Washington EPA Final report February 2006.

Griffin FJ, Vines CA, Pillai MC, Yanagimachi R, and Cherr GN. 1996 Sperm motility initiation factor is a minor component of the Pacific herring egg chorion *Develop Growth Differ*. 38:193-202

Dinnel, PA, Paisano L, Shi A, Elphick J, Bergmann K, Alaimo J. 2006. Development of Embryo and Larval Pacific Herring, *Clupea pallasi*, Bioassay Protocols: Phase V Final Report for the Washington Department of Ecology and U.S. Environmental Protection Agency, Region 10

E. Lab preparation of cage devices with fertilized eggs

1. Carefully place the Nitex sheet into the cage with the fertilized eggs facing inward.
2. Secure the corners of the cage device using the long white zip tie (14 inches long), using at least two zip ties at opposite corners of the cage.
3. If needed, attach the data logger to the inner lid of the cage, following the protocol outlined below.
4. Place the lid onto the top of the cage and zip tie the two opposite sides of the lid with small zip ties (4" long) in a position midway along the length of the lid.
5. Ensure that there are no loose Nitex edges or zip tie ends protruding into the cage, these could damage or scrape eggs off the mesh.
6. Attach the security zip tie (red zip tie with numbers) to the lid of the cage device.
7. Place the cage device into the zip loc bag (18"x20") and fill the zip loc bag with 50% full strength seawater diluted with Milli-Q water (16 ppt salinity) for transport to the site.

F. Deployment, Monitoring and Retrieval of prepared cage devices

1. Vessel powered with 4-stroke engine is used for all operations, and approaches anchor-buoy installations downwind. Vessel is fueled in advance of all operations, never on the day of operations. Vessel is to be decontaminated after fueling and before operations.
2. All work related to deployment/retrieval of cages/PEMDs is from the bow or center of the vessel, never the stern. A designated boat operator handles all mechanical systems on board and never touch anchors, cages, or PEMDs during any operation.

3. Upon arrival at the site, the cage device to be attached to the anchor and buoy system is lowered to the subsurface water, followed by removal of zip loc bag.
4. Cages and PEMDs are deployed and retrieved at low tide.
5. Bags containing cages/PEMDs will be handled below the surface either over the side of a vessel with low freeboard (e.g. an inflatable) or by a free diver floating on the surface if necessary. Deployment method will be documented in the logbook.
6. Attach the cage device to the anchor and buoy system using two heavy duty zip ties, interlaced into the strands of the mooring line, just below the secondary float.
7. Record the unique number of the security zip tie that is used in the cage device.
8. Lower the cage to the required depth (1 ft from the bottom, approximately 4" above the stainless steel ring attached to the top of the mooring anchor).
9. Upon completion of deployment, boat will drift clear of anchor before starting engine again.
10. Repeat processes 2 - 5 for each additional cages to be deployed.
11. The retrieval of the cage devices is the reverse of the deployment. Bring up the cage to the subsurface water.
12. Place the zip lock bag (18"x20") over the cage.
13. Check that the security zip tie has the same number on it as when the cage was deployed, and has not been tampered with.
14. Remove the zip ties used to attach the cage to the anchor buoy system.
15. Close the zip lock bag with the cages and fertilized eggs within, fill the zip lock bag with ambient sea water from the site, seal the zip lock bag and bring up the immersed cage above the water.
16. Label the bag with site information including the date and time, and place the cage device in the cooler for transfer to the laboratory.
17. Collect additional ambient water from the site as needed for laboratory procedures.

G. Salinity and Temperature data collection with SearStar DST CTDs

1. **Activation & Data Retrieval Logbook:** Note date, time, DST CTD recorder number, and name of operator for activation of data collection and retrieval of data.

2. **Chain of Custody Forms:** Start a chain of custody form for transport of each DST CTD recorder. Chain of custody will begin with computer operator that activates the recorder and will remain with the recorder until it is deployed San Francisco Bay. Custodian who deploys recorder will retain possession of the chain of custody form until the recorder has been retrieved from the Bay, at which time the chain of custody form will be rejoined with the recorder.

3. **Salinity & Temperature Recorder Background Information:** SearStar DST CTD (Data Storage Tag Conductivity, Temperature, Depth) remote recorders were manufactured by Star-Oddi, Vatnagardar, Iceland. Set-up and activation of

the recorders was conducted using software supplied by Star Oddi and followed set-up and activation instructions in the SeaStar User's Manual. The software was installed on and a recorder communication box connected to the UVCHERRLAB computer in Dr. Cherr's lab at the Bodega Marine Lab following installation and connection instructions on pages 7-12. Each of six recorders was programmed to record temperature and conductivity, and activated. SeaStar recorders are calibrated internally, however, each recorder was activated for testing with salinities and temperature confirmed using a refracotometer, (SOP #) and hand held thermometer.

4. **Recorder Activation:** DST CTD recorders will be activated 12-24 hours before transport to and deployment in the Bay. They will be programmed to collect temperature and conductivity data at 30 minute intervals.

5. **Transport and Deployment of Recorders:** Within 24 hours of activation recorders will be transported to RTC, in a protective box out of water. Recorders will remain dry with an RTC custodian until deployment in the Bay. On the day of deployment custodian will secure recorder into a nitex oyster bag and attach bag inside of embryo chamber. At the deployment site salinity and temperature readings will be taken using a hand held YSI and recorded on the chain of custody form; this will provide an external check on DST CTD recorder accuracy. At recorder retrieval a final set of YSI readings will be taken and noted on the chain of custody form. After retrieval, recorders will be rinsed in freshwater, dried and returned to BML. At BML they will be cleaned in freshwater according to User Manual instructions.

6. **Data Retrieval:** Temperature and Salinity (based on temperature and conductivity data) data will be downloads from cleaned recorders. Data will be saved in DAT (SeaStar data file) format and in EXCEL format to the hard drive of the UVCHERRLAB computer. The files will also be copied to a dedicated USB memory stick.

2.23 In situ deployment of passive sampling devices

Lab preparation, deployment, monitoring and retrieval of PEMDs

For each cage site PEMDs will be deployed as follows:

3 Units deployed in the water;
1 Field blank at time of Deployment;
1 Field blank at time of Retrieval.

Deployment will follow procedures previously used by NOAA, NMFS, Alaska Fisheries Science Center, Auke Bay Lab, Juneau, Alaska. Procedures are attached in Appendix C.

2.24 Collection of subtidal and intertidal sediments

Sediment collection

Objective: Sediments will be collected at herring spawning sites, egg caging sites and collection sites of adult herring for detailed chemical analysis of PAHs.

Platforms:

SUBTIDAL - small boat with hydraulics for locations of adult herring collections and spawning site occurring below 20 meters

INTERTIDAL - collections by hand and or with SCUBA

Station location:

Data Format : degrees, decimal minutes (4 places) e.g., 120° 12.3950 W, 48° 34.9065 N

Personnel needed:

1 - Data recorder/manager and Sample custodian /QA manager

2 - Grab operators/sample collectors

Subtidal sediment collection

Equipment needed:

GPS unit **Van Veen (0.1m^2) or Ponar grab sampler**

The sampling device should be attached to the hydrowire of the vessel boom using a ballbearing swivel. The swivel will minimize the twisting forces on the sampler during deployment and ensure that proper contact is made with the bottom. For safety, the hydrowire, swivel, and all shackles should have a load capacity at least 3 times greater than the weight of a full sampler. In addition, screw-pin shackles should have wire through the eye and around one side of the to prevent the pin from rotating.

Stainless steel bowls, spoons

Foil

Pre-rinsed glass jars (I-Chem 300 or better)

two 4 oz for PAHs/POPs, total organic carbon (TOC)

one 8 oz for grain size

Sample labels

Cooler with ice

Phosphate free liquid soap

Scrub brush

Isopropyl alcohol in squirt bottles

Clear tape

Security tape

COC Forms

Logbook

Logsheets

Ballpoint pens **Permanent ink markers**

Procedure:

1. **Prepare equipment and sample containers**:

Prior to deployment of grab rinse grab interior, all stainless steel bowls and spoons to be used, with isopropyl alcohol. Allow to air dry, then cover bowls and spoon with aluminum foil until use.

Prepare sample containers. Place labels on jars and keep dry. Labels can be pre-printed with the information above except for date and time and name or initials of collector(s).

Label Example:

NWFSC-ECD **Cosco Busan Oil Spill Assessment 2007/08**	Date: Time: Collected by:
Sample Type:	Site Name:
Analysis :	Site Number:
Preservative : 4°C or Freeze	

2. **Deploy grab at site**. The sampler should be lowered through the water and retrieved at a controlled speed of approximately one foot per second. Under no circumstances should the sampler be allowed to "free fall" to the bottom, as this may result in premature triggering, an excessive bow wake, or improper orientation upon contact with the bottom. The sampler should contact the bottom gently, and only its weight or piston mechanism should be used to force it into the sediment.

3. **Record position when grab sampler hits bottom**. Record GPS unit daily accurately. Data format: degrees, decimal minutes (4 places) e.g., 120° 12.3950 W, 48° 34.9065 N)

4**. Bring grab up slowly to minimize disturbance to sample**.

5. **Determine if grab is successful.** Visually observe that grab is closed, and no excessive leakage is occurring as it ascends, this may cause sample disturbance or wash out. If grab is ascending open, redeploy. Any sediment in an unsuccessful grab should be discarded in a way that it will not be collected in any subsequent grabs, i.e. away from sampling location.

6. **Retrieve grab.** Bring closed or successfully deployed grab onboard the vessel and place on a level surface, minimizing disturbance.

7. **Examine grab contents.** Open flaps and gently drain or decant off any overlying water, then examine sample condition to determine if grab sample is acceptable for collection.

criteria for acceptable grab:

- grab is not overfilled and extruding with sample such that the sediment surface is pressed against the top of the sampler;
- penetration depth is a minimum of 5.0 cm;
- sediment surface appears relatively flat and not washed out.

8. **Record grab characteristics**. If the grab sample is acceptable, record the following on the logsheet:

- Gross characteristics of the surficial sediment (texture, color, biological structures (e.g., shells, tubes, macrophytes)
- Presence of debris (e.g., wood chips, wood fibers, human artifacts)
- Presence of oily sheen
- Obvious odor (e.g., hydrogen sulfide, oil, creosote)
- Penetration depth

9. **Collect sediment sample from grab.** In ensure that only relatively recent sediments are collected from only the top 2.0-3.0 cm. Scrape the top 2.0-3.0 cm with a rinsed stainless steel spoon and place in a stainless steel bowl. Recover bowl and spoon with foil and redeploy grab.

10. **Collect additional sediment from site as needed**. Redeploy grab following procedure above as needed in order to obtain adequate volume for each sample type. Usually 2-3 total grabs are necessary.

11. **Homogenize sample.** Once all sediment has been collected from each grab and placed in the stainless steel bowl, mix the sediment for 1 minute to homogenize it.

12. **Transfer sample to containers**. Transfer homogenized sediment into glass jars filling jars no more than ¾ full. The sample should be free of debris, such as large twigs and rocks. Cap the jar securely, then write the date, time and collector's initials on the label with a permanent marker.

13. **Place seals on jars.** Tape around the entire jar label with clear tape to further secure the label then place a security seal on jar.

14. **Place all jars in cooler with ice**. Samples should be transferred from ice within 3 hours of collection and stored as follows: **refrigerate** (4°C) grain size samples – DO NOT FREEZE; **freeze** POPs/PAHs and TOC samples in a -20°C or lower temperature freezer.

15. **Clean all sampling equipment.** After samples have been collected, rinse the grab, spoons and bowls with site water and scrub with phosphate free soap using a scrub brush, then rinse again with site water.

16. **Fill out the Chain of Custody Form following instructions found in this SOP**.

17**. Follow shipping guidelines written in section 1.47 COC Procedures for Sample Shipment.**

Intertidal sampling

Equipment needed:

- **GPS unit**
- **Template – stainless steel cookie cutter ~ 4 cm diameter, 2 cm depth minimum dimensions**
- **Stainless steel spatula, bowls, spoons**
- **Foil**
- **Pre-rinsed glass jars (I-Chem 300 or better)**
  - two 4oz for PAHs/POPs, TOC
  - one 8oz for grain size
- **Sample labels**
- **Cooler with ice**
- **Phosphate free liquid soap**
- **Scrub brush**
- **Isopropyl alcohol in squirt bottles**
- **Clear tape**
- **Security tape**
- **COC Forms**
- **Logbook**
- **Logsheets**
- **Ballpoint pens**
- **Permanent ink markers**

Procedure:

1. **Prepare equipment and sample containers**:

Prior to deployment of grab, rinse cookie cutters, spatulas, all stainless steel bowls and spoons to be used with isopropyl alcohol. Allow them to air dry then cover them with foil until use.

Prepare sample containers. Place labels on jars and keep dry. Labels can be pre-printed with the information above except for date and time and name or initials of collector(s).

Label Example:

NWFSC-ECD **Cosco Busan Oil Spill Assessment 2007/08**		Date: Time: Collected by:
Sample Type:	Site Name:	
Analysis :	Site Number:	
Preservative : 4°C or Freeze	Sample #:	

2. **Determine collection site**. Establish an area within the intertidal zone upslope of where the subtidal spawning sites are located and in the immediate vicinity of the area of the subtidal egg collection.

3. **Collect sample**. Press the cookie cutter down no more than 5.0 cm into desired area, insert spatula underneath cookie cutter to retain sediment sample. Place the sample in a stainless steel bowl. Cover it with foil.

4. **Record position**. Record GPS unit daily accuracy.
Data format : degrees, decimal minutes (4 places) e.g., 120°12.3950 W, 48° 34.9065 N)

5. **Record the following on Datasheet**:

- Gross characteristics of the surficial sediment (Texture, Color, Biological structures (e.g., shells, tubes, macrophytes)
- Presence of debris (e.g., wood chips, wood fibers, human artifacts)
- Presence of oily sheen
- Obvious odor (e.g., hydrogen sulfide, oil, creosote)
- Penetration depth

6. **Obtain additional sediment as described in step 3 in order to collect adequate volumes needed for each sample required**.

7. **Homogenize sample**. Once enough sediment has been collected and placed in the stainless steel bowl, mix the sediment for 1 minute to homogenize it.

8. **Fill sample containers**. Transfer homogenized sediment into glass jars filling jars no more than ¾ full. The sample should be free of debris, such as large twigs and rocks. Cap the jar securely, then write the date, time and collector's initials on the label with a permanent marker.

9. **Place seals on jars**. Tape around the entire jar label with clear tape to further secure the label then place a security seal on the jar.

10. **Place all jars in cooler with ice**. Samples should be transferred from ice within 3 hours of collection and stored as follows: **refrigerate** (4°C) grain size samples – DO NOT FREEZE; **freeze** POPs/PAHs and TOC sediment samples in a -20°C or lower temperature freezer.

11. **Clean all sampling equipment.** After samples have been collected rinse grab, spoons, bowls with site water and scrub with phosphate free soap using a scrub brush, then rinse again with site water.

12. **Fill out Chain of Custody Form following instructions found in this SOP.**

13. **Follow shipping guides written in section 1.47 COC Procedures for Sample Shipment.**

Datasheet for Sediment collected for chemical analyses:

NOAA/NMFS/NWFSC/ECD
CBOS 2008 Herring Injury Assessment Study
SEDIMENT FOR CHEMICAL ANALYSES

Page __of __

Date (MM/DD/YYYY)	**Site**			**Vessel/Platform Info:**		
				GPS Info:		
Grab /Collection#	1	2	3	4	5	6
Time (12hr; HH:MM am/pm)						
Latitude (Degrees, Decimal minute)						
Longitude(Degrees, Decimal minute)						
Acceptable(yes/no)						
Comments						
Sediment Characteristics						
Gross characteristics of the surficial sediment (Texture, Color, Biological structures (e.g., shells, tubes, macrophytes)						
Presence of debris (e.g., wood chips, wood fibers, human artifacts)						
Presence of oily sheen						
Obvious odor (e.g., hydrogen sulfide, oil, creosote)						
Penetration depth (nearest cm)						
Sample collected from grab (yes/no)						
SAMPLE INFORMATION			**Sample #**			
SAMPLE COLLECTED FOR: Yes/No	**PAHs, POPs**					
	TOC					
	Grain size					
Additional Comments				Data reviewed by: Signature:		

2.25 Analyses of PAHs in herring eggs, PEMDs and sediments

Detailed analyses of herring eggs and sediments for PAHs

Herring eggs and sediments will be extracted and analyzed for PAHs using a gas chromatography/mass spectrometry method described in Sloan *et al*., 2004. This method involves: (1) extraction of tissues or sediments using an accelerated solvent extraction procedure, (2) clean-up of the entire methylene chloride extract on a single stacked silica gel/alumina column, (3) separation of PAHs from the bulk lipid and other biogenic material by high-performance size exclusion liquid chromatography, and (4) analysis on a low resolution quadrupole GC/MS system equipped with a 60-meter DB-5 GC capillary column. The instrument will be calibrated using sets of up to six multi-level calibration standards of known concentrations.

Sum "low molecular weight PAHs" (LMWAHs) will include summing the concentrations of naphthalene, C1- through C4-naphthalenes, acenaphthylene, acenaphthene, fluorene, C1- through C3-fluorenes, phenanthrene, C1- through C4-phenanthrenes, dibenzothiophene, C1- through C4-dibenzothiophenes and anthracene. Sum "high molecular weight PAHs" (HMWAHs) will include adding the levels of fluoranthene, pyrene, C1-fluoranthenes/pyrenes, benz[a]anthracene, chrysene/ triphenylene, C1- through C4-chrysenes/ benz[*a*]anthracenes, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene/ benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, indenopyrene, dibenz[*a,h+a,c*]anthracene, benzo[*ghi*]perylene. Total PAHs will be calculated by summing the concentrations of LMWAHs and HMWAHs.
The herring egg samples will also be analyzed for lipid classes (i.e., sterol esters/wax esters, triglycerides, free fatty acids, cholesterol, polar lipids) and percent lipid by thin-layer chromatography/flame ionization detection (TLC/FID) using an Iatroscan Mark 6 as described in Ylitalo et al., 2005. Prior to sample cleanup on the alumina/silica column, a 1-mL aliquot of sample extract will be transferred to a clean vial. The volume of each 1-mL egg extract will be reduced to ~100µL using nitrogen gas to ensure that each lipid class will be in the linear range of the FID. Each lipid sample extract will be spotted on a Type SIII Chromarod and developed in a chromatography tank containing 60:10:0.02 hexane:diethyl ether:formic acid (v/v/v). The percent lipid values will be calculated by summing the concentrations of five lipid classes for each sample. The dry weight determinations for the egg and sediment samples will be determined as described in Sloan et al., 2004.

References

Sloan, C.A., Brown, D.W., Pearce, R.W., Boyer, R.H., Bolton, J.L., Burrows, D.G., Herman, D.P. and Krahn, M.M. 2004. Northwest Fisheries Science Center Procedures for Extraction, Cleanup and Gas Chromatography/Mass Spectrometry Analysis of Sediments and Tissues for Organic Contaminants. F/NWC-59, NOAA Tech Memo, NMFS, U S Department of Commerce, Seattle, WA. 47pp. http://www.nwfsc.noaa.gov/publications/displayallinfo.cfm?docmetadataid=4330

Sloan, C.A., D.W. Brown, G.M. Ylitalo, J. Buzitis, D.P. Herman, D.G. Burrows, G.K. Yanagida, R.W. Pearce, J.L. Bolton, R.H. Boyer, M.M. Krahn. 2006. Quality assurance plan for analyses of environmental samples for polycyclic aromatic compounds, persistent organic pollutants, fatty acids, stable isotope ratios, lipid classes, and

metabolites of polycyclic aromatic compounds. U.S. Dept. of Commerce, NOAA Tech. Memo., NMFS-NWFSC-77, 30 pp. http://www.nwfsc.noaa.gov/publications/displayinclude.cfm?incfile=technicalmemorandum2007.inc

Ylitalo, G.M., Yanagida, G.K., Hufnagle Jr, L. and Krahn, M.M. 2005. Determination of lipid classes and lipid content in tissues of aquatic organisms using a thin layer chromatography/flame ionization detection (TLC/FID) microlipid method. p. 227-37. In: G.K. Ostrander (eds.) Techniques in Aquatic Toxicology. Volume 2. CRC Press, Boca Raton, FL, USA.

Detailed analyses of PEMDs for PAHs

PEMDs will be extracted and analyzed for PAHs using GC/MS. An internal standard solution will be applied to each sample, and a spiking solution of analytes will be added to the spiked samples. The samples will then be extracted in 20%/80% methylene chloride/pentane by sonication. The sample extracts will be eluted through silica columns using 20%/80% methylene chloride/pentane to remove any interfering biogenic compounds. The cleaned up extracts will be concentrated to 100 µL and analyzed for PAHs by GC/MS in a series which includes six concentration levels of calibration standards ranging from approximately 0.001 to 1 ng/µL for each analyte. The concentrations of analytes (ng per sample) will be calculated using internal standards and point-to-point calibration. The percent recoveries of the internal standards will be calculated for each sample using the GC internal standard. The analyte percent recoveries for the spiked lab blank strips and spiked solvent method blanks will be calculated relative to the analysis of the spiking solution. Sum "low molecular weight PAHs" (LMWAHs) will include summing the concentrations of naphthalene, C1- through C4-naphthalenes acenaphthylene, acenaphthene, fluorene, C1- through C3-fluorenes, phenanthrene, C1- through C4-phenanthrenes, dibenzothiophene, C1- through C4-dibenzothiophenes and anthracene. Sum "high molecular weight PAHs" (HMWAHs) will include adding the levels of fluoranthene, pyrene, C1-fluoranthenes/pyrenes, benz[a]anthracene, chrysene/ triphenylene, C1- through C4-chrysenes/ benz[*a*]anthracenes, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene/ benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, indenopyrene, dibenz[*a,h*+*a,c*]anthracene, benzo[*ghi*]perylene. Total PAHs will be calculated by summing the concentrations of LMWAHs and HMWAHs.

2.26 Measurement of maternal contributions of PAHs and other CYP1A-inducing compounds in eggs and tissue

Field Collection of Pre-Spawn Adult Pacific Herring

Fish will be collected by either hook and line, dipnets or cast nets in cooperation with the UC Davis Bodega Marine Lab – Aquatic Resources Group (CDFG Scientific Collecting Permit SC - 001821)

Collection of Tissues from Adult Herring

Objective: Bile, ovaries and whole bodies will be collected from pre-spawning adult females at herring spawning sites to determine recent PAH exposure through measurement of bile FACs and to determine levels of POPs and PAHs in ovaries and whole bodies to evaluate maternal transfer as necessary.

Personnel needed:

1 - Data recorder/manager and Sample custodian /QA manager
2-3 - Fish necropsiers

Equipment needed:

4-ml amber vials with limited volume inserts and springs (10 per site)
8-oz pre-rinsed glass jar (I-Chem 300 or better) (10 per site)
Foil
Ziploc bags
Isopropyl alcohol in squirt bottles
Teflon cutting boards
Phosphate –free liquid soap
Distilled water
Measuring board or ruler (cm scale)
Flow through live tank with aeration
Scalpel handles and blades
Scissors and forceps
Datasheets
Ice and icebath
1-cc Tuberculin syringe, 27 gauge
Sample labels
Lighted magnifying glass

Methodology for sampling ovaries for PAHs and POPs analysis, extracting bile for bile FACs analysis, and creating composite samples for adult Pacific Herring

Cleaning procedures for equipment. All equipment (e.g., forceps, hemostat, scalpel handle, cutting board) that may come in contact with the fish tissue samples should be thoroughly cleaned in the following way:

1. Handle all parts with latex or nitrile gloves
2. Use a soap solution and a small brush to remove small particles.
3. Rinse with water
4. Rinse with isopropyl alcohol
5. Rinse thoroughly with distilled water
6. Air dry and take precautions against contamination (cover with rinsed foil)

Fish holding procedures. After capturing the fish, place them in holding tanks or large coolers containing fresh aerated seawater, until the fish can be necropsied aboard the research vessel. Refresh the water in the tanks periodically and ensure that the water temperature is at a comfortable level for the animals. The water temperature can be

cooled with frozen seawater in plastic bottles. **The bile samples must be collected from "freshly sacrificed" fish as soon as possible or degradation of the bile occurs which interferes with the bile FACs analysis.**

Procedure:

1. Female herring will be captured from prespawn aggregations in San Francisco Bay and held alive in a flow-through holding tank described previously, until resection.

2. Up to ten bile-FAC and ten ovary-POP composite samples will be created from each station, with each composite sample represented by ten fish.

3. For resection, a live fish will be taken from the holding tank and delivered to the cutting table where it is killed immediately by inserting a scalpel into the brain.

4. The fish is then opened from vent to mandible using angled scissors, taking care to avoid cutting gonads. After exposing the viscera sex fish, and discard males.

5. For female fish, if the ovaries are ripe, and the gall bladder appears to have enough bile to extract, the ovaries are removed and placed on a clean square of foil, laid on ice for later compositing.

6. The gall bladder is immobilized using forceps; bile ducts may be pinched slightly to concentrate bile into the gall bladder. Bile is removed from the gall bladder by using a 27- gauge tuberculin syringe (with the aid of a lighted magnifying glass.)

7. The needle is inserted into the center of the gall bladder, and bile drawn using the syringe. Care must be taken to draw only bile into the needle, avoiding tissues and exterior fluids.

8. From the bile extracted from each fish, one or two drops (approx. 5 µl) are placed into a bile vial. This maintains equal representation of all fish. Drops are added serially from each of ten fish into a single vial, resulting in a composite sample of between 50 and 100 µl. One syringe should be used per composite. If bile volume is low in the gall bladders, the needle may need to be "primed" with bile from the first fish. In that case, bile can only be expelled from the syringe after the second fish has been drawn, in which case the first fish is discarded.

9. Dispense bile into a pre-labeled 4-ml amber vial glass, with a clear glass limited volume insert, spring, and Teflon disk in the cap. The amber vial is held in an ice bath with the cap screwed on tight between individual fish. After the sample is complete (bile from ten fish combined), the bile vial is moved to the freezer as soon as possible.

10. For each of the ten fish in a composite where bile extraction was successful, a single ovary is placed into a pre-cleaned and labeled 8-oz jar. The ovary composite should

contain ovaries from the same ten fish that contributed to a Bile FAC sample. Ovary composite samples should be placed in the freezer immediately after they are created.

11. Each bile/ovary fish is then measured for standard and total length, placed in an individual ziplock bag along with a FishID tag, and either frozen or placed on ice for transport to the lab where scales or otoliths will be removed for aging. Each whole fish sample is then frozen until analyzed for PAHs and POPs.

12. Record the following on Logsheet.

- Collection Date
- Unique Sample ID Number
- Site animal was captured
- Composite Number
- Bile collected
- Ovary collected
- Whole body collected
- Total length (to the nearest mm)
- Total weight (to the nearest g)
- Standard/Fork length
- Comments/observations

13. Fill out Chain of Custody Form following instructions found in this SOP.

14. Follow shipping guides written in section **1.47 COC Procedures for Sample Shipment**.

Datasheet for Tissues collected for chemical analyses:

NOAA/NMFS/NWFSC/ECD
CBOS 2008 Herring Injury Assessment Study
Tissues for Chemical Analyses

Fish No.	Sample ID #	Collection Date	Site	Fork Length (mm)	Length (mm)	Weight (g)	Bile		Ovary		Eggs		Whole Body
							Collected	Comp #	Collected	Comp #	Collected	Comp #	Collected
1	20080001												
2	20080002												
3	20080003												
4	20080004												
5	20080005												
6	20080006												
7	20080007												
8	20080008												
9	20080009												
10	20080010												
11	20080011												
12	20080012												
13	20080013												
14	20080014												
15	20080015												
16	20080016												
17	20080017												
18	20080018												
19	20080019												
20	20080020												

Reviewed by _____
Signature:

Weight - nearest gram
Collected - YES or NO
Comp# - 1...10

Record data as follows
Collection Date - DD/MM/YYYY
Fork Length - nearest mm
Total Length - nearest mm

COMMENTS:

Page 1

Analysis of herring bile for PAH metabolites

Bile samples will be analyzed for PAH metabolites using high performance liquid chromatography (HPLC) with fluorescence detection (Krahn *et al*., 1984; Johnson *et al*., 2007). Bile will be injected directly onto a C18 reverse- phase column (Phenomenex Synergi Hydro) and eluted with a linear gradient from 100% water (containing a trace amount of acetic acid) to 100% methanol at a flow of 1.0 mL/min. Chromatograms will be recorded at the following wavelength pairs: 1) 293/335 nm where several 2-3 ring compounds (e.g., naphthalene) fluoresce, 2) 260/380 nm where several 3-4 ring compounds (e.g., phenanthrene, dibenzothiophene) fluoresce and 3) 380/430 nm where 4-5 ring compounds (e.g., benzo[a]pyrene) fluoresce. Peaks eluting after 5 minutes will be integrated and the areas of these peaks will be summed. The concentrations of fluorescent PAHs in bile will be determined using naphthalene (NPH), phenanthrene (PHN) and benzo[a]pyrene (BaP) as external standards and converting the fluorescence response of bile to naphthalene (ng NPH equivalents/g bile), phenanthrene (ng PHN equivalents/g bile), and benzo(a)pyrene (ng BaP equivalents/g bile) equivalents. (add full citations?)

Detailed analyses of herring whole bodies and ovaries for PAHs and POPs

Herring whole bodies and ovary samples will be extracted and analyzed for PAHs and POPs using a gas chromatography/mass spectrometry method (Sloan *et al*., 2004). This method involves: (1) extraction of tissues using an accelerated solvent extraction procedure, (2) clean-up of the entire methylene chloride extract on a single stacked silica gel/alumina column, (3) separation of PAHs and POPs from the bulk lipid and other biogenic material by high-performance size exclusion liquid chromatography, and (4) analysis on a low resolution quadrupole GC/MS system equipped with a 60-meter DB-5 GC capillary column. The instrument will be calibrated using sets of up to six multi-level calibration standards of known concentrations.

Sum "low molecular weight PAHs" (LMWAHs) will include summing the concentrations of naphthalene, C1- through C4-naphthalenes, acenaphthylene, acenaphthene, fluorene, C1- through C3-fluorenes, phenanthrene, C1- through C4-phenanthrenes, dibenzothiophene, C1- through C4-dibenzothiophenes and anthracene. Sum "high molecular weight PAHs" (HMWAHs) will include adding the levels of fluoranthene, pyrene, C1-fluoranthenes/pyrenes, benz[a]anthracene, chrysene/ triphenylene, C1- through C4-chrysenes/ benz[*a*]anthracenes, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene/ benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, indenopyrene, dibenz[*a,h+a,c*]anthracene, benzo[*ghi*]perylene. Total PAHs will be calculated by summing the concentrations of LMWAHs and HMWAHs. Sum PCBs will include the sum of congeners 17, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 82, 87, 95, 99, 101/90, 105, 110, 118, 128, 138/163/164, 149, 151, 153/132, 156, 158, 170, 171, 177, 180, 183, 187/159/182, 191, 194, 195, 199, 205, 206, 208, 209. Sum DDTs will be the sum of *o,p*'-DDD, *p,p'*-DDD, *o,p*'-DDE, *p,p*'-DDE, *o,p*'-DDT and *p,p*'-DDT. Sum chlordanes will include the sum of oxychlordane, *gamma*-chlordane, nona-III-chlordane, *alpha*-chlordane, *trans*-nonachlor and *cis*-nonachlor. Sum hexachlorocyclohexanes (HCHs) will be the sum of *alpha*-, *beta*-, and *gamma*-HCH isomers, and sum PBDEs will include the sum of congeners 28, 47, 49, 66, 85, 99, 100, 153, 154, 183.

The herring ovary and whole body samples will also be analyzed for lipid classes (i.e., sterol esters/wax esters, triglycerides, free fatty acids, cholesterol, polar lipids) and percent lipid by thin-layer chromatography/flame ionization detection (TLC/FID) using an Iatroscan Mark 6 as described in Ylitalo et al., 2005 Prior to sample cleanup on the alumina/silica column, a 1-mL aliquot of sample extract will be transferred to a clean vial. The volume of each 1-mL extract will be reduced to ~100µL using nitrogen gas to ensure that each lipid class will be in the linear range of the FID. Each lipid sample extract will be spotted on a Type SIII Chromarod and developed in a chromatography tank containing 60:10:0.02 hexane:diethyl ether:formic acid (v/v/v). The percent lipid values will be calculated by summing the concentrations of five lipid classes for each sample. The dry weight determinations for the egg and sediment samples will be determined as described in Sloan et al., 2004.

2.27 Morphological assessment of herring embryos

Nitex membranes with herring embryos will be collected and placed in pre-labeled individual bags in the field and transferred according to COC requirements. Embryos will be delivered from the field in cold seawater held at constant temperature.

Field/Operations Person – function as liaison between field team and lab teams to coordinate transfer of samples, data collection and COC requirements.

1. Immediately upon arrival *Embryologist #1* will verify condition of delivered embryos
2. Nitex membrane can be cut into thirds with clean isopropanol rinsed scissors and divided among the three embryologists in 150-mm plastic Petri dishes filled with cold seawater and placed in ice buckets.
3. Embryos not actively in use will be kept at constant temperature (10-12˚C, depending on ambient SF Bay temperatures) in a digital cooling incubator.
4. Subsamples of embryos are prepared for transfer to Bodega Marine Lab for larval assays, following appropriate COC procedures.

DAY1

Embryo Processing:

Do NOT image or process any embryos damaged during dechorionation.

1. *Embryologist #1*: Concurrently process embryos for freezing. Two samples are retained.
   Sample A: Remove approximately 100 embryos in chorions from Nitrex mesh using clean isopropanol-rinsed forceps
   a. Place embryos in pre-labeled, RNase-free tubes
   b. Rinse once with cold PBS
   c. Flash freeze closed labeled tube in liquid nitrogen
   d. Log samples in electronic database

   Sample B: At least 3 grams of embryos are scraped with a clean stainless steel spatula from the Nitrex mesh into a preweighed and precleaned IChem jar. Jars are labeled according to standardized nomenclature and stored at -20˚C in a locked freezer. Samples are recorded in data log and Chain of Custody form is filled out.

2. *Embryologist #2*: Concurrently assist with imaging and PFA fix embryos.
   a. Embryos are dechorionated with fine forceps under a stereomicroscope. Embryos chilled by placement of 100-mm Petri dish surrounded by an ice bath within a 150-mm dish. Dechorionated embryos are provided to Embryologist #3 for imaging.

b. Collect embryos from Embryologist #3 post-imaging and fix 30 pooled embryos in 1.5 ml 4% Millonig's-buffered PFA. Tubes are labeled following the standardized nomenclature.
c. Log samples and images
d. See Day 2 duties.

Microscopic imaging:

3. *Embryologist #:3* Visually inspect and photo document 30 dechorionated embryos using a stereomicroscope fitted with a phototube and Fire-i400 1394 camera connected via firewire to laptop with BTV Pro, iMovieHD. Embryos are placed in a 150-mm Petri dish with 1% agarose (in 16 ppt seawater) slot matrix, with temperature held at 10-12˚C with cooling stage apparatus. Embryos are acclimated for 10 min. Magnifications for imaging will be identical for all samples collected, and will be determined during the initial data collection. Total magnifications are most likely to be in the range of 60X for imaging of pericardial area and 10X for overall morphology. At the beginning and end of each data collection period, a JPEG image will be captured of a stage micrometer at each magnification.
   a. Image capture 1 – With anterior to the left and dorsal to the top of the frame, focus on cardiac/pericardial region under high power magnification. 20-sec video clips are captured for each of 30 embryos iMovieHD. A single iMovieHD file is saved containing 30 clips. Files are backed-up from the capture drive to each of two external hard drives. Single JPEG frames will be exported during the data analysis phase for import into ImageJ for measurement of pericardial space.
   b. Image capture 2 – A low power magnification image is taken with the entire embryo in focus. Embryo is oriented for a left-lateral view (anterior to left, dorsal at top). Single JPEGs are captured with BTVCarbon Pro. A folder with the 30 low-magnification images is paired with the iMovieHD file, and backed-up to the two external hard drives.
   c. Documentation: A standardized nomenclature is used to record all file names. In addition to back up of folders and files, text files are exported listing all file names.

DAY 2

Embryologist #2:

1. After 24 hours of PFA fixation, wash embryos into PBS with one fast wash and then several washes (minimum 3) over the course of the day.
2. Record samples on Logsheet and fill out Chain of Custody form.
3. Pack samples for shipment and store at 4 degrees until shipping.
4. Follow shipping guides written in section **1.47 COC procedures for Sample Shipment**.

2.28 Hatching Success and Larval Survival

Embryo Culture:

1. After receiving embryos collected 24-48hrs. prior to hatching, field samples (n=5 from each site) will be placed in 250 ml glass culture dishes containing 150ml of 0.45 µm filtered ½ strength seawater (16 ppt). Embryos will be incubated at 12^{o}C until initiation of hatching ("Day 0") at concentrations of no more than 100 embryos/100 ml. For outplanted embryos, Nitex mesh will be cut such that ~200 embryos/dish will be used. For natural spawns, collected embryos on their natural substrates will be placed into each dish with a maximum of 100 embryos/100 ml. Hatching and larval morphology will be quantitated each day for 5 days ("Days 0- 5"). Water will be changed on a daily basis.
2. Hatching will be scored at the same time each day by counting the number of hatched larvae in each dish after they are removed with the water changes. The number of dead larvae will be counted and remaining live larvae anesthetized using MS-222 and assessed for normal morphology and for the total number of live larvae. Abnormal live larvae will include those showing classic effects of oil PAH exposure such as cardiac edema, spinal curvature, inability to straighten, etc.
3. The final percentages of hatch will be calculated at 5 days after counting unhatched embryos and adding together the number of hatched larvae over the 5 day period.
4. The temporal distribution of hatching over the 5-day period will be determined.

2.29 Larval Feeding Success

Larval Culture (up to 20 days post hatch):

1. Hatched larvae are transferred to clean containers (50-75 larvae/500 ml) containing16 ppt seawater)
2. Larvae are incubated at 12^{o}C (incubator) or in flowing seawater with an ambient light:dark cycle
3. Daily water changes of 50-75% of culture water (16 ppt seawater) are performed for larvae kept under static conditions.
   Larvae gravitate towards light, so shining a light source at one end of the container allows for suctioning of water at the other end.
4. Larvae are observed daily for evidence of mortalities, abnormalities (skeletal abnormalities, edema) or disease and those exhibiting such abnormalities are removed using a pipet and euthanized.
5. Larvae are fed rotifers (*Brachionus plicatilis*) ad libidum
6. At the termination of the experiments, larvae are euthanized in >150 mg/L MS222.

2.30 Analyses of CYP1A induction in herring embryos

Detailed SOPs for CYP1A induction by 1) immunofluorescence and confocal microscopy and 2) quantitative real-time RT-PCR will be provided upon trustee decision to proceed with this analysis.

3.0 Quality Assurance

Quality Assurance procedures will be followed to monitor (1) the performance of the measurement systems to maintain statistical control, and provide rapid feedback so that corrective measures can be taken before data quality is compromised and (2) verify that reported data are sufficiently complete, comparable, representative, unbiased and precise so as to be suitable for their intended use.

Field collection of spawned herring eggs Samples will be placed in bags prior to breaking the surface of the water, and will remain in bags while in transport to prevent contamination from boat engine exhaust and other sources of PAHs. Basic water quality conditions will be maintained during transport. For chemical analyses, a sufficient quantity of samples will be collected to allow triplicate analyses for the purpose of QA.

Artificial spawning of herring eggs Eggs will be incubated 16 ppt filtered seawater held at ambient SF temperature in a digitally controlled cooling incubator. Fertilization rate will be inspected at the stage of first cleavage– batches of eggs with low fertilization rate will not be used

Artificial spawning of eggs and deployment to the field (should this be combined with paragraph above?) See SOP(something missing here?) and will only be opened after eggs are placed underwater in the field locations to prevent contamination by boat exhaust or other sources of PAHs.

Deployment and retrieval of passive sampling devices *See SOP*

Analysis of PAHs and POPs in herring eggs, adult herring (maternal transfer), PEMDs and sediment As part of the laboratory quality assurance program, the accuracy of our methods are monitored using National Institute of Standards and Technology (NIST) standard reference materials (contaminated sediment SRM 1941b or blue mussel homogenate SRM 1974b). These quality assurance materials will be analyzed with each sample set of eggs, whole bodies or sediments, as appropriate, and the results must meet the laboratory criteria described in Sloan *et al*. (2006). Spiked blank PEMDs will be analyzed with each sample set of PEMDs in lieu of an SRM. Approximately 10% of the samples will be analyzed in replicate to measure precision of the method and the laboratory quality assurance criteria will be met for all analytes detected in the samples. Method blanks must also meet laboratory criteria. In addition, percent recoveries of surrogate standards must be within the acceptable range listed in NWFSC's Laboratory Quality Assurance Plan (Sloan *et al*., 2006)

Analysis of bile in adult herring To ensure that the HPLC/fluorescence system is operating properly, a NPH/PHN/BaP calibration standard will be analyzed numerous times ($n \geq 5$) until a relative standard deviation < 15% is obtained for each PAC. As part of our laboratory quality assurance (QA) plan, two QA samples [a method blank and a fish bile control sample (bile of Atlantic salmon exposed to 25 µg/mL of Monterey crude oil for 48 hours)] will be analyzed with the fish bile samples (Sloan *et al*., 2006).

Morphological assessment of herring development Morphological features will be compared with normal developmental features described in Hill and Johnston 1997, J. Fish Biol. 51:960-977. Embryos will be held at ambient temperature in a digitally controlled incubator. Temperature during microscope observations will be controlled with a Peltier-cooled stage insert (Brooks industries, Lake Villa, Il,) designed to maintain the content of a 100-mm petri dish as low as 5C. Quality assurance and control measures will be put in place to ensure consistent analysis. Consistent magnification of images will be ensured by documentation with a stage micrometer. Proper embryo orientation and focus will be ensured by comparing to standardized high-quality images supplied to the embryologists on the laptop used for data collection. All images will be encrypted and analyses conducted in a blinded manner. Several qualified scientists will conduct a blinded image analysis and calculate pericardial edema based on the calculated area surrounding the heart (pericardial space). A percentage of all pericardial space calculations determined by each staff member will be cross-checked by either the lead scientist or distributed evenly across the other analysis staff members. Alternately, a randomized subset of all analyses could be re-analyzed by the lead scientist to measure consistency. At the conclusion of image analysis all data will be un-encrypted and statistically evaluated.

Chain of Custody Procedures Chain of custody procedures will be used for all samples and for all data and data documentation, whether in hard copy or electronic format. Each container is considered to be an individual sample and is assigned a unique ID number. A sample is considered in "custody" if: a) it is in the custodian's actual possession or view, b) it is retained in a secured place (under lock) with restricted access, or c) it is placed in a container and secured with an official seal(s) such that the sample cannot be reached without breaking the seal(s).Samples are kept in the custody of designated sampling and/or field personnel until transfer to the laboratory. The original signed and dated chain of custody record accompanies the sample(s). The laboratory sample custodian or designee maintains a laboratory sample-tracking record, similar to the COC record, which follows each sample through all stages of laboratory processing.
All unanalyzed samples and unutilized sample aliquots or extracts are held by the laboratory in a manner to preserve sample integrity at a secure location with chain of custody procedures for one (1) year after the QA Contractor has validated the data package for that particular set of samples.
All data and data documentation, whether in hard copy or electronic format, are the responsibility of the QA Coordinator acting on behalf of Counsel to the Case Management Team. These materials will all be clearly marked with "Attorney Work Product."

APPENDIX A. CHAIN OF CUSTODY FORM

Northwest Fisheries Science Center
Environmental Conservation Division

CHAIN OF CUSTODY FORM

(Use ball point pen only)

Form No. : 1				
PROJECT or CASE TITLE AND REMARKS: 2				
DATE SAMPLES COLLECTED: 3		SAMPLE CUSTODIAN: 4		
ITEM NO. 1	**SAMPLE TYPE/PURPOSE** 5	**SAMPLE ID NUMBERS** 6	**SAMPLE CONTAINER** 7	**TOTAL NUMBER OF SAMPLES** 8
Comments 9			Continued from Form No: Item No. from previous form	10
ITEM NO. 2	**SAMPLE TYPE/PURPOSE**	**SAMPLE ID NUMBERS**	**SAMPLE CONTAINER**	**TOTAL NUMBER OF SAMPLES**
Comments			Continued from Form No: Item No. from previous form	
ITEM NO. 3	**SAMPLE TYPE/PURPOSE**	**SAMPLE ID NUMBERS**	**SAMPLE CONTAINER**	**TOTAL NUMBER OF SAMPLES**
Comments			Continued from Form No: Item No. from previous form	
ITEM NO. 4	**SAMPLE TYPE/PURPOSE**	**SAMPLE ID NUMBERS**	**SAMPLE CONTAINER**	**TOTAL NUMBER OF SAMPLES**
Comments			Continued from Form No: Item No. from previous form	
ITEM NO. 5	**SAMPLE TYPE/PURPOSE**	**SAMPLE ID NUMBERS**	**SAMPLE CONTAINER**	**TOTAL NUMBER OF SAMPLES**
Comments			Continued from Form No: Item No. from previous form	

TRANSFER SIGNATURES

ITEM NO: 11	FROM: PRINT NAME, AGENCY 12	RELEASE SIGNATURE:	RELEASE DATE:	DELIVERED VIA: ☐ IN PERSON ☐ FED EX ☐ OTHER
	TO: PRINT NAME, AGENCY	RECEIPT SIGNATURE:	RECEIPT DATE:	Sample Condition/Comments 13
ITEM NO:	FROM: PRINT NAME, AGENCY	RELEASE SIGNATURE:	RELEASE DATE:	DELIVERED VIA: ☐ IN PERSON ☐ FED EX ☐ OTHER
	TO: PRINT NAME, AGENCY	RECEIPT SIGNATURE:	RECEIPT DATE:	Sample Condition/Comments
ITEM NO:	FROM: PRINT NAME, AGENCY	RELEASE SIGNATURE:	RELEASE DATE:	DELIVERED VIA: ☐ IN PERSON ☐ FED EX ☐ OTHER
	TO: PRINT NAME, AGENCY	RECEIPT SIGNATURE:	RECEIPT DATE:	Sample Condition/Comments
ITEM NO:	FROM: PRINT NAME, AGENCY	RELEASE SIGNATURE:	RELEASE DATE:	DELIVERED VIA: ☐ IN PERSON ☐ FED EX ☐ OTHER
	TO: PRINT NAME, AGENCY	RECEIPT SIGNATURE:	RECEIPT DATE:	Sample Condition/Comments
ITEM NO:	FROM: PRINT NAME, AGENCY	RELEASE SIGNATURE:	RELEASE DATE:	DELIVERED VIA: ☐ IN PERSON ☐ FED EX ☐ OTHER
	TO: PRINT NAME, AGENCY	RECEIPT SIGNATURE:	RECEIPT DATE:	Sample Condition/Comments

INSTRUCTIONS FOR FILLING OUT Chain of Custody (COC) FORM

- **The original copy of the Chain of Custody (COC) form must accompany samples, or be in the possession of the field or lab sample custodian.**

- **Samples relinquished to the laboratory for analyses must begin a new COC**

There are 4 Copies of the COC form. Copies are designated as follows:

WHITE – Original stays with sample
GOLDENROD – Field COC Notebook
PINK – Lab
CANARY – Lab or Study QA Coordinator

1. FORM No. A pre-printed sticker should be affixed to the White Copy of the COC form. Write the number printed on the sticker. DO NOT USE a form without a pre-printed sticker.

2. Project or Case Title and Remarks. e.g. CBOS Spill Assessment 2008

3. Date Samples Collected. Use full date i.e. Day, Month, Year (DD/MM/YYYY)

4. Sample Custodian. Samples Taken By, Project POC, Lab POC etc**.** This can be the designated sample custodian for the project or lab who has knowledge of sample origin. Examples: Chief Scientist, Lab Manager or coordinator, Principal Investigator.

5. Sample Type and Purpose. e.g. Eggs –Chemistry Analyses, Bile-FACs etc.

6. Sample ID Numbers. This should be number range if appropriate e.g. 20080001 through 20080023

7. Sample Container e.g., cryovial, 4oz jars

8. Total Number of Samples. Total number for sample set on this form

9. Comments. Describe any issues anything out of the ordinary etc. with the samples.

10. Continued from Form No: and **Item No. from previous form:** Samples that have been relinquished to another party such as the Lab will begin a new form. Write the previous Form number and Item number from the original form.

11. Item No. This will be Item number 1 through 5, itemized on the top half of the form.

12. Transfer Signatures. Fill in printed name and signatures of both person relinquishing and person receiving samples and dates. Check method of delivery.

13. SAMPLE CONDITION/COMMENTS. **NOTE IF ANY SAMPLES ARE MISSING, IF CUSTODY SEALS ARE INTACT, SAMPLES FROZEN OR THAWED ETC.**

APPENDIX B: QUICK GUIDE ON COC PROCEDURES FOR CBOS SAMPLE COLLECTION AND PROCESSING:

FIELD COLLECTION OF HERRING EGGS OR SEDIMENT:

Field logbook:

- Record everything (who, where, when, why) about sample collection and storage. Reference COC form #'s in the logbook.

Custody forms:

- confirm COC sample #'s match those recorded on COC form
- sign and date

Seal sample containers

- if samples will be out of sight of "custodian" (placed in a freezer, stored overnight, shipped, etc.)

DEPLOYING AND COLLECTING CAGES HERRING EGGS

Seal the cages in a tamper proof method: (e.g. cable ties)

Field logbook:

- Record everything (who, where, when, how) about sample deployment or collection

Custody Forms:

- confirm COC sample #'s match those recorded on COC form
- sign and date

Seal sample containers

- **i**f samples will be out of sight of "custodian" (placed in a freezer, stored overnight, shipped, etc.

TRANSFER OF SAMPLES FROM THE FIELD, TO THE ON-SITE LAB:

Custody forms:

- receiving personnel should confirm COC sample #'s match those recorded on COC form
- both parties (receiving and relinquishing) sign and date forms

Lab Notebook:

- Note everything (who, where, when, how) about transfer, storage, condition, processing, repackaging, etc.

Start new Custody forms if repackaged:

- Indicate original COC form # so COC can be tracked to its origin.
- confirm COC sample #'s match those recorded on COC form
- sign and date

Seal sample containers

- if samples will be out of sight of "custodian" (placed in a freezer, stored overnight, shipped, etc.

ADULT HERRING, (FOR TISSUE COLLECTION OR ARTIFICIAL SPAWNING)

Field Logbook:

- Record everything (who, where, when, how) about adult herring capture and transport to the lab.

Custody forms:

- although sample numbers may not be appropriate here, provide sample information such as "Site Name adult herring collected 1/07/08"
- sign and date

Seal sample containers

- if samples will be out of sight of "custodian" (placed in a freezer, stored overnight, shipped, etc.

Transfer custody of adult fish to laboratory personnel for processing

- relinquishing and receiving people sign COC form.
- assign sample numbers to tissue samples or spawned eggs, and start a new COC form .
- Refer to original COC form # when preparing new COC documentation

ARTIFICIALLY SPAWNED EGGS:

Logbook:

- Record everything (who, where, when, why) about activities, storage, etc.

Custody forms and security of samples:

- will need to work out COC methods depending on workflow, however COC tracking needs to be maintained during storage of spawned eggs at the laboratory and transport to the field cages

LABORATORY PROCESSING: (E.G. MICROSCOPY EXAMINATION, OTHERS)

Chain of Custody- accepting samples:

- Check samples received, are they the same as on the COC forms?
- Sign and date forms.

Logbook:

- Record when samples were unsealed
- record what activities were conducted and by whom
- record when returned to secure packaging
- record what data was collected, how data is stored, including file names!
- -convert image file directories to electronic files (excel) on a daily basis
- -create back-up electronic database files on external hard drives

- -create hardcopy versions and paste into the logbook (sign, witness)
- if samples were destroyed, (e.g. beyond useful developmental stage) note who, how and especially when they were destroyed)
- Basically record everything (who, where, when, why) about activities, storage, etc.

Storing samples:

- maintain COC at all times

Record keeping

- Maintain all records on a password protected computer or locked up
- Keep master database to link all files (images, data sheets, sample numbers)
- Backup files on external database
- Print final hardcopy and put into notebook

Discarding samples:

- Frozen and fixed, - do not discard
- finite samples (e.g., live fish embryos) record in logbooks when and why discarded.

COC of Data: maintain on a password-protected computer, or locked in a file cabinet.

APPENDIX C: SOP FOR PEMDS

Introduction

Passive accumulation devices (PADs) are typically hydrophilic membranes with or without hydrophilic reservoirs and they are designed to sample non-polar hydrophobic hydrocarbons, including polynuclear aromatic hydrocarbons (PAH) and persistent organic pollutants (POPs) from air, water, and sediment. A commercially available PAD commonly available in the USA is the semi-permeable membrane device (SPMD); its central reservoir is triolein (e.g., Huckins et al. 1990). Hydrocarbons in SPMDs diffuse through pores in the membranes and are trapped in the central triolein matrix, mimicking uptake by living organisms. Advantages of passive sampling are that they can sample large volumes of water, amplify trace hydrocarbon quantities (part-per-billion or part-per-trillion) to detectable levels, and average the signal over time. In addition, they are cheaper and easier to analyze than biological tissue and can be deployed over a greater range of environmental conditions.

At low ambient hydrocarbon concentrations, low-density polyethylene membrane devices (PEMDs) deployed without inclusion of the central hydrocarbon reservoir are simpler and less expensive sampling devices than SPMDs, yet provide the same benefits (Carls et al. 2004). Loss of accumulated PAH is slow, thus PEMDs reliably capture sporadic or fluctuating events. Composition of PAH accumulated by PEMDs can be used to identify hydrocarbon sources. At the Auke Bay Laboratory, we also refer to PEMDs as LDPEs (low density polyethylene devices) or PMDs (polyethylene membrane devices). A universal moniker has not yet been established in the literature.

Laboratory preparation

Low-density polyethylene tubing (98 μm • 4.9 cm • 50 cm) is sonicated twice in pentane to remove hydrocarbons, placed in aluminum samplers (11.5 diameter • 6.6 cm with perforated endplates, 3 mm holes spaced 4.8 mm apart, precleaned in dichloromethane), wrapped with two layers of aluminum foil, heat-sealed in two plastic bags, and frozen until shipment (Fig. 1; Carls et al. 2004).

Fig. 1. Example PEMD wrapped in foil and placed in ziplock bags. This example was wrapped with a single layer of foil which has torn, illustrating the need to be careful and double wrap each canister.