The 2007 *Cosco Busan* oil spill: Field and laboratory assessment of 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* allided 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 during the 2007-2008 herring spawning season 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; and (2) characterize the exposure of herring embryos to polycyclic aromatic hydrocarbons (PAHs) potentially derived from *Cosco Busan* oil. Because the findings from the 2007-2008 season strongly suggested impacts to embryos incubated at oiled sites, follow-up field and lab studies were performed during the following two spawning seasons.

During the 2007-2008 spawning season, herring embryos developing in situ in San Francisco Bay were assessed for PAH exposure, sublethal cardiac toxicity, developmental abnormalities, and hatching success. Cages containing artificially fertilized embryos were moored together with passive water sampling devices for PAHs (polyethylene membrane devices; PEMDs) at six sites. Four of these sites were visibly oiled immediately after the spill, while two sites were not oiled but contiguous with the same heavily urbanized shoreline (reference sites). Caged embryos were in the subtidal zone, at a common depth at least 1 m below the surface throughout the tidal cycle. Naturally spawned embryos were collected from five mid to low intertidal sites, four of which were adjacent to the caged embryos. Embryos from all sites were transported to a laboratory for live imaging using digital photo- and videomicroscopy and for incubation to hatching. Chemical analysis of embryos collected in 2008 and 2010 included PAHs and a suite of persistent organic pollutants (POPs) routinely found in urban environments, including polychlorinated biphenyls (PCBs) and organochlorine pesticides. Additionally, ovaries and whole bodies of pre-spawning adult herring entering San Francisco Bay in 2008 were analyzed for PAHs and POPs to evaluate the potential for maternal transfer of contaminants.

Whereas embryos incubated in the turbid subtidal zone at oiled sites in 2008 showed heart rate defects and pericardial edema consistent with sublethal petroleum toxicity, the vast majority of embryos developing in the intertidal zone at oiled sites died just before the hatching stage, with major disruption of tissues. No toxicity was observed in natural spawn or caged embryos from unoiled reference sites. Very few larvae with normal morphology hatched from natural spawn samples collected at oiled sited in 2008. The composition of PAHs at oiled sites in embryos and PEMDs was consistent with oil exposure against a background of urban PAH sources, although tissue concentrations were too low to explain the dramatic lethality. Concentrations of other pollutants typically associated with urbanization were also too low to cause lethality. In a series of laboratory studies in 2009, Cosco Busan oil demonstrated a potent phototoxic effect, whereby tissues are disrupted through an interaction between as yet identified compounds and sunlight. This phototoxic activity remained potent after two months of weathering. Embryos developing in the subtidal zone at oiled sites were presumably protected from this effect by the highly turbid water above them, while more intense exposure to sunlight in the intertidal zone led to lethality. Natural spawn sampled two years later from oiled sites showed no elevated necrosis or mortality, indicating that phototoxic activity was eliminated by much more prolonged weathering.

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 response 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

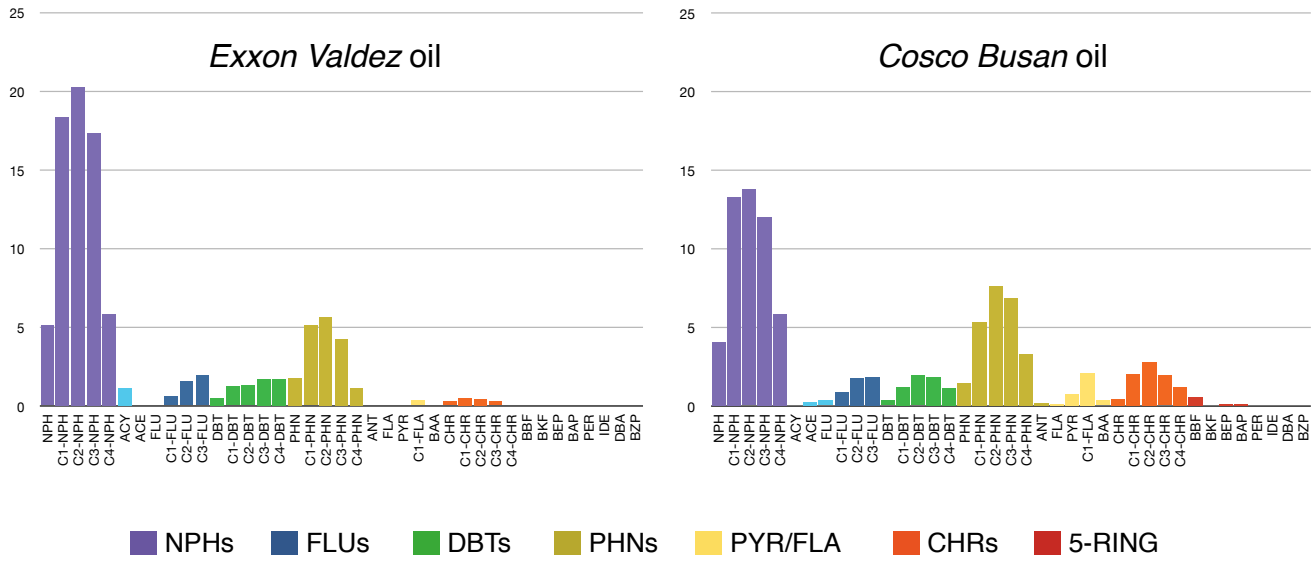


Figure 1-2: PAH composition of *Exxon Valdez* hold oil compared to *Cosco Busan* bunker oil. The x-axis is percentage of total PAHs. PAH subclasses are color-coded and degree of alkylation increases to the right (C1-, C2-, etc.). NPHs, naphthalenes; FLUs, fluorenes; DBTs, dibenzothiophenes; PHNs, phenanthrenes; 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.

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 adjacent to but not necessarily in direct contact with an oiled shoreline 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.

Many chemical and elemental components of crude oil are much more highly concentrated in residual oils (Table 1-1) [1, 2, 86]. 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 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

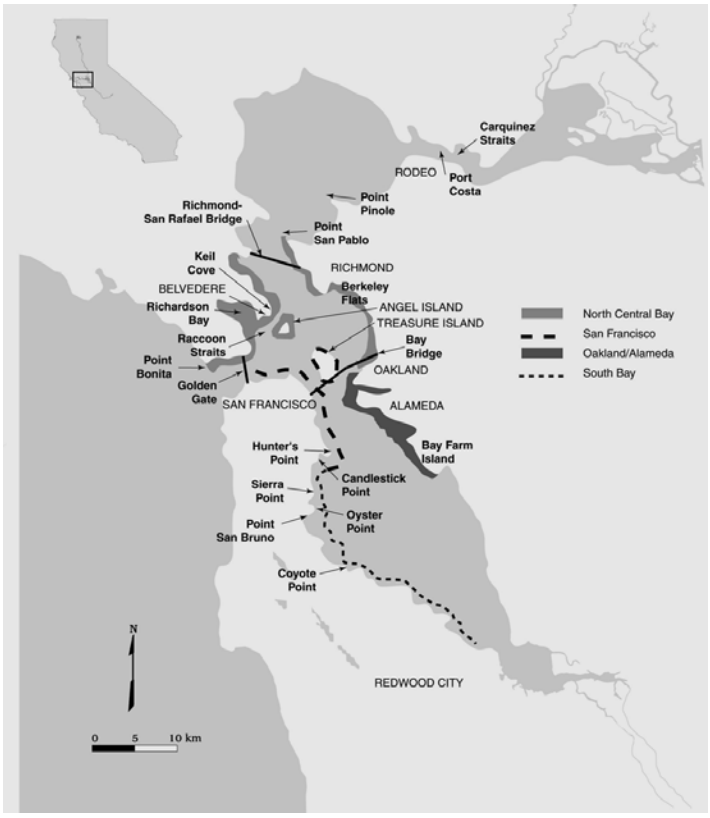


Figure 1-3: Historical Pacific herring spawning regions within San Francisco Bay. (From reference 11)

December through March with the peak of spawning occurring during January and February, although it has begun as early as October [5, 7-9].

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

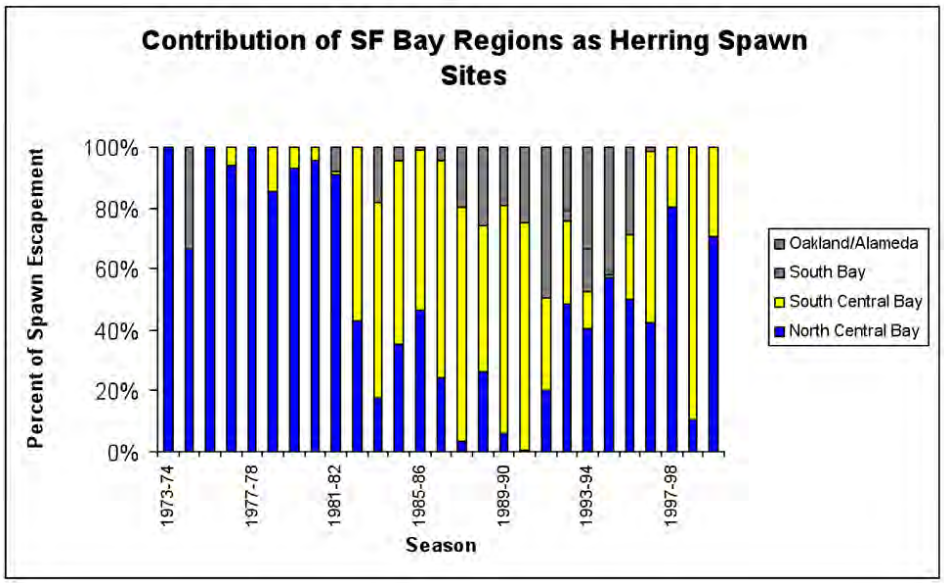


Figure 1-4: Percent of Pacific herring spawning adult biomass (i.e., escapement) by region for each season in San Francisco Bay, 1973–2000. Average percent biomass for each region was 54.9% North Central bay, 34.2% South Central Bay, 9.8% Oakland–Alameda, and 1.1% South Bay. (From reference 11; San Francisco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

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].

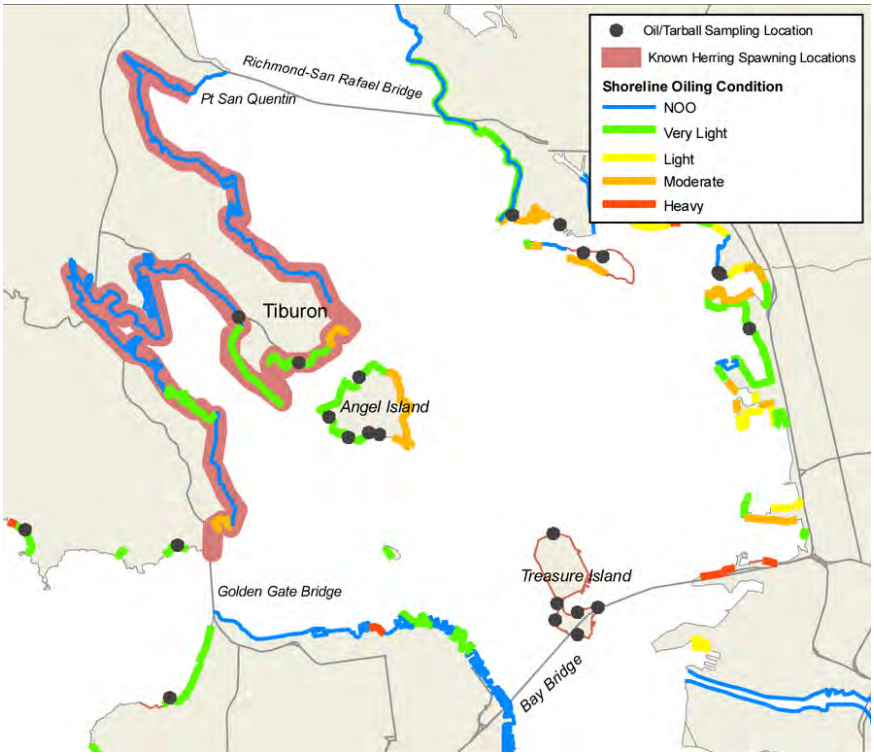


Figure 1-5: Relationship of shoreline oiling to recent herring spawning grounds in the Central Bay.

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 ≥ 0.1% caused premature hatching but no mortality in embryos. There were otherwise no significant effects at concentrations ≤ 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 the 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

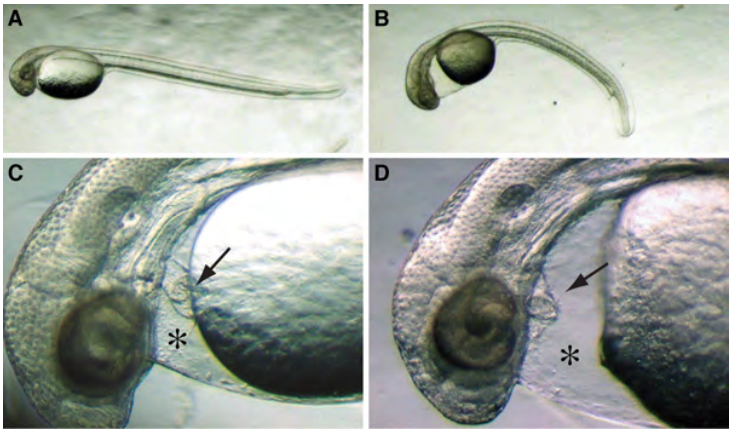


Figure 1-6: Cardiogenic edema in Pacific herring embryos exposed to ANSCO. (A, B) Gross morphology of embryos at 7 days post-fertilization exposed to clean (A) or oiled (B) gravel effluent. (C, D) Higher magnifications showing the heart (arrows) and pericardial space (asterisks) in embryos exposed to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

particulate oil [48].

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.

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].

The effects of PAHs and petroleum products described above are all based on studies of oil effects in the absence of other stressors. An additional pathway of toxicity identified for individual PAHs and whole oils involves interactions with ultraviolet (UV) wavelengths of sunlight. Specifically, a large body of literature demonstrates that certain PAHs are capable of producing cellular phototoxicity through the UV-mediated activation of bioaccumulated compounds and subsequent generation of reactive oxygen species and membrane damage [87,88]. This has been raised as a mechanism that is putatively important in the environment, due to the potential susceptibility of unpigmented organisms to UV exposure from solar radiation in shallow waters [89]. Most of the studies on PAH phototoxicity in aquatic systems have focused on planktonic invertebrates e.g. [90-92], while a few have focused on phototoxicity of individual PAHs and ANSCO preparations in fish early life history stages [93-96]. A recent study compared the phototoxicity of bunker oils to ANSCO in zebrafish embryos and found that bunker oils had much greater phototoxic potential, and that the phototoxicity was largely from compounds other than the typically measured PAHs [97].

In summary, the literature on the toxicity of different types of petroleum products (i.e. crude and bunker oils) indicates that there is likely to be considerable overlap with the types of toxicity observed with ANSCO, but also that there may be novel effects associated with the more chemically complex bunker oils.

1.7 General goals and approach for the assessment of injuries in the field. 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 only describes the results of the Tier 1 studies. An adequate assessment was obtained with the completion of Tier 1 studies, and part from analysis of sediments, Tiers 2 and 3 were not implemented.

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 maps Shoreline Cleanup and Assessment Teams; SCAT). 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 to 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).

Because significant biological effects were observed in herring embryos incubated at oiled sites in February 2008, follow-up sampling was performed in 2009 and 2010. The goal of these studies was to collect natural spawn from the same locations sampled in 2008. In 2009, there was no overlap of natural spawn in intertidal zones sampled in 2008, but spawning occurred in the intertidal zone in a new reference site on the Tiburon Peninsula, Paradise Cove Park. In 2010, intertidal spawning occurred at the exact same GPS coordinates sampled in 2008 at Sausalito, Peninsula Point, and Keil Cove, and at the 2009 reference site, Paradise Cove. A summary of all the sites and the types of samples analyzed are provided in Table 1-2.

Table 1-2: Physical shoreline characteristics of sample sites and types of samples collected each year.

Site	SCAT rating	Cleanup	Adjacent land use/maritime use	Subtidal sampling		Intertidal sampling
				Incubated Caged Embryos[2]	PEMDs	Natural spawn sampled[1]
Keil Cove (KC)	oiled; heavy	extensive wiping, removal of rock	residential, undeveloped forest	2008	yes	2008, 2010
Horseshoe Cove (HC)	oiled; moderate-light	extensive wiping of rip-rap	marina, major highway	2008	yes	ND
Sausalito (SA)	oiled; very light-light	some wiping	marina, commercial, residential	2008	yes	2008, 2010
Peninsula Point (PP)	oiled; light	some wiping	residential	2008	yes	2008, 2010
San Rafael Bay (SRB)	no oil	NA	commercial parking lot, major highway	2008	yes	2008
Paradise Cove (PC)	no oil	NA	residential, public green space	not sampled	no	2009, 2010
Point San Quentin (PSQ)	no oil	NA	commercial/industrial parking lots, major highway	2008	yes	not sampled

[1]All caged and naturally spawned embryos were assessed for sublethal exposure to PAHs and POPs, except the 2009 samples.
NA = not applicable, ND = no spawn detected

1.8 Laboratory studies supporting interpretation of field injury assessment. Contemporary research on oil toxicity, largely in response to the Exxon Valdez spill, has focused on crude oil, and in particular petrogenic polycyclic aromatic hydrocarbons (PAHs). The hallmark of "canonical" crude oil toxicity in fish embryos is cardiogenic edema, attributable to the tricyclic PAH fraction of unrefined petroleum such as Alaska North Slope crude oil (ANSCO). It was anticipated that if any lingering oil toxicity followed the Cosco Busan spill, it would be observed as a small increase in the detection of pericardial edema in herring embryos incubated near oiled shoreline. The 2007-2008 Fish Injury studies were designed to detect such differences. While there were statistically significant increases in measures of sublethal pericardial edema in caged embryos incubated in the subtidal zone of oiled shorelines, embryos that incubated in the intertidal zones of oiled shoreline apparently succumbed to a dramatically different type of lethal toxicity. The complete absence of this lethality at the non-oiled site, plus the inability to associate lethality with other chemical or abiotic stressors, strongly suggests a link to exposure to Cosco Busan oil. While canonical petrogenic PAH toxicity is sublethal, previous laboratory studies with ANSCO and herring larvae showed that oil can produce acutely lethal toxicity when combined with exposure to ambient sunlight or UV wavelength light. At the same time, modern residual fuel oils such as that carried on board the Cosco Busan have distinct chemical differences from unrefined crude oil that could result in different types of toxicity. On this basis, the novel lethal effect observed in 2007-2008 natural spawn samples and the differences in effects observed in subtidal vs. intertidal incubation leads to these specific aims: **(1) Does the inherent toxicity of Cosco Busan bunker oil differ significantly from unrefined Alaska North Slope crude oil? (2) Did sunlight exposure of beached Cosco Busan bunker oil produce**

novel toxic compounds through photo-oxidation? (3) Was the observed necrosis in natural spawn samples due to phototoxicity of PAHs or other bunker oil constituents?

To test these specific aims, a laboratory study was designed by investigators at NOAA's Northwest Fisheries Science Center and the UC-Davis Bodega Marine Laboratory, and implemented at the Bodega Marine Laboratory December 2008 through March 2009. Oiled gravel columns were used to generate water contaminated with dissolved-phase oil constituents in a way that mimics intertidal conditions following an oil spill. The basic principle was to expose herring embryos to oil during weathering by initiating weathering of the columns in January with continuously flowing seawater, and incubating herring embryos in the column effluents at different points between January and whenever the availability of gametes ceased (potentially April). A replicate design tested effluents from columns containing clean gravel, gravel from a non-oiled urbanized beach in San Francisco Bay, gravel coated with three concentrations of ANSCO as a positive control, and gravel coated with three concentrations of Cosco Busan oil. Both the columns and the incubation reservoirs for embryos were exposed outdoors to either full sunlight or sunlight with reduced UV wavelengths with the use of covers constructed from UV transmitting (UVT) or UV blocking (UVB) plastic.

Due to the constraints of obtaining sufficient masses of herring gametes and the time to analyze the embryos from a single experiment, only Aim 3 was rigorously tested. The study as it was executed could not rule in or out a contribution of photo-oxidation to toxicity. The toxicity of the two oils was not directly compared in the laboratory without the additional stressor(s) of outdoor exposure. Embryos were incubated in the column effluents at four points between late January and late March 2009. After incubation to 8 days post-fertilization (just before hatch), embryos were examined for signs of necrosis. In order to verify the oiled gravel dose response relationships, PAHs were measured in water samples at the start and end of embryo incubation, and in embryos tissues at the end of incubation. However, PAH concentrations were not intended to be used as the sole determinant in the interpretation of toxic effects, as there may be other unmeasured compounds contributing to toxicity of a given oil.

An additional lab study addressed whether incubation at higher than optimal salinity could account for some of the abnormalities observed in embryos from oiled sites in 2008. This study is described in Section 4.7.

Section 2: Methods and Implementation

2.1 Field studies. The major aim of field studies was to opportunistically sample herring embryos naturally deposited at a variety of oiled and reference shorelines. In the event that herring did not spawn along oiled shoreline, but moved to other locations in greater San Francisco Bay, herring embryos fertilized in vitro were incubated in cages placed on replicate moorings in the shallow subtidal zones of four oiled and two reference shorelines. Sites selected in January 2008 for mooring caged embryos included four oiled (Horseshoe Cove, Sausalito waterfront, Peninsula Point, Keil Cove) and two reference sites (Point San Quentin, San Rafael Bay). In 2008 three oiled sites and one reference site had natural spawn depositions that could be sampled. Methods described here were established for those sites, and were also applied to follow-up sampling taken in 2009 and 2010. An additional reference site was available in 2009 and 2010, Paradise Cove on the Tiburon Peninsula.

2.1.1 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	2/27/08 12:04 PM	2/27/08 12:18 PM	2/27/08 12:33 PM

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	2/28/08 12:05 PM	2/28/08 12:11 PM	2/28/08 12:17 PM	2/28/08 12:24 PM	2/28/08 12:36 PM	2/28/08 12:36 PM
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	2/29/08 12:15 PM	2/29/08 12:29 PM	2/29/08 12:36 PM	2/29/08 12:47 PM	2/29/08 12:54 PM	2/29/08 1:02 PM	2/29/08 1:11 PM	2/29/08 1:17 PM

Figure 2-1: Collection of natural spawn

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

Figure 2-2: Construction of moorings

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.

Samples taken in 2009 and 2010 followed identical procedures (Table 2-2). However, in 2009 intertidal spawning occurred at only a single site, Paradise Cove. In this case spawning was higher in the intertidal zone and on substrate dissimilar to previous samples (about half the samples were collected from rocks). Although this deviated from the Standard Operating Procedures manual (SOP, Section 7.4) established in 2008, samples were processed accordingly. In 2010 intertidal spawning occurred at the same three oiled sites in 2008 and at the Paradise Cove reference site, at the same intertidal depth as the original samples. However, only three of the eight transects at Sausalito had observable spawn deposition.

Table 2-12: Summary of 2009 and 2010 Natural Spawn Sampling Sites

Site	N1	N2	N3	N4	N5	N6	N7	N8
Paradise Cove (MRS003b)	37⁰53.644 x 122⁰ 27.448	37⁰53.643 x 122⁰ 27.441	37⁰53.642 x 122⁰ 27.436	37⁰53.641 x 122⁰ 27.428	37⁰53.646 x 122⁰ 27.423	37⁰53.638 x 122⁰ 27.415	37⁰53.636 x 122⁰ 27.380	37⁰53.641 x 122⁰ 27.386
Date/Time begun	1/27/09 2:17 PM	1/27/09 2:38 PM	1/27/09 3:16 PM	1/27/09 3:32 PM	1/27/09 3:50 PM	1/27/09 4:08 PM	1/27/09 4:18 PM	1/27/09 4:36 PM
Paradise Cove (MRS003b)	37⁰53.641 x 122⁰ 27.403	37⁰53.641 x 122⁰ 27.397	37⁰53.639 x 122⁰ 27.395	37⁰53.638 x 122⁰ 27.368	37⁰53.633 x 122⁰ 27.358	37⁰53.638 x 122⁰ 27.359	37⁰53.634 x 122⁰ 27.353	37⁰53.633 x 122⁰ 27.346
Date/Time begun	2/06/10 11:42 AM	2/06/10 11:48 AM	2/06/10 11:59 AM	2/06/10 12:18 PM	2/06/10 12:23 PM	2/06/10 12:42 PM	2/06/10 12:50 PM	2/06/10 12:57 PM
Sausalito (N1-5, MRQ10 N6-8, P01)	37⁰51.688 x 122⁰ 29.174	37⁰51.691 x 122⁰ 29.181	37⁰51.693 x 122⁰ 29.185	37⁰51.696 x 122⁰ 29.191	37⁰51.697 x 122⁰ 29.199 & 37⁰51.698 x 122⁰ 29.205	37⁰51.482 x 122⁰ 28.724	37⁰51.487 x 122⁰ 28.718	37⁰51.493 x 122⁰ 28.718
Date/Time begun	no spawn	no spawn	no spawn	no spawn	no spawn	2/04/10 11:45 AM	2/04/10 11:54 AM	2/04/10 12:04 PM
Keil Cove (MRR20)	37⁰52.826 x 122⁰ 26.411	37⁰52.824 x 122⁰ 26.405	37⁰52.821 x 122⁰ 26.392	37⁰52.816 x 122⁰ 26.391	37⁰52.815 x 122⁰ 26.387	37⁰52.811 x 122⁰ 26.380	37⁰52.806 x 122⁰ 26.369	37⁰52.803 x 122⁰ 26.362
Date/Time begun	2/05/10 12:51 PM	2/05/10 12:55 PM	2/05/10 1:00 PM	2/05/10 1:04 PM	2/05/10 1:11 PM	2/05/10 1:16 PM	2/05/10 1:19 PM	2/05/10 1:23 PM
Peninsula Pt. (MRQ01)	37⁰52.056 x 122⁰ 27.994	37⁰52.051 x 122⁰ 27.988	37⁰52.046 x 122⁰ 27.987	37⁰52.042 x 122⁰ 27.980	37⁰52.039 x 122⁰ 27.976	37⁰52.036 x 122⁰ 27.973	37⁰52.032 x 122⁰ 27.969	37⁰52.030 x 122⁰ 27.965
Date/Time begun	2/04/10 9:58 AM	2/04/10 10:07 AM	2/04/10 10:13 AM	2/04/10 10:22 AM	2/04/10 10:25 AM	2/04/10 10:32 AM	2/04/10 10:37 AM	2/04/10 10:40 AM

2.1.2 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 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.

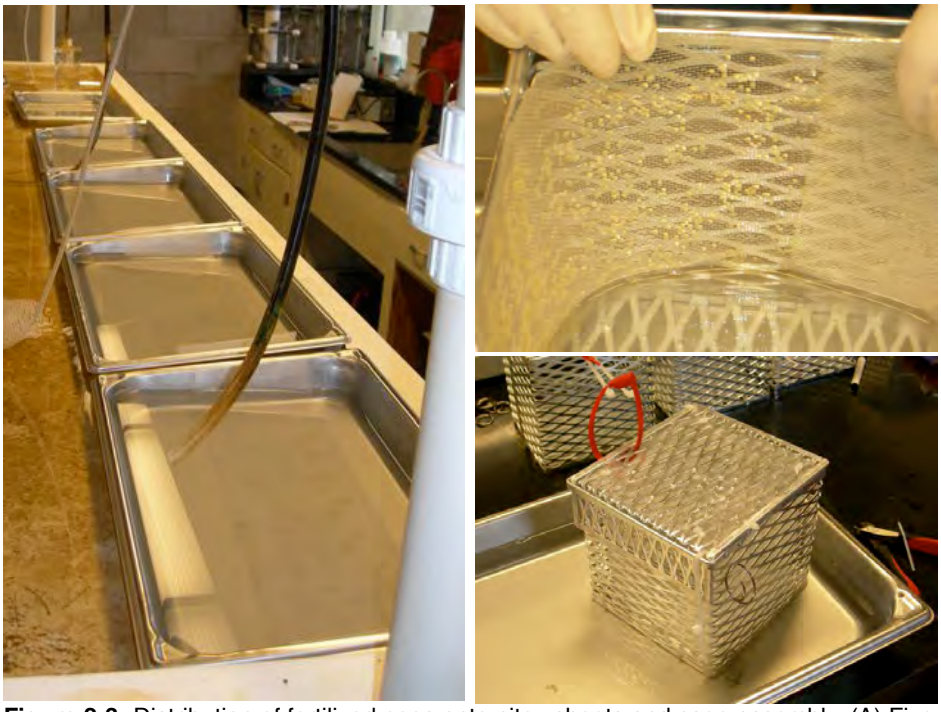


Figure 2-3: Distribution of fertilized eggs onto nitex sheets and cage assembly. (A) Five replicate sheets with monolayers of eggs incubating in milt. (B) Insertion of nitex sheet with fertilized eggs into cage. (C) Fully assembled cage with numbered security tag.

2.1.3 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.

2.1.4 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.

2.1.5 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.

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

2.1.7 Laboratory assays of hatching and larval swimming behavior.

2.1.7.1 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.

2.1.7.2 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.

2.1.7.3 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 $p < 0.05$.

2.1.7.4 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.

2.1.8 Selection of sediment collection sites and sediment collection.

2.1.8.1 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.

2.1.8.2 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.

2.1.9 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).

2.1.10 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 $\alpha \leq 0.05$.

2.2 Laboratory studies. These methods are described in detail in the Laboratory SOP manual (Section 7.5)

2.2.1 Laboratory oiled gravel column exposure and phototoxicity study

2.2.1.1 Exposure system. Exposures were conducted outdoors on a south-facing concrete pad. Six water tables to holed eight columns each were constructed in a terraced array to prevent tables from shading each other. Ambient full-strength seawater was mixed with fresh water to 22 ppt ("treatment water") in a 3800-l holding tank. Fine clay silt present in the laboratory's well-water supply was removed with a 5-micron bag filter. Treatment water was delivered from the holding tank via a manifold to a peristaltic pump (Masterflex L/S variable speed drive with 8-channel cartridge, Cole-Parmer, Vernon Hills, IL) beneath each table, which distributed water to each of eight columns at a rate of 0.8 to 1 l/hr. Water was pumped into the bottom of each column via a tube (a section of 5-ml borosilicate glass pipet) and up-welled over the lip of the beaker and was collected in custom made 20 X 41 X 8 cm aquaria serving as embryo exposure reservoirsFigure. A standpipe in each aquarium held the steady-state volume at 4-l.

Three replicates of each oil dose and control gravel weathered either under a cover of UV-transmitting plastic or UV-blocking plastic (to allow exposure to visible sunlight wavelengths). Covers were designed to block typical southerly or northerly rainfall, but were open on either end to allow air circulation and prevent heat trapping. Columns were randomly distributed by dose and oil type across all the tables, and half of each table (e.g. four columns) was randomized for a UV-blocking or UV-transmitting cover. Water flow to the columns was initiated 14 January 2009. Embryo incubations were initiated on 23 January, 13 February, 26 February, and 18 March.

2.2.1.2 Preparation of oiled gravel columns. Oiled gravel was prepared by tumbling in a portable cement mixer using a modification of previously published methods [1]. Locally obtained river gravel obtained from a landscaping supplier was washed on 1-cm plastic sieves, and spread into monolayers on cardboard sheet to dry. Final drying was achieved with a heat gun. Aliquots (~20 ml) of each oil were held in brown glass bottles briefly at 65°C in a water bath to maintain fluidity. A 5-ml glass pipet was calibrated to deliver desired masses of oil by adding oil drop-wise to a pre-weighed 25-ml beaker on an analytical balance. The number of drops required to deliver 1 gram of oil was calibrated in triplicate and was 52 drops for ANSCO and 47 drops for CBBO. For the lowest doses (0.1 g/kg and 0.3 g/kg) oil was scattered drop-wise over gravel in the mixer drum. 11 kg batches of gravel were oiled using a separate mixer for each oil. For ANSCO at 0.1 g/kg, 54 drops (1.1 g) were added before tumbling. For CB bunker at 0.1 g/kg, drops were added in three groups (16, 16, and 17; 1.1 g total) with brief tumbling between. For the CB bunker 0.3 g/kg dose, 147 drops were added in five groups with tumbling between each. For the 10 g/kg doses, heated oil was weighed into a pre-weighed beaker, then poured onto tumbling gravel. The beaker was re-weighed after pouring to ensure delivery of 11 g total. Each batch of gravel was tumbled for 10 minutes after all oil was added. Gravel was spread out on aluminum foil-covered cardboard to dry up to 12 hours before packing into columns. Each dose of gravel was divided equally among 6 replicate columns (1-liter glass beakers, 1.8 kg gravel each). Columns were covered with aluminum foil and stored indoors at room temperature until used.

Three of four experiments included two negative controls; clean gravel of the same batch used to generate oiled gravel, and gravel collected from a San Francisco Bay beach outside of the spill zone ("urban" gravel). Gravel of similar grain size was selected from the beach at China Camp State Park on the north side of Point San Pablo. The urban gravel was processed in the same way as the commercially obtained gravel.

2.2.1.3 Water quality monitoring. During incubation daily water quality measurements collected manually included temperature, salinity and dissolved oxygen. In addition, the aquarium for one control column (e.g. clean or urban gravel) on each table contained a continuous temperature probe that recorded every 10 min. Each water table also contained a continuous temperature recorder to monitor the water bath. Ammonia levels were measured colorimetrically for each aquarium prior to addition of embryos and at the end of incubation.

2.2.1.4 Embryology. Capture of ripe adults, preparation of gametes, and fertilizations were carried out as described in the SOP (Appendix). Test fertilizations were conducted with eggs from individual females, and those with high fertilization success (≥ 90%) were pooled for large-scale fertilizations. Mean (± SEM) female weights for each experiment were 103.6 ± 4.6 g (n = 5), 59.7 ± 3.8 g (n = 26), 61.2 ± 2.8 g (n = 33), and 62.7 ± 4.8 (n = 13). Milt was pooled from five males for each experiment. Eggs were kept from clumping prior to fertilization with polyvinyl alcohol (see SOP), and were distributed onto two substrates for exposure. For morphological observations, eggs were deposited onto frosted microscope slides targeting ~100 eggs per slide. For analytical chemistry samples, 2-3 grams of eggs were deposited onto 10 X 20 cm sheets of nylon mesh. Embryos were distributed to exposure aquaria within two hours of fertilization.

Embryos and larvae were observed with oblique coherent contrast illumination using Nikon SMZ800 stereomicroscopes fitted with diascopic bases, and digital images captured using Fire-i 400 industrial cameras (Unibrain, Inc., San Ramon, CA) and BTV Pro 5.4.1 (www.bensoftware.com) on Apple PowerBook G4 computers. Pre-hatch embryos were imaged without anesthesia either through the chorion or after manual dechorionation with fine forceps, while larvae were anesthetized with MS-222. In the first (13 January) experiment, embryos were subsampled, dechorionated and examined in detail daily beginning at 5 dpf. During this experiment, cytolytic phototoxicity was observed by 8 dpf. Because this phenotype

was readily visible through the chorion, and affected embryos did not remain intact with dechorionation, in subsequent experiments phototoxicity was quantified by counting cytolysed embryos through the chorions. In the 26 January experiment, counts on one slide from each column included total eggs attached, unfertilized eggs, embryos that died during or before gastrulation, necrotic (cytolyzed) eyed embryos, and viable eyed embryos. Percentage of embryos showing necrotic phototoxicity was then normalized to total eyed embryos by subtracting unfertilized eggs and early lethal embryos from total eggs. In the 18 March experiment, counts for each slide included total eggs, unfertilized eggs, viable eyed embryos, and dead embryos irrespective of stage. Embryos that died during early development were quantified in the laboratory controls and averaged 13%. This value was indistinguishable from the rate of early mortality in the earlier experiments, where it was found that early mortality rates were independent of any treatment. The value for necrotic late embryos was obtained by subtracting the average early mortality (13%) from total mortality normalized to fertilized embryos.

2.2.1.5 Assessment of larval hatching and abnormalities. On the day of embryological observations (8 dpf), one replicate slide from each column was placed into 250 ml glass culture dishes containing 200 ml 16 ppt seawater and incubated in a 12˚C incubator. Hatched larvae were collected daily up to 8 days after retrieval from column effluent (i.e. 15 dpf), anesthetized with MS-222 and examined microscopically using an Olympus SZH stereo zoom microscope. Selected images were collected using a Pixel Link camera and PixelLinkCapture software. Treatments were evaluated for the following: unhatched eyed embryos, unfertilized embryos (1st day only), non-viable embryos (dead plus unfertilized), partial hatched embryos, hatched larvae live, hatched larvae normal, hatched larvae abnormal. For the 1/23/09 and 2/13/09 column experiments unfertilized embryos were not counted separately from dead embryos. Abnormal morphology included the following: scoliosis, edema, opaque yolk, opaque head, opaque tail, kinked tail, bent heads, jaw abnormalities. Other types of abnormalities were not observed on a consistent basis: For the 1/23/09 experiment, hatching commenced on 2/1/09 for one bowl and on 2/2/09 for 7 bowls, facilitating observation of abnormal motor activity in hatchlings prior to anesthesia in MS-222. Subsequent days resulted in a large amount of hatching in most bowls, increasing the time necessary to score morphology. As a result, larvae were transferred to bowls for anesthesia in MS-222 prior to observation of motor activity and abnormal motor activity was observed only in extremely abnormal larvae (i.e. severely deformed larvae continue to twitch despite the presence of MS-222). For the 1/23/09 experiment, hatchlings were observed and photographed for abnormal cardiac function (bradycardia, arrhythmia) or morphology during the first few days of hatching at high magnification on a dissecting microscope. Due to the amount of time required for these observations, counts on all bowls were not performed on a daily basis. In subsequent experiments, daily counts were performed and hatchlings were primarily observed at low magnification, thus cardiac abnormalities were not always quantified.

Percent normal hatch was calculated as normal hatch as a percentage of total embryos/larvae (the sum of eyed unhatched, non-viable, partial hatched, hatched larvae dead, and hatched larvae live).

2.2.2 Salinity study.

2.2.2.1 Preparation of incubation media:

½ strength seawater was prepared by diluting Bodega Bay 0.45 μM filtered seawater (FSW) 1:1 with distilled water

22 ppt seawater was prepared by diluting 647 ml of 34 ppt Bodega Bay FSW with distilled water QS to 1000 ml

30 ppt seawater was prepared by diluting 882 ml of 34 ppt Bodega Bay FSW with distilled water QS to 1000 ml.

Salinity was checked with a refractometer

2.2.2.2 Fertilization and incubation:

Trial fertilization:

Herring eggs from each female were distributed individually into 1 compartment of 4 quadrant petri plates in ½ strength FSW.

A drop from a diluted suspension of sperm was added to each quadrant and the plates placed into a 12^0C incubator for 15 minutes

Percent fertilization for each female was assessed by observing for elevation of the chorion

2.2.2.3 Salinity treatments

Treatment	Fertilization salinity	Incubation Salinity (7 days)	Incubation Salinity (through hatching)
16-16	16 ppt	16 ppt	16 ppt
16-22	16 ppt	22 ppt	16 ppt
16-30	16 ppt	30 ppt	16 ppt
22-22	22 ppt	22 ppt	16 ppt
30-30	30 ppt	30 ppt	16 ppt

Using a spatula, eggs from females with 90% or greater fertilization rate in the trial fertilization were suspended in approximately 250 ml of ½ Calcium Magnesium free artificial seawater (CaMgFSW) containing 0.25% polyvinyl alcohol (PVA). This prevents the normally adhesive eggs from adhering to the nalgene beaker or to each other.

A sperm suspension was prepared by macerating the testis from 2-3 males in approximately 100 ml ½ FSW 7"x3" loaf pans containing 4 microscope slides each were filled with approximately 200 ml of ½ FSW, 22 ppt FSW or 30 ppt SW and 5 ml of the sperm suspension added to the pans. The pan were gently swirled to ensure dispersal of the sperm suspension.

50-100 eggs from the beaker of ½ CaMgFSW were dropped onto each slide and the pans gently swirled to maximize contact of the eggs with sperm.

Eggs were incubated for 30 min at 120C in the water table or in the incubator, then rinsed with fresh FSW of the same salinity to remove sperm.

Slides were transferred to 100 x 50 mm finger bowls (2 slides/bowl) containing the appropriate salinity solutions (16, 22, or 30 ppt) and incubated in a 120C incubator with daily water changes of the appropriate salinity.

On the 7th day of incubation, 4 slides from each treatment (2 from each bowl) were shipped to Northwest Fisheries Science Center, NOAA in Seattle, WA.

From the 8th day of incubation through final hatching, all treatments were incubated in 16 ppt FSW.

2.2.2.4 Scoring of embryos

Duplicate slides were received at NWFSC from BML on 3/13/09 (8 dpf), along with BML 16 ppt seawater for processing. All slides were examined upon receipt and showed similar numbers of viable eyed embryos. Due to time constraints, the most relevant treatments were selected for dechorionation, i.e. optimal laboratory salinity regimen (fertilized at 16 ppt, incubated at 16 ppt) and high salinity regimen (fertilized at 30 ppt, incubated at 30 ppt). For dechorionation, slides were transferred to 16 ppt seawater and held at 12 °C on a cooling stage. At least 20 embryos were dechorionated at 16 ppt from a single slide for each treatment, and held at 16 ppt for imaging.

At commencement of hatching at BML, culture were counted as follows:

Unhatched eyed embryos
Unfertilized embryos (1st day only)
Non-viable embryos (dead plus unfertilized)
Partial hatched embryos
Hatched larvae live
Hatched larvae normal
Hatched larvae abnormal

Abnormal larvae were evaluated for scoliosis, edema, opaque yolks, heads, or tail, kinked tails, bent heads, jaw abnormalities.

Percent normal hatch was calculated as normal hatch as a percentage of total embryos/larvae (the sum of eyed unhatched, non-viable, partial hatched, hatched larvae dead, and hatched larvae live).

Section 3: Results of Field Assessment

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

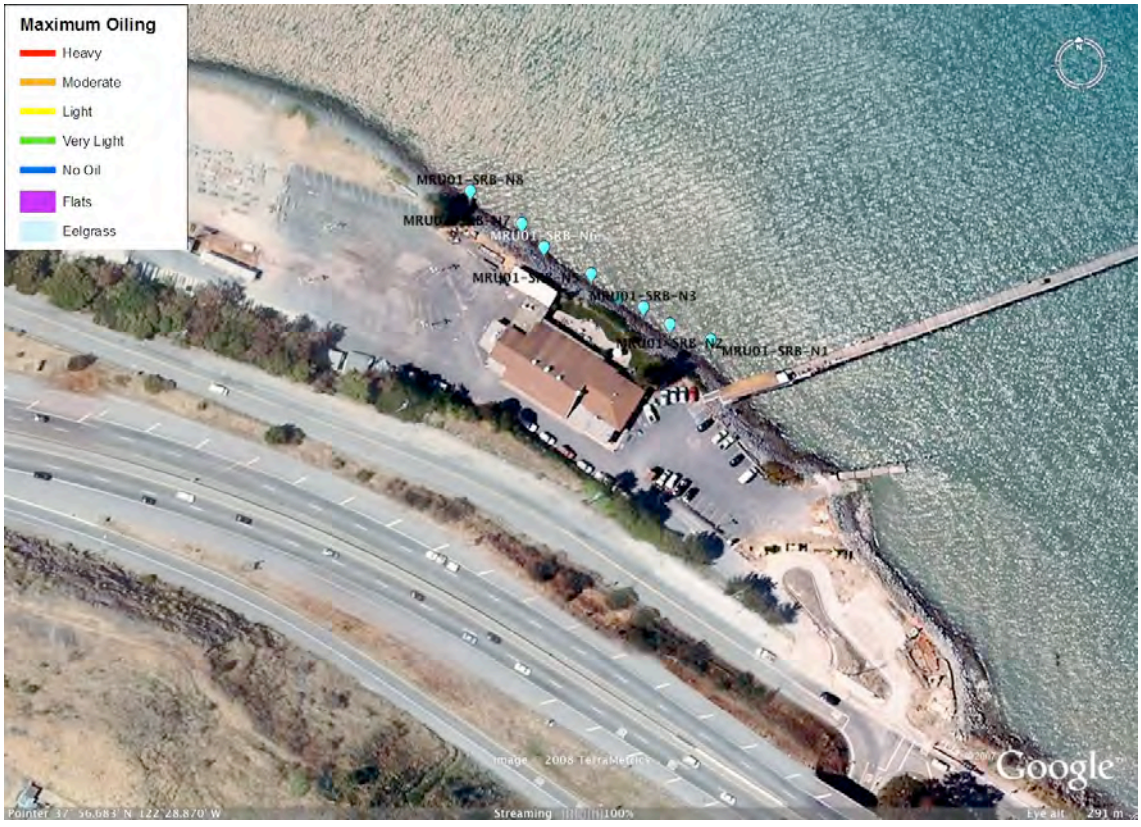


Figure 3-1G: Close-up of San Rafael Bay natural spawn grabs

3.2 High rates of body axis defects, neural tissue necrosis, and cardiac arrhythmia in 2008 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

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*

(*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).

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

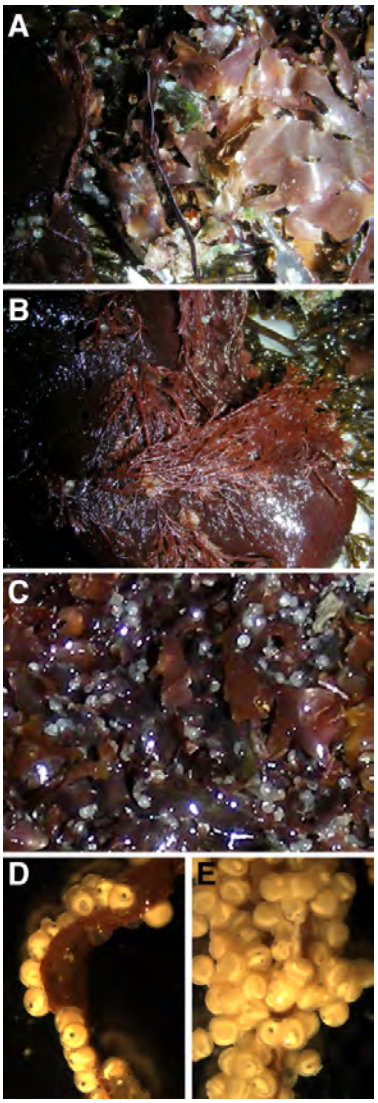


Figure 3-2: Representative 2008 natural spawn samples. (A) and (B) Mixed algae typical of Peninsula Point and Keil Cove. (C) Close-up of fresh natural spawn sample from Peninsula Point with "salt-and-pepper" density. (D) Light single-layer spawn sample from Keil Cove fixed in Stockard's. (E) Medium density spawn from San Rafael Bay (Stockard's fixed), up to four embryos deep at points.

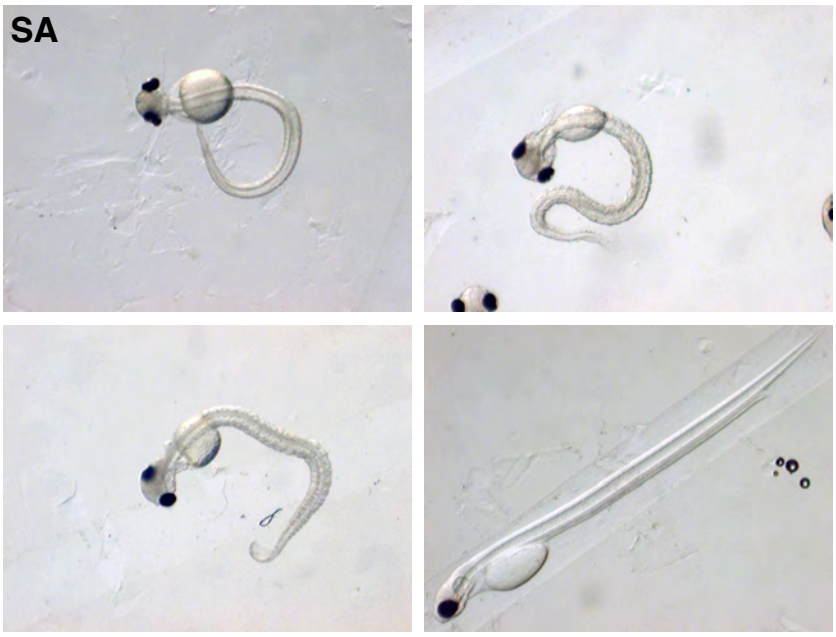


Figure 3-3: Body axis defects in 2008 natural spawn samples from oiled sites compared to typical normal morphology from San Rafael Bay samples

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% (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

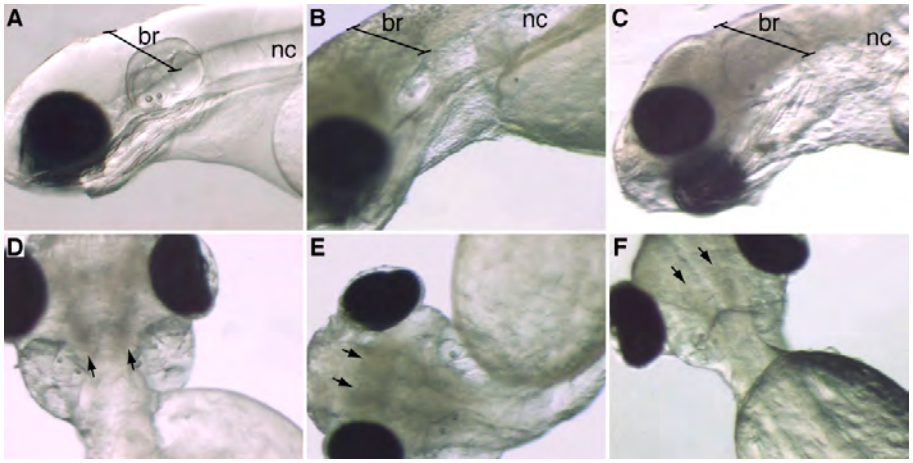


Figure 3-4: CNS opacity in natural spawn samples from oiled sites. (A-C) lateral views of the head. (A) Typical translucent embryo from San Rafael Bay. The line indicates the span of brain tissue (*br*), which lies above anterior portion of the notochord (*nc*). (B) Embryo from Peninsula Point. (C) Embryo 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.

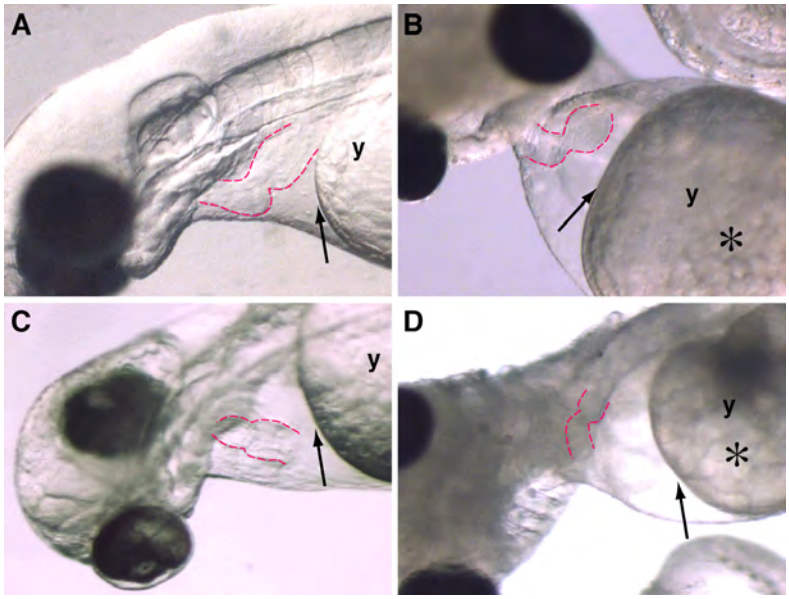


Figure 3-5: Pericardial edema in 2008 natural spawn samples from oiled sites. (A) San Rafael Bay, (B) Sausalito, (C) Peninsula Point, (D) Keil Cove. Red dashed lines indicate the outline of the heart. In the embryos from SRB (A), the heart takes up most of the pericardial space, and the anterior end 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).

from 5-76% (mean 33%, $p < 0.05$). At Peninsula Point, edema was 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

Table 3-3: Overall scores for abnormalities in 2008 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

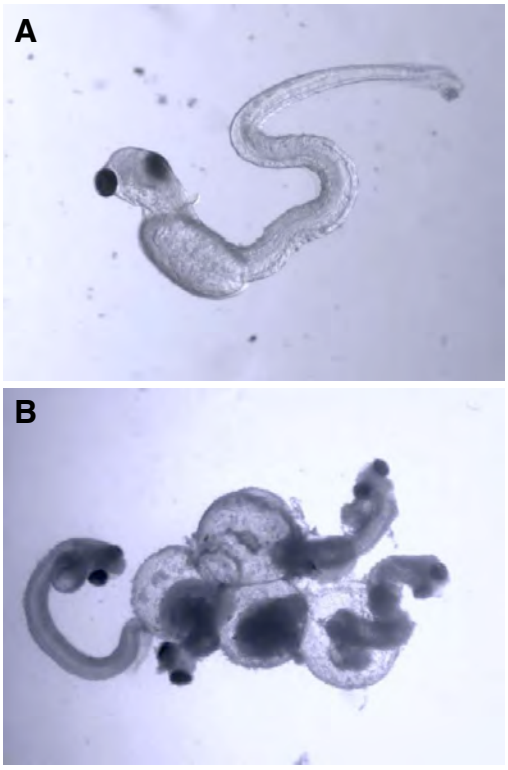


Figure 3-6: (A) Hatched abnormal larva with typical body axis defects; (B) Partially hatched dead larvae

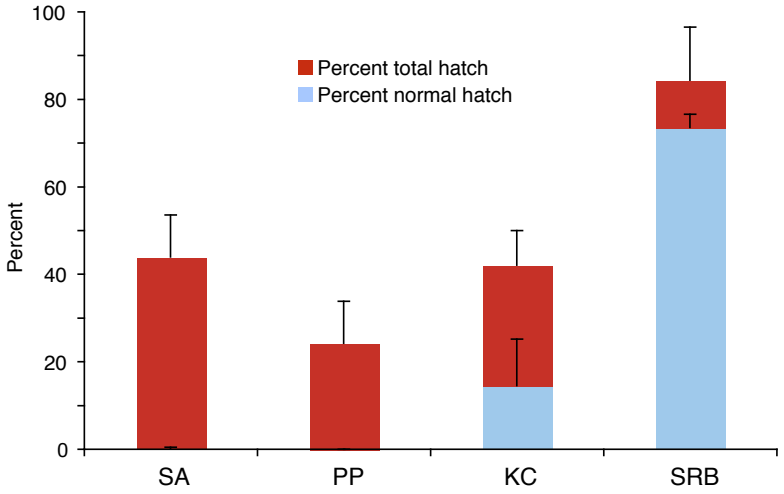


Figure 3-7: Laboratory hatching in samples of natural spawn in 2008. Red bars, percent total larvae hatched; blue bars, percent hatched larvae with normal morphology.

abnormalities in cardiac function were observed in samples from San Rafael Bay. 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 a quantification of mortality.

3.3 Reduced hatching success and high rates of abnormal morphology in larvae from 2008 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

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

Peninsula Point, 24 ± 10% of embryos hatched, with none 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 2008 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).

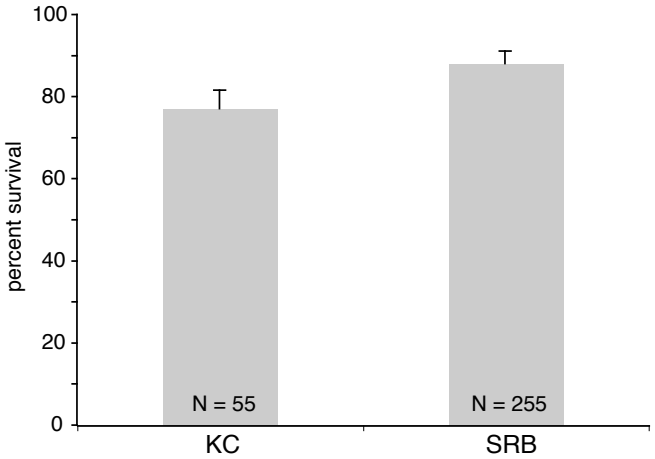


Figure 3-8: Percent survival of hatched larvae from naturally spawned embryos. Dead or abnormal larvae (abnormalities included body axis defects, edema, or opacity) were removed each day. Percent survival was determined at the end of the 5 day incubation period. (KC is significantly different from SRB;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

3.5 Behavior in larvae from 2008 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).

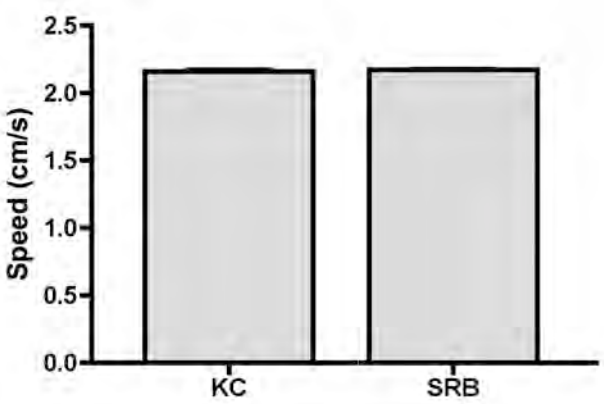


Figure 3-9: Critical swimming speed (U_{crit}) in larvae hatched from natural spawn samples. Keil Cove provided the only surviving oiled-site larvae that satisfied the requirements for swimming performance (see Section 2.7.4). There is no statistical difference between the two sites for Ucrit based on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

3.6 Recovery of normal natural spawn at intertidal zones of oiled sites in 2010. Herring did not spawn at any of the same sites in 2009, but they did spawn at another unoiled reference location (Paradise Cove; PC; sampled in January). The natural spawn from PC was higher in the intertidal zone than the naturally spawned sites samples in 2008, distributed on rocks as well as macroalgae. A relatively high proportion of embryos from this site failed to gastrulate (28 ± 7%), nevertheless, viable embryos from PC produced a normal hatch rate of 77 ± 4% (Figure 3-10).

In 2010, herring once again spawned at several locations where natural spawn was collected in 2008. The overlap was exact at two oiled sites (PP, KC) and partial at a third oiled site (SA). (The SA site was subsquently divided into an inner marina SA1 subsite and outer SA2 subsite, Figure 3-1b). Moreover, herring spawned again at the PC reference site, at an intertidal depth equivalent to the samples collected in 2008. Spawn densities were higher than in 2008, with all sites showing medium-high densities (3-6 layers of embryos). Larvae at all three oiled sites had normal cardiac and spontaneous motor activity. Furthermore, hatching rates were similar among the oiled and reference sites (Figure 3-10), and these were similar to the relatively high hatching rates for non-oiled reference sites sampled in previous years (SRB in 2008 and PC in 2009). However, there was a small but significant increase in pericardial edema in larvae from oiled KC site (3.5± 0.7%) relative to the other sites (SA, 1.6 ± 0.3%; PP, 1.0 ± 0.3%; PC, 1.2 ± 0.3%; ANOVA $p < 0.003$, Tukey-Kramer HSD).

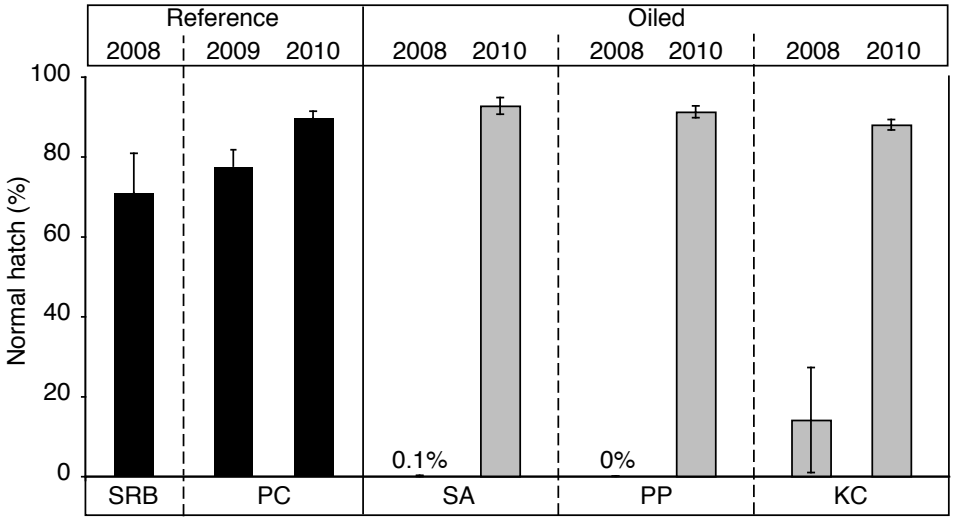


Figure 3-10: Hatching rates of larvae from natural spawn samples across all three years of field sampling. Values represent mean ± s.e.m. normal hatch rates, calculated as percent morphologically normal larvae hatched from total eggs in 8 transect subsamples per site. Grand totals of eggs assessed for each site were 535 (SRB 2008), 820 (PC 2009), 683 (PC 2010), 968, (SA 2008), 385 (SA 2010), 549 (PP 2008), 919 (PP 2010), 469 (KC 2008), 726 (KC 2010).

3.7 Reduced heart rate (bradycardia) in caged embryos incubated adjacent to oiled sites in 2008. Heart rates were quantified in 20-sec digital video clips. Pronounced bradycardia was observed at all four oiled sites, with significant variability among cages at a given site (Figure 3-11). At three of the oiled sites, all five cages showed a significant bradycardia in the range of 90 beats/min (HC, 90 ± 1; SA, 92 ± 1; PP 92 ± 1). Only one of five cages at the KC oiled site showed significant bradycardia (cage 1, 95 ± 1 beats/min), and the overall mean (114 ± 2 beats/min) was thus similar to reference sites. There was no significant variation among heart rates within the five cages from each of the two reference sites (Figure 3-11a), and both sites had an overall mean heart rate of 116 ± 1 beats/min (Figure 3-11b). Mean temperatures were indistinguishable among sites (Table 3-5), indicating that differences in heart rate were not due to incubation temperatures.

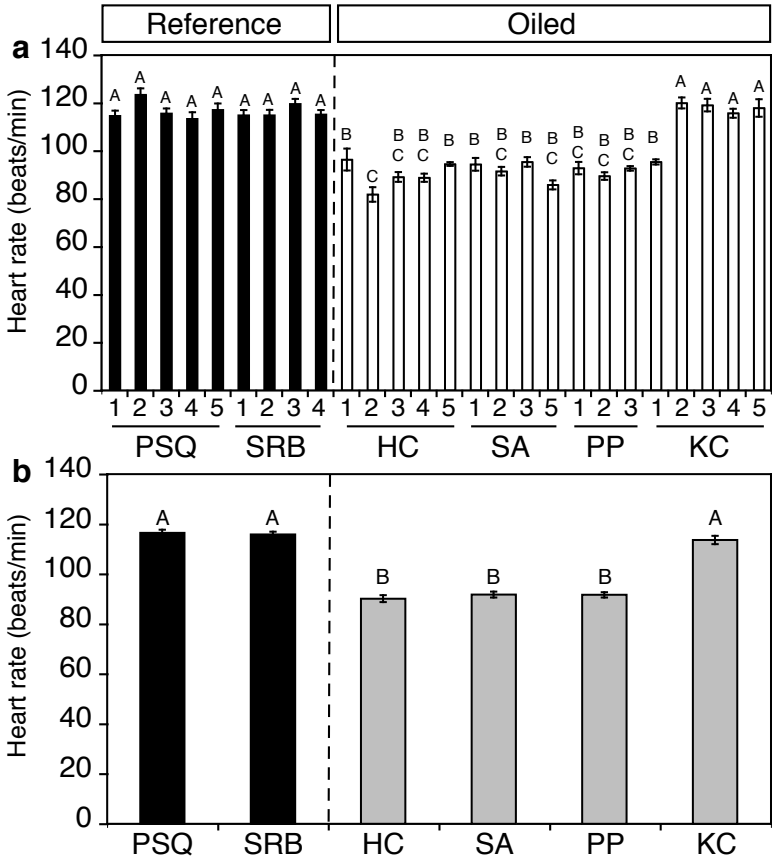


Figure 3-11: Reduced heart rate in embryos incubated in subtidal cages at oiled sites in 2008. (a) Mean heart rate (N = 30) by cage. Reference (non-oiled) and oiled sites indicated by black and grey, respectively. (b) Mean heart rate by site. Mean heart rates from individual cages were pooled for a site mean. Error bars in (b) and (c) are s.e.m. Nested ANOVA (cages nested under site, nested under oiled state) indicated effects of cage ($P < 0.001$), site ($P < 0.001$), and oiled state ($P < 0.001$). In (a) letters A-C indicate statistically similar cages identified by post-hoc means comparison with Tukey-Kramer HSD ($\alpha = 0.05$). In (b) oiled sites HC, SA, and PP were statistically different than oiled site KC and reference sites PSQ and SRB (Tukey-Kramer HSD, $\alpha = 0.05$). Overall, oiled sites were statistically different than non-oiled sites (T test, $\alpha = 0.05$).

Table 3-5: Temperature and salinity measurements collected at cage moorings

site[1]	mean[2] temperature	maximum temperature	mean salinity	maximum salinity
PSQ	11.2 ± 0.3	12.1	20.2 ± 2.9	26.1
HC	10.9 ± 0.6	11.8	27.6 ± 1.4	30.1
SA	11.0 ± 0.4	12.2	24.8 ± 0.8	27.6
PP	11.0 ±0.6	13.6	26.7 ± 1.8	30.5
KC	10.9 ± 0.4	12.4	26.4 ± 1.6	29.9

[1]Data absent from site SRB due to logger failure
[2]means ± s.e.m.

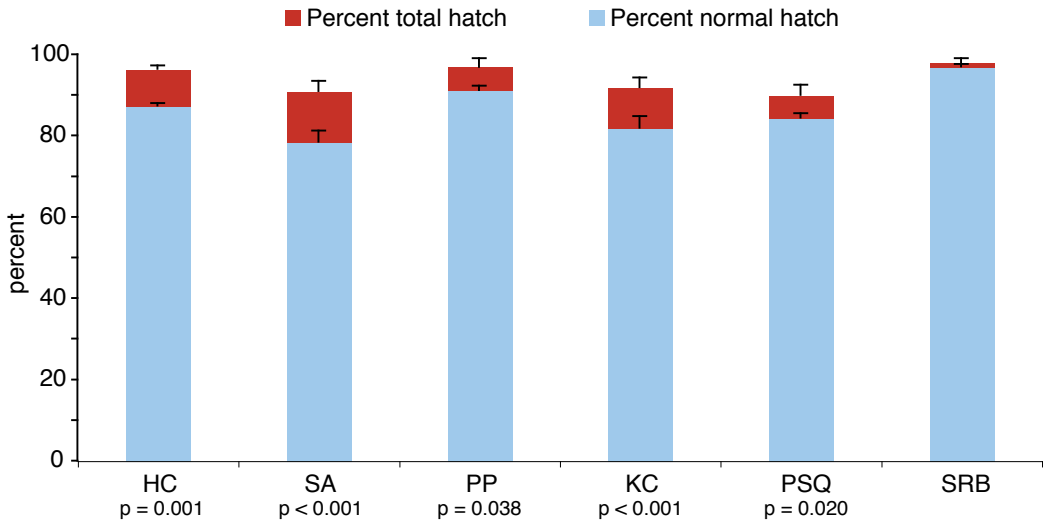


Figure 3-12: Mean hatching success of embryos incubated in deployed cages, Feb 2008. Total hatched larvae as percent of embryos are indicated by red bars, percent of total with normal morphology indicated by blue bars. Total number of embryos indicated at the bar base. P-value in comparison to SRB indicated below other site names.

3.8 Hatching success and pericardial edema in larvae from 2008 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. However, when abnormal morphology was specifically categorized, larvae with pericardial edema were detected only from cages incubated at oiled sites (Figure 3-13). Cardiogenic edema, produced by more severe impairment of cardiac rhythm in developing fish embryos, was observed in a small but significant percentage of larvae hatched from each oiled site, ranging from 0.9 to 2.5% (Figure 3-12). No edema was observed among 652 larvae hatched in cages at reference sites (PSQ, n = 308; SRB, n = 344).

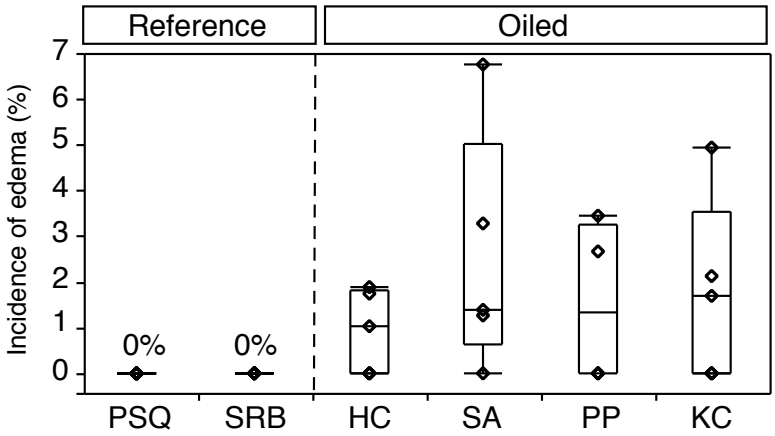


Figure 3-13: Incidence of pericardial edema in larvae hatched in the laboratory after incubation to 7 days post-fertilization in subtidal cages. Box plots encompass individual values for each cage, error bars show 95% confidence interval and upper and lower box limits show 75th and 25th percentiles, respectively. Edema tended to be observed more frequently at oiled sites (Wilcoxon rank sums test p = 0.06).

3.9 Larval survival from caged embryos in 2008. 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).

3.10 Lower critical swimming speed in larvae hatched from cages incubated at oiled sites in 2008. Results of the critical swimming speed analysis were combined for each set of cages at each station. For the

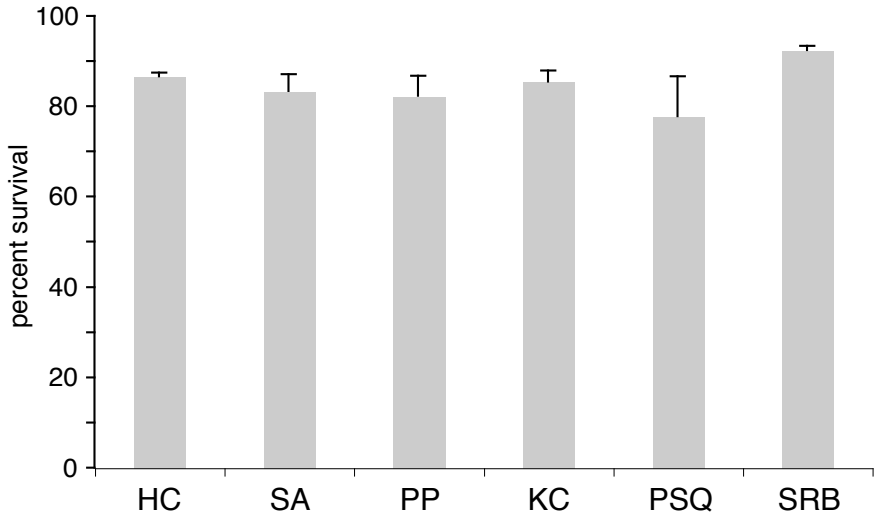


Figure 3-14: Percent survival of hatched larvae from 2008 caged embryos. Dead or abnormal larvae (abnormalities included body axis defects, edema, or opacity) were removed each day. Percent survival was determined at the end of the 5 day incubation period. (KC is significantly different from SRB; $p<0.05$; ANOVA with Tukey-Kramer HSD test for all pairwise multiple comparisons).

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.

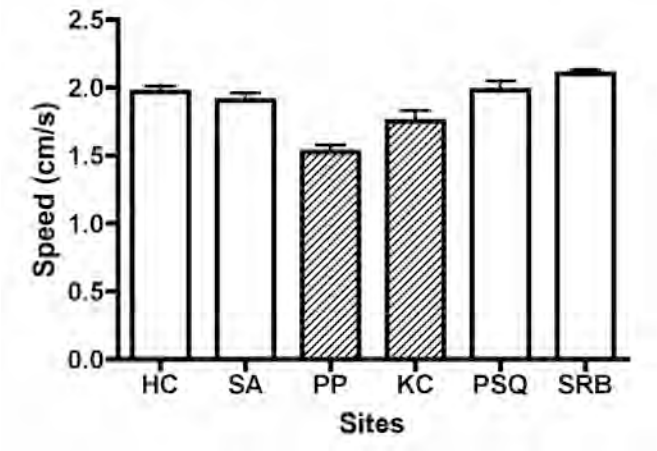


Figure 3-15: Mean critical swimming speed in larvae from 2008 caged embryos by site. Sites PP and KC are statistically different from the remaining locations. The individual cage results were combined for each station. A Kruskal-Wallis One Way ANOVA of Variance on Ranks was applied to determine differences between stations (P = <0.001).

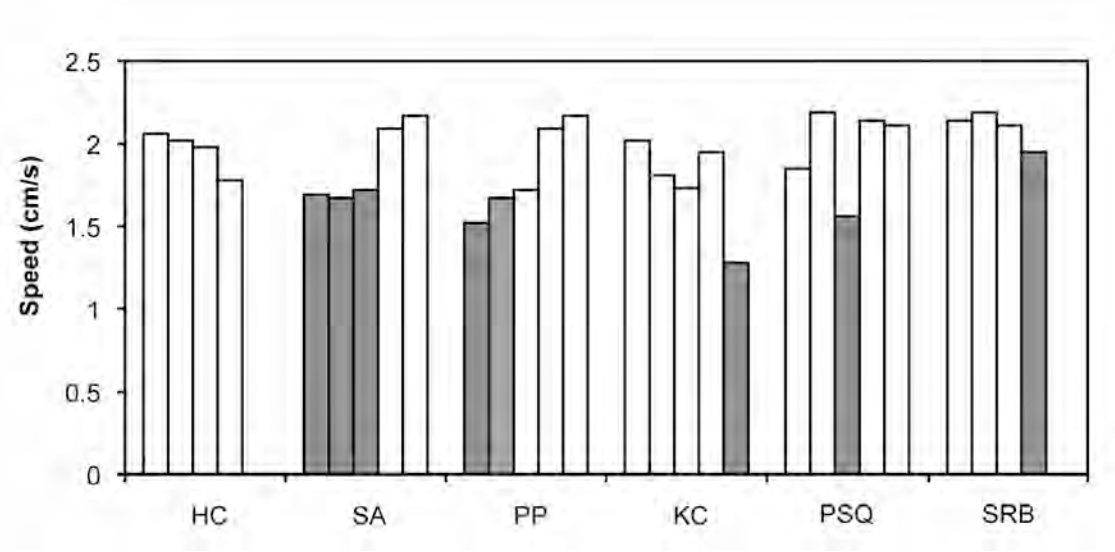


Figure 3-16: Mean critical swimming speed in larvae for each cage. Dark bars indicate a statistically significant difference between cages within the station. A one way analysis of variance was conducted on all the stations groups except for station SRB where a Kruskal-Wallis ANOVA on Ranks was used.

3.11 Background levels of PAHs and POPs in reproductively mature Pacific herring from San Francisco Bay in 2008. 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 3-6), concentrations of sum PAHs ranged from 23 – 52 ng/g in whole bodies and

Table 3-6: PAHs and POPs in maternal tissues of herring in San Francisco Bay and Puget Sound

Tissue	Region	Site	Year	Percent lipid					
Whole body	SF	Richardson Bay (n=6)	2008	3.4 ± 0.24	44 ± 1.7	3.2 ± 0.3	47 ± 11	33 ± 4.0	12 ± 0.61
	PS	Port Orchard (n=56)	1999-2004*	6.4	NA	NA	NA	19	160
	PS	Quartermaster Harbor (n=10)	1999-2004*	8.1	NA	NA	NA	19	120
	PS	Cherry Point (n=20)	1999-2004*	3.3	NA	NA	NA	11	41
Ovary	SF	Richardson Bay (n=6)	2008	0.40 ± 0.10	11 ± 0.7	0.5 ± 0.1	12 ± 0.6	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	5.5 ± 0.57	51 ± 7.1
	PS	Quartermaster Harbor (n=1)	2001†	2.9 ± 0.0	24	1.1	25.1	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	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

8.6 – 13 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 3-6). 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 3-6). 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.

3.12 PAHs detected in embryos from natural spawn samples (2008-2010) and deployed cages (2008). 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

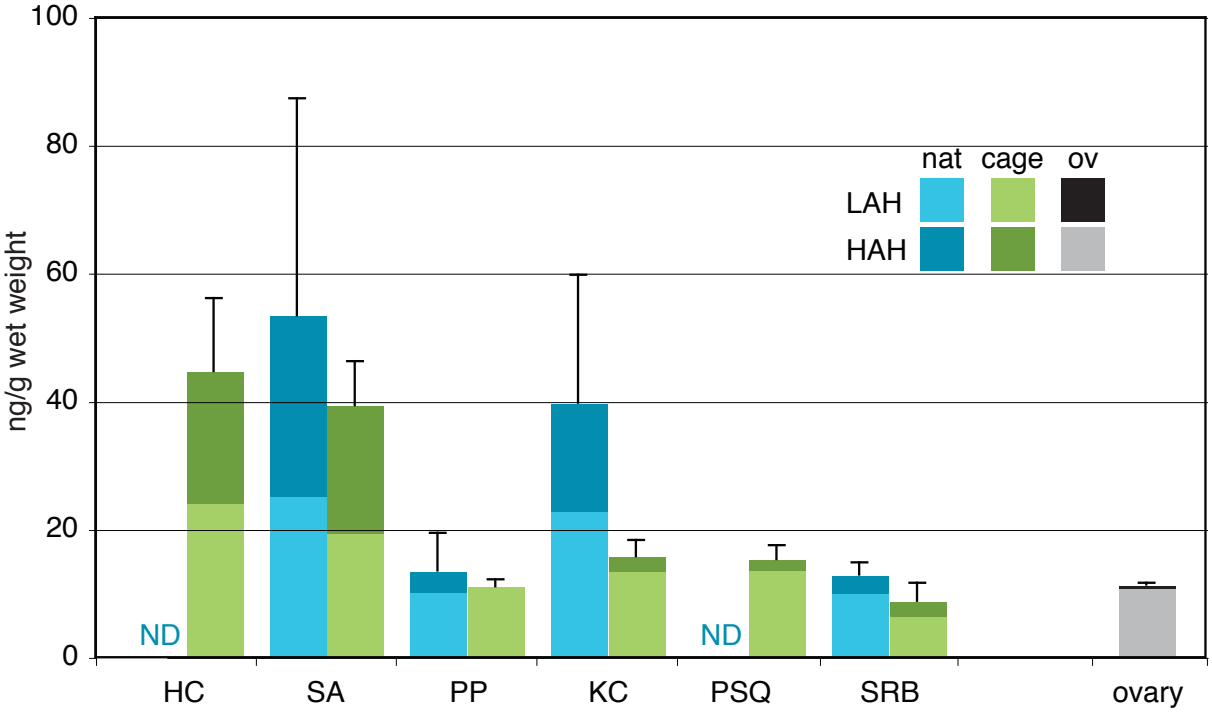


Figure 3-17: Mean sums of PAHs in embryos from natural spawn samples, caged embryos, and prespawn ovaries from SF Bay adults in 2008. Blue bars are natural spawn embryos, green bars are caged embryos; lighter shades represent sum of low molecular weight PAHs, darker shades sum of high molecular weight 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 in 2008. 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 3-7). Embryos from Horseshoe Cove, Peninsula Point and Keil 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).

Differences were apparent in the mean sum alkyl-phenanthrenes detected in embryos (Figure 3-18).

Table 3-7: 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 ($p < 0.0001$).

These are the most abundant of the toxicologically relevant tricyclic PAHs in *Cosco Busan* bunker oil (Fig ure 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 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

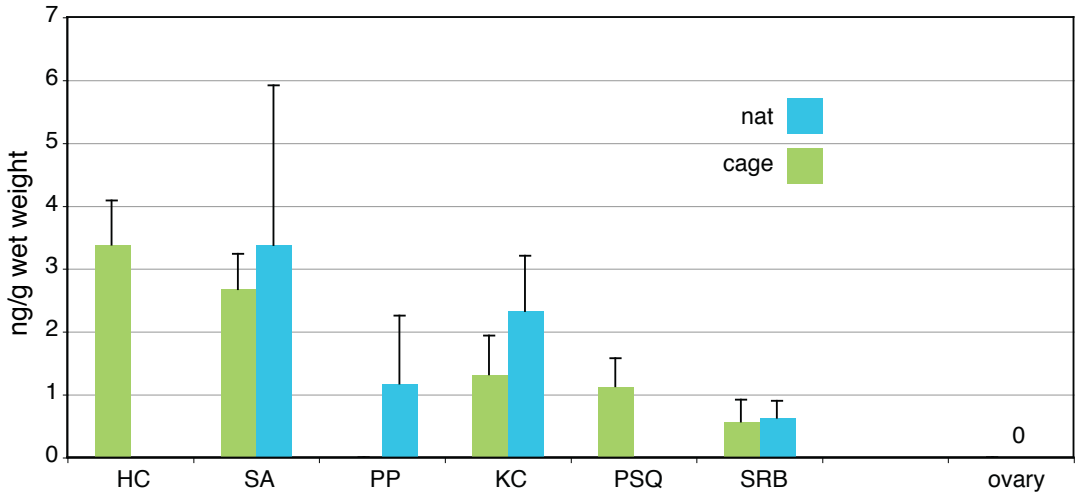


Figure 3-18: Sum alkyl-phenanthrenes (C1- through C4-PHN) in embryos from natural spawn samples, cages, and prespawn ovaries in 2008. Values are mean and standard error. Alkyl-phenanthrenes were below detection limits in ovaries.

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.

When comparing caged and naturally spawned embryos across all three years of sampling, analyses of individual classes of PAHs representing petrogenic or pyrogenic sources also revealed significant differences among sites (Table 3-8), despite generally low mean ∑PAH concentrations that were in many cases statistically indistinguishable from maternal background levels using standard parametric tests. Sulfur-containing dibenzothiophenes are petrogenic PAHs and often used for characterizing sources of oil in the environment [78-81]. Although Cosco Busan bunker oil is low in sulfur compared to other fuels, with a C2-dibenzothiophene/C2-phenanthrene (D2/P2) ratio of 0.26, the alkyl-dibenzothiophenes are nevertheless one of this bunker oil's most abundant PAH classes. Total alkyl-dibenzothiophenes tended to be higher in natural spawn embryos from each oiled site and in caged embryos from three of four oiled sites than from non-oiled sites in 2008 (Wilcoxon rank sums test $P = 0.08$). Co-detection of C2- and C3-dibenzothiophene was also higher in both caged and naturally spawned embryos from oiled sites in 2008. In 2010, alkyl-dibenzothiophenes in natural spawn were not detected at oiled site PP but remained significantly elevated at oiled site KC (ANOVA $P = 0.03$, Tukey-Kramer HSD $\alpha = 0.05$). The pyrogenic PAH fluoranthene did not show the same relationship. Slightly lower fluoranthene levels at reference sites were not statistically significant ($P = 0.28$, Wilcoxon rank sums test), and there was only a weak correlation between mean fluoranthene and total alkyl-dibenzothiophene concentrations ($r^2 = 0.6$).

Table 3-8: Summary of selected PAH concentrations (ng/g wet weight) measured in naturally spawned and caged embryos collected in 2008 and 2010

Matrix/yr (*N*)	Site[1]	Mean ∑PAHs[2]	Mean FLA	Mean alkyl-DBTs	Frequency C2/C3-DBT (%)[3]
Spawn/08 (8)	SRB	21 ± 2	1.1 ± 0.2	0.05 ± 0.03	0
Spawn/08 (5)	SA1[4]	81 ± 40	2.9 ± 0.7	0.49 ± 0.29	20
Spawn/08 (3)	SA2	18 ± 3	0.6 ± 0.1	0	0
Spawn/08 (8)	PP	19 ± 5	0.8 ± 0.1	0.12 ± 0.08	25
Spawn/08 (8)	KC	45 ± 18	3.8 ± 2.2	0.28 ± 0.09	75
Spawn/10 (8)	PC	28 ± 3	0.5 ± 0.1	0.05 ± 0.05	13
Spawn/10 (3)	SA2	27 ± 1	1.4 ± 0.1	0	0
Spawn/10 (8)	PP	23 ± 1	0.6 ± 0.1	0	0
Spawn/10 (8)	KC	34 ± 9	1.8 ± 1.0	0.48 ± 0.16	100
Cage/08 (5)	PSQ	23 ± 2	1.0 ± 0.2	0.09 ± 0.06	0
Cage/08 (4)	SRB	17 ± 3	0.8 ± 0.3	0	0
Cage/08 (4)	HC	52 ± 10	3.7 ± 0.9	0.48 ± 0.23	50
Cage/08 (5)	SA	48 ± 6	2.7 ± 0.5	0.51 ± 0.13	80
Cage/08 (4)	PP	21 ± 1	0.8 ± 0.1	0	0
Cage/08 (5)	KC	24 ± 3	0.7 ± 0.1	0.21 ± 0.10	40

[1]Reference sites = PC, PSQ, SRB; Oiled sites = HC, SA, PP, KC
[2]Mean and s.e.m. values from given *N*
[3]frequency of samples with both C2- and C3-DBT detected
[4]
FLA = fluoranthene
DBTs = dibenzothiophenes

3.13 Distinct patterns of PAH inputs evident from analysis of PEMD passive samplers. A comparison of PAH patterns in PEMDs deployed along side the caged embryos indicated distinct PAH chemical fingerprints among the six subtidal sites, reflecting their surrounding upland development (Figure 3-19). Moreover, as expected for an urbanized estuary, all sites had mixed inputs of petrogenic and pyrogenic PAHs. Non-metric multidimensional scaling (nMDS) and an analysis of similarity (ANOSIM) revealed that PAHs patterns were highly significantly different among sites (Figure 3-19; P = 0.001, R = 0.69). A pair wise

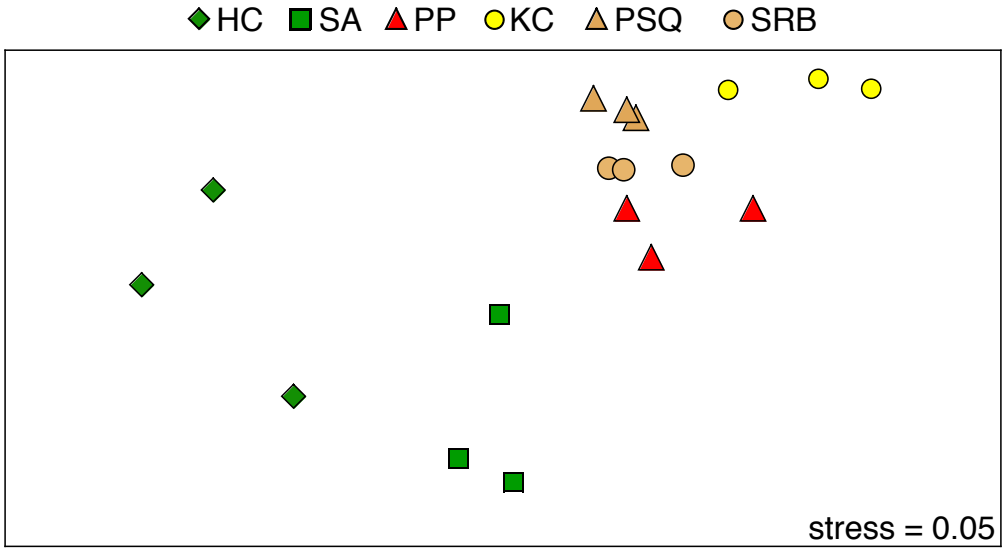


Figure 3-19. Comparison of PAH patterns in PEMDs deployed at six sites indicates distinct PAH sources. Non-metric multidimensional scaling (nMDS) was used to represent a large number of PAH compounds in low dimensional (2D) space. nMDS analysis was carried out using Primer 6.0 as described in the Methods. Axes surround a unitless space within which samples are placed according to the degree of similarity in the relative abundance of five influential PAH compounds (pre-selected from 34 compounds using the Primer BEST routine). Similarity in PAH patterns determines the distance between points in the space; samples with similar patterns are placed close together and dissimilar patterns farther apart. The observed patterns are statistically different from a random configuration of points (Stress = 0.05). PSQ, SRB are reference sites; HC, SA, PP, and KC are oiled sites.

ANOSIM test showed that sites HC and SA, both of which have marinas and were oiled, were similar (R=0.33) and different from all other sites (R range from 0.85 to 1.0). Similarly, reference sites PSQ and SRB, which are on opposite sides of a major highway (Richmond Bridge; Figure 31E, F), were indistinguishable from each other (R=0.26) and were isolated from all other sites (R range from 0.85 to 1.0). Oiled sites PP and KC, which are residential or minimally developed, were isolated from each other and all other sites (R range from 0.85 to 1.0). Diagnostic PAH ratios also showed differences among sites (Table 3-9). The ratio of sum alkyl-phenanthrenes to phenanthrene (MP/P) is widely used to distinguish pyrogenic from petrogenic sources [82], with the latter having MP/P ratios > 2. Conversely, the ratio of fluoranthene + pyrene to sum C2- through C4-phenanthrene (Fl + Py/C24P) increases with increasing pyrogenic composition [81]. The MP/P ratio was > 2 for only the most heavily oiled site, KC (MP/P = 2.6), while the FL+PY/C24P ratios clustered into four groups that matched the nMDS analysis. This analysis is consistent with heterogeneous background PAH inputs among the six sites that are associated with the adjacent upland development and land use, as well as an elevation of petrogenic signal above the background level at the heavily oiled site KC.

Table 3-9: Diagnostic PAH ratios for PEMD samples.

Site (description)	MP/P (diagnosis)	FL+PY/C24P (diagnosis)
KC (heavily oiled/residential	2.6 petrogenic	2.1 (least pyrogenic)
PP (lightly oiled/residential)	1.5 petrogenic/pyrogenic	2.7 (less pyrogenic)
PSQ (non-oiled/ developed)	1.9 petrogenic/pyrogenic	2.9 (intermediate pyrogenic)
SRB (non-oiled/ developed)	1.9 petrogenic/pyrogenic	3.0 (intermediate pyrogenic)
HC (oiled/ marina)	1.1 petrogenic/pyrogenic	4.2 (high pyrogenic)
SA (oiled marina)	1.0 petrogenic/pyrogenic	4.7 (high pyrogenic)

MP/P is the ratio of the sum of alkyl-phenanthrenes to parent non-alkylated phenanthrene; Fl + Py/C24P is the ratio of fluoranthene + pyrene to sum C2- through C4-phenanthrenes.

Section 4: Results of Laboratory Studies

4.1 Phototoxicity Trial 1, January 22-30 2009. This trial was considered a "dry run" in which there would not be a full work-up of samples for chemical analysis. The primary purpose was to check that the oiled gravel doses were correctly targeted, and that the positive controls were producing the expected sublethal toxicity. Embryos on randomly selected replicate slides were examined daily from 5 dpf through 8 dpf. Representative images were collected from most but not all dose and light combinations.

Results at 8 dpf are shown in Figure 4-1. In general, the positive and negative controls produced expected results. Normal development was observed in clean and urban gravel effluents. Embryos exposed to ANS under UVB plastic showed dose-dependent pericardial edema (not shown), as did embryos exposed to ANS under UVT plastic (arrows, Figure 4-1, second column, top). In addition, at the highest dose of ANS under UVT plastic, there was a reduction in the size of the embryos, and a slight dorsal curvature. This novel effect of oil + UV exposure was also observedFigure in the CB 0.3 g/kg UVT treatment (fourth column, middle). Dose-dependent pericardial edema was also observed in embryos exposed to CB oil under UVB plastic (arrows, Figure 4-1, third column). While embryos exposed to CB oil under UVT plastic appeared viable at 6 dpf (Figure 4-2), by 8 dpf embryos exposed to 1 g/kg CB oil and UV were completely necrotic (Figure 4-1, third column), making them impossible to dechorionate.

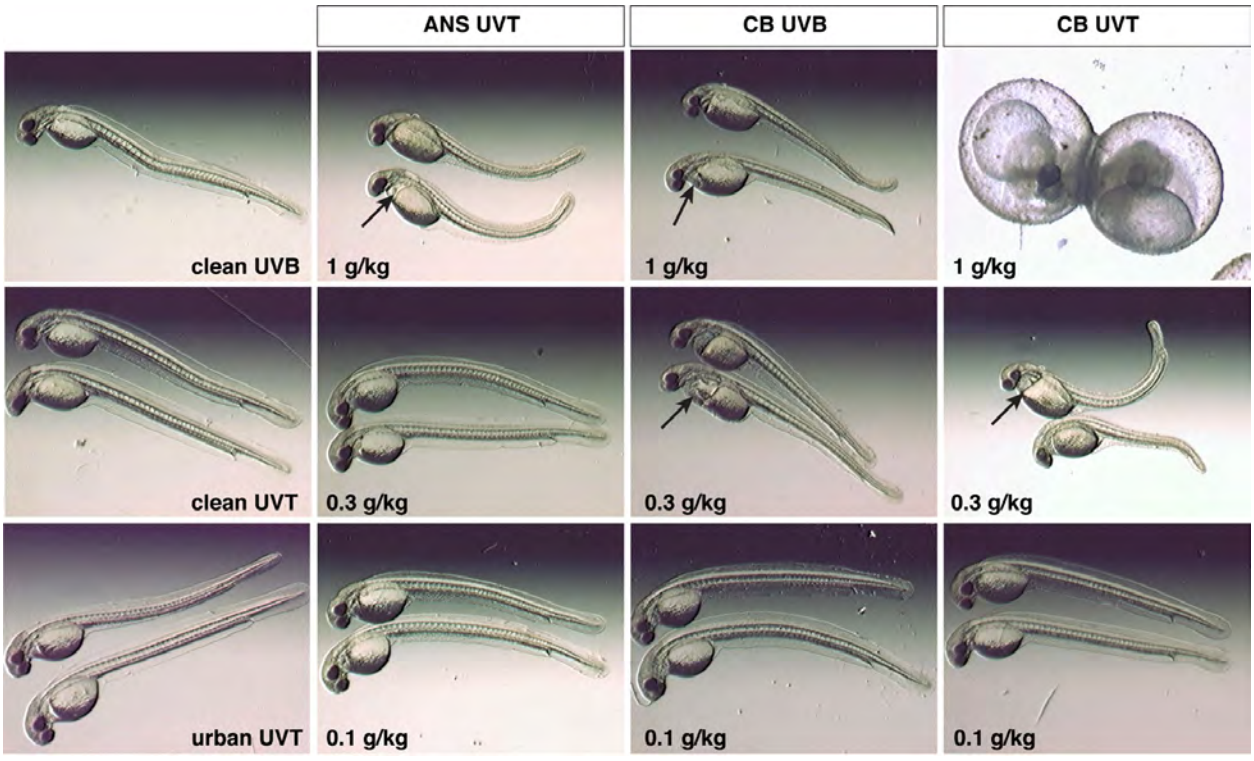


Figure 4-1: Morphology of 8 dpf embryos, Trial 1. Arrows indicate pericardial edema

During this trial, continuous temperature loggers were placed in one control effluent aquarium on each table. Temperature in the exposure aquaria fluctuated around the desired 12˚C incubation temperature

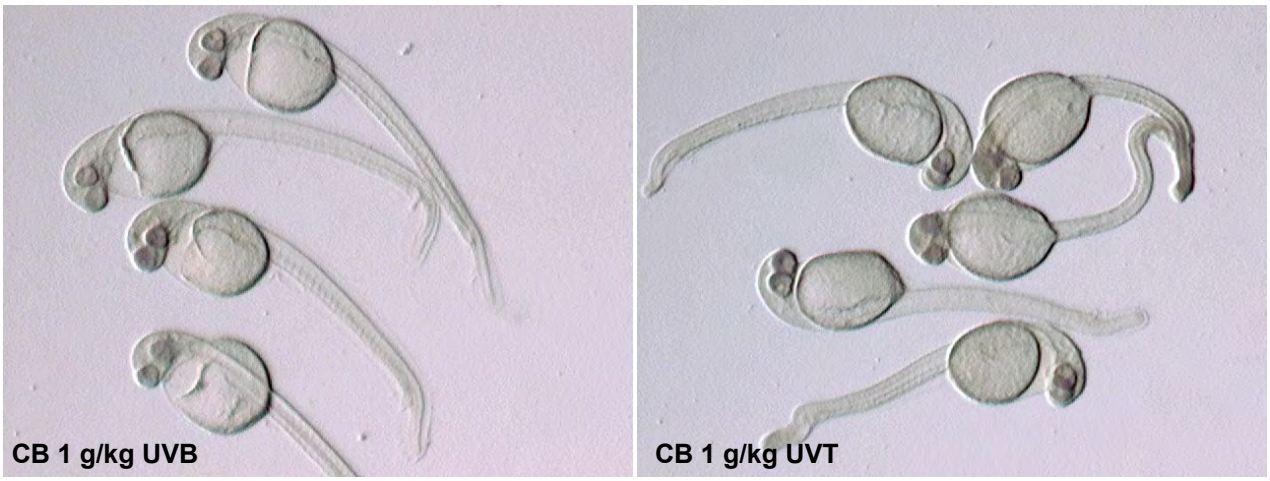


Figure 4-2: Morphology of high dose CB-exposed embryos at 6 dpf, Trial 1

on a diurnal basis, peaking around 18°C between 12:00 and 14:00 (Figure 4-3). However, all tables showed very similar temperature profiles. Spot checking temperatures randomly in other column effluents showed that the logger data were broadly representative of all columns in a table. Data was not collected from Table 4 due to failure of the logger.

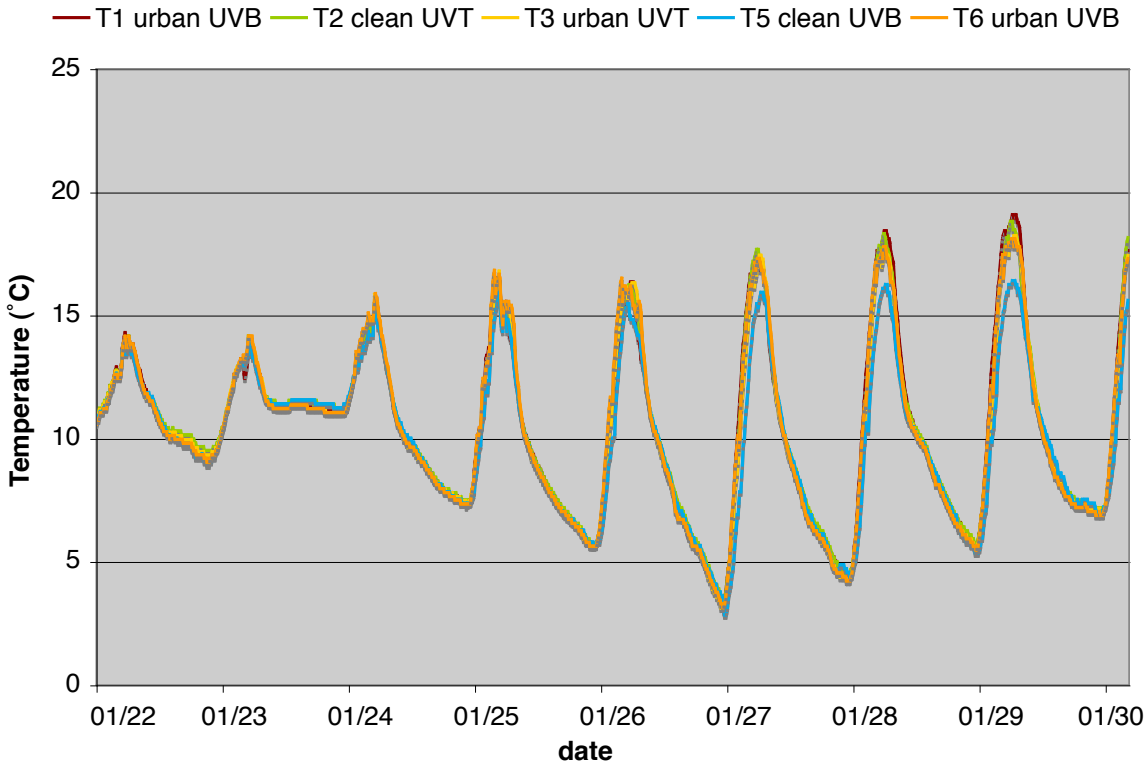


Figure 4-3: Trial 1 temperature logger data from control column effluents, recorded every 10 min.

4.2 Phototoxicity Trial 2, February 13-22 2009. During this trial, it was noted at 5 dpf that there were high rates of abnormal early embryos in lab incubator controls. These embryos had gastrulation defects. Because of this, it was decided to abort this trial due to the time it would take to distinguish oil-associated lethality from background. However, a small-scale analysis was completed. So that the columns could be prepared for a new batch of embryos, one or two replicates of each treatment were removed from the

column effluents at 7 dpf and placed in 20-gal aquaria with 22 ppt seawater, immersed in a 4-ft tank for temp control, and incubated an additional 2 days under the appropriate UVT or UVB plastic. Thus oil exposure stopped at 7 dpf, while sunlight exposure continued to 9 dpf when embryos were analyzed.

Results are shown in Figure 4-4. Embryos were scored as viable eyed embryos. Similar to what was observed in Trial 1, high rates of cytolyzed eyed embryos were observed in the CB 1 g/kg UVT treatment. Only a few embryos from the CB 1 g/kg UVT treatment were sufficiently intact to dechorionate (Figure 4-5).

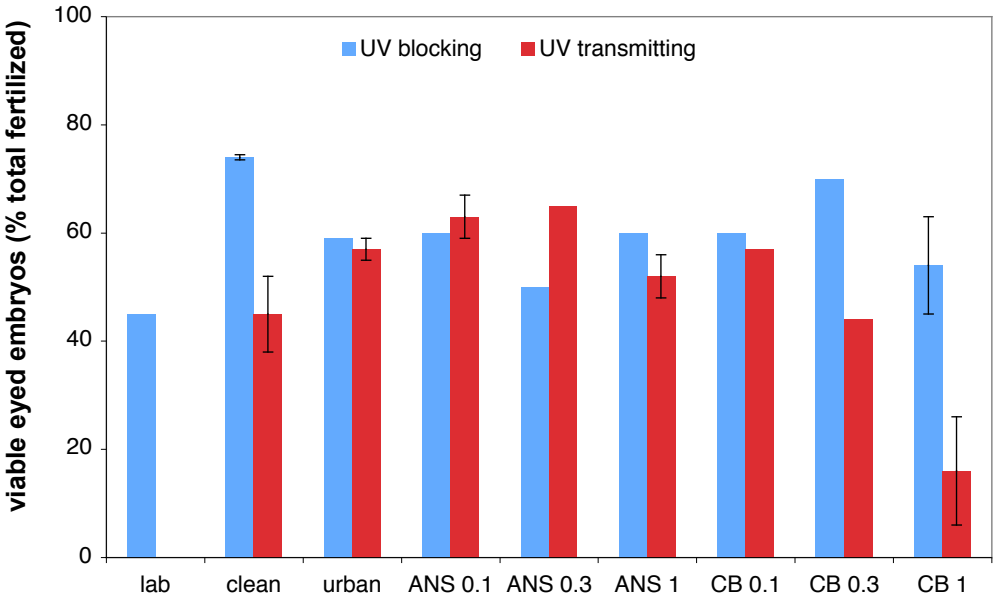


Figure 4-4: Viable eyed embryos, Trial 2. Error bars present for samples with n = 2.

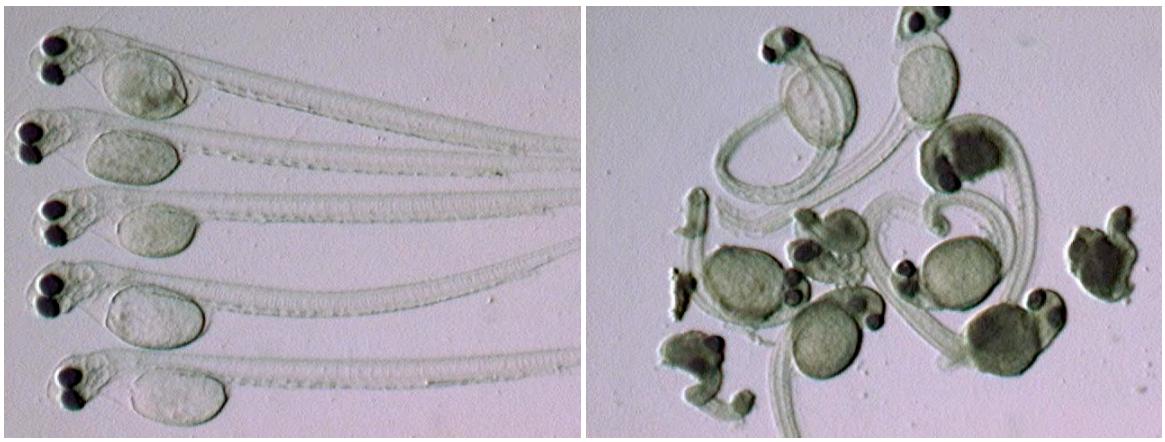


Figure 4-5: Morphology of non-cytolyzed CB 1 UVT embryos compared to CB 1 UVB

Some changes were made for this trial to improve the temperature control. A chiller was added to cool the water leaving the head tank providing source water for the columns. The water baths were switched from freshwater to seawater to dampen the night time chilling effect. Incubation temperatures still fluctuated several degrees around the desired 12°C, with higher temperature peaks (20°C) coming with warmer sunny days. Consistent with Trial 1, all tables showed similar temperature trends (Figure 4-6). The last two

days of incubation for this trial were carried out in 20-gal aquaria submerged in 4-foot tanks held at 12˚C. It was noted during this run that diatom growth was becoming visible in the incubator tanks and tubing feeding the columns. Tanks were scrubbed prior to placement of embryo slides.

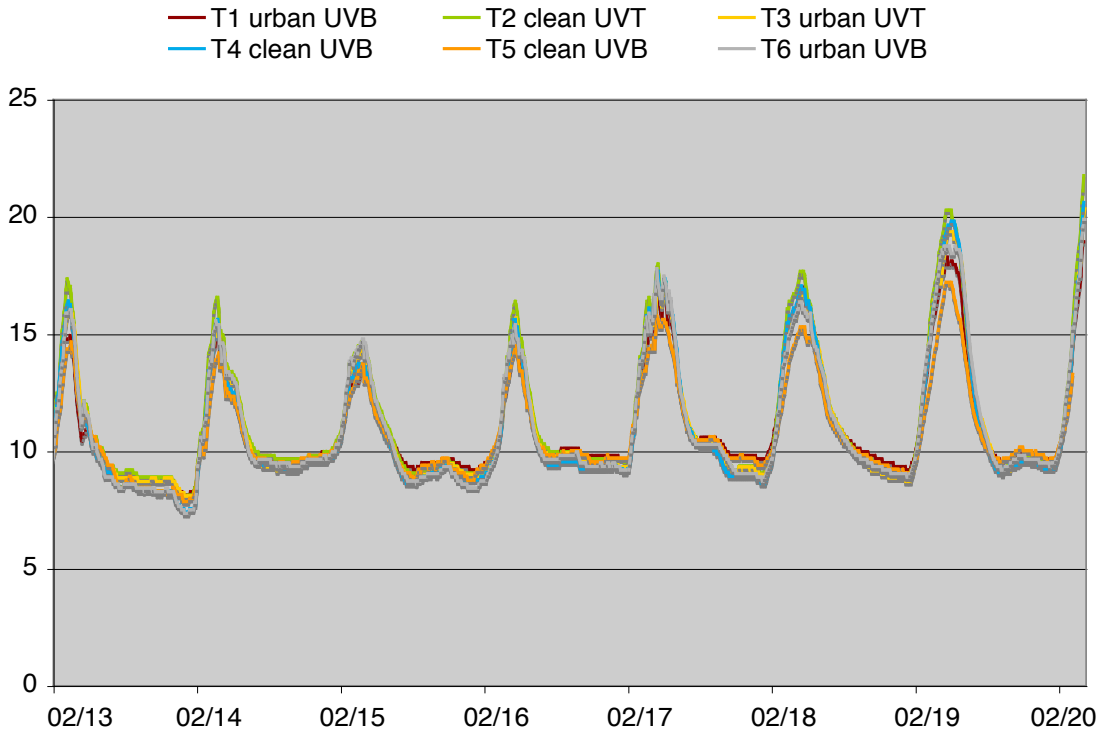


Figure 4-6: Trial 2 temperature logger data from control column effluents, recorded every 10 min.

4.3 Phototoxicity Trial 3, February 26 - March 7 2009. Laboratory controls were satisfactory for this trial (about 10% background embryos with abnormal early development), and a full experiment was completed with samples taken for water and tissue PAH analysis. Embryos were incubated to 9 dpf and analyzed. Due to higher daytime temperatures then the previous trials, development was accelerated, and some hatching had occurred by 9 dpf. Therefore, embryos were scored as unfertilized eggs, dead during segmentation or earlier (no eye pigment), viable with eye pigment, necrotic with eye pigment, or hatched (empty chorion). During this trial, heavy growth of singular and filamentous diatoms had occurred between days 6 and 9 of incubation, coating the outer chorions. Embryos on slides were gently scraped with forceps to allow visual scoring through the chorion.

This was the first trial for which there was a complete set of samples taken for PAH analysis in both water and tissue. Two duplicate sets of column effluent samples (200-ml) were collected and analyzed for PAHs. One was analyzed by the NOAA NWFSC without filtering. The second set was analyzed by the Alpha Analytical Woods Hole Division (Mansfield, MA), after filtration ostensibly to remove particulate oil or oil droplets. The Alpha Analytical data are reported here. Aqueous PAH levels correlated well with oil doses loaded on gravel (Figure 4-7). PAH levels were trace, with the highest CB oil dose producing total PAHs (TPAH) less than 1 ppb (µg/l). The lowest oil doses (0.1 g/kg) produced aqueous TPAH that were nominally higher than but difficult to distinguish from background levels of about 100 ppb.

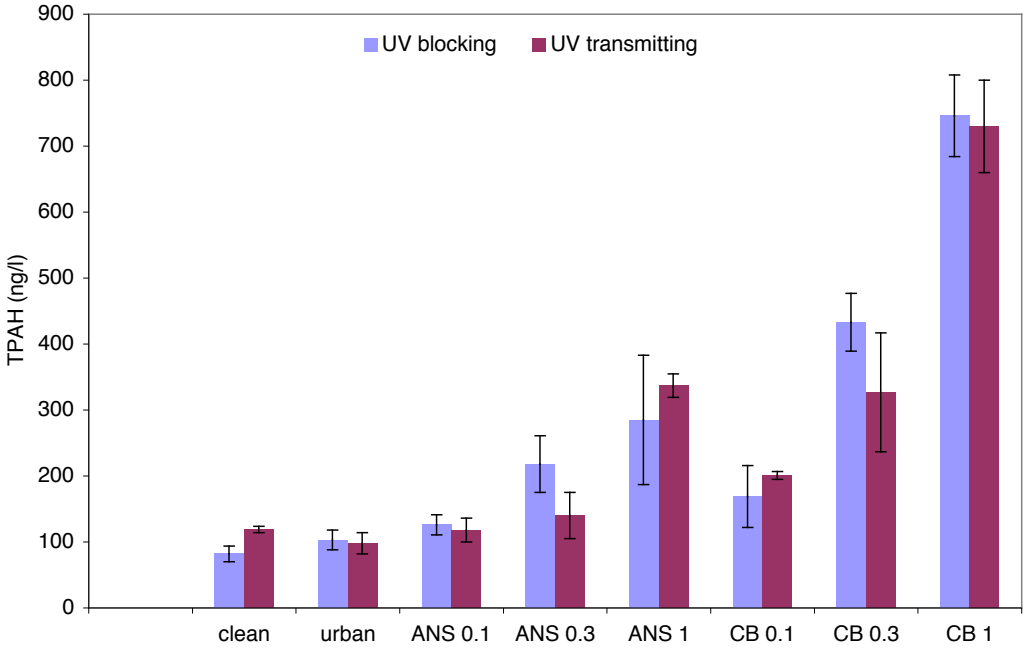


Figure 4-7: Total aqueous PAHs in column effluents (38 analytes). Values are mean ± SE for three replicate columns from samples taken at the start of incubation.

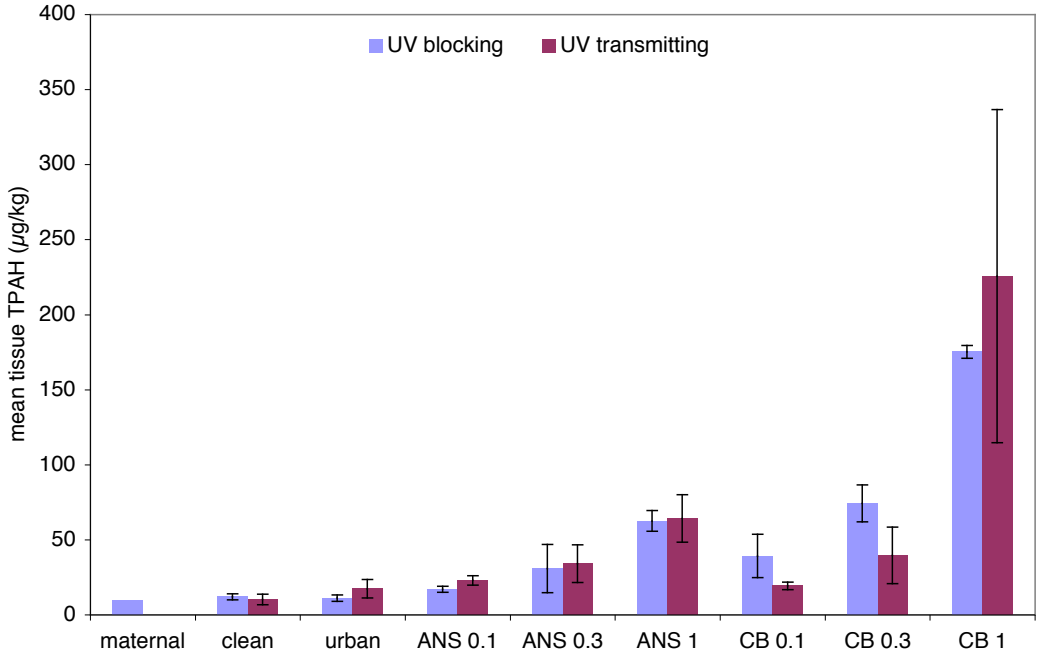


Figure 4-8: Total PAHs in embryos (38 analytes). Values are wet weight mean ± SE for three replicates.

TPAH measured in embryo tissues correlated with aqueous TPAH and oil loading dose (Figure 4-8). The highest TPAH values were found in embryos exposed to the highest (1 g/kg) dose of CB oil, where both UVB and UVT treatments accumulated about 200 ppb TPAH. For reference, the doses of ANSCO producing sublethal effects on heart rate in herring resulted in TPAH at 480 ppb at the lowest tested dose

(0.4 g/kg) at an earlier phase of weathering (Incardona et al., 2009 *Environ Sci Technol* 43:201). The lower doses of both CB oil and ANS produced TPAH below 75 ppb.

Embryos were examined beginning at 5 dpf. As was observed in the first two trials, embryos progressed through development to the eye pigmentation stage, with high numbers of deteriorating, cytolyzed embryos in the CB 1 g/kg UVT treatment appearing close to hatching. Results for necrotic eyed embryos are shown in Figure 9. For the CB 1.0 g/kg dose under UV transmitting plastic, a mean of 91% was found to be cytolyzed by the late eyed stage, consistent with the effect observed in Trial 1. The middle dose of CB oil (0.3 g/kg) under UV transmitting conditions produced a statistically significant increase in necrotic lethality at 16% compared to 3% under UV blocking conditions. The high dose of ANSCO also produced a statistically significant increase in necrotic lethality at 16% compared to 6% under UV blocking conditions. The percentage of necrotic embryos did not correlate with tissue PAH levels (Figure 4-8 vs.

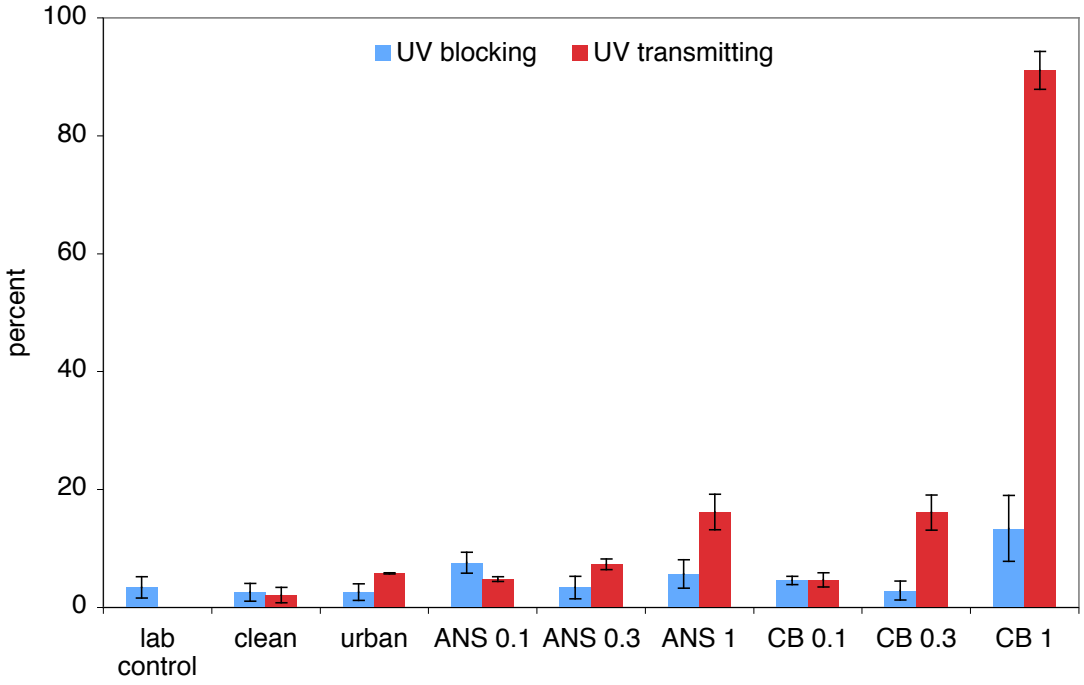


Figure 4-9: Necrotic eyed embryos, Trial 3. Mean and SE for n = 3, except CB 0.3 g/kg UVT treatment, n = 2. Denominator for percentage is total eyed eggs per slide (necrotic eyed + viable eyed + hatched)

Figure 4-9): While the CB 0.3 g/kg UVT treatment and the ANS 0.3 g/kg UVT treatment had indistinguishable PAH levels (40 ± 19 ng/g vs. 34 ± 13 ng/g, respectively), only the CB 0.3 g/kg UVT treatment produced a significantly higher percentage of necrotic embryos (16 ± 3% vs. 4.8 ± 0.4%).

With increasing daytime temperatures and day length, incubation temperatures continued to have diurnal variation and diatom growth became much heavier than the previous trial. Temperature peaked on two days at 22.5 - 24°C (Figure 4-10). All tables showed similar temperature trends. Algae growing in the columns and incubation aquaria was examined microscopically and found to consist of green filamentous (stacking) and brown singular diatoms, with some flagellated forms. Chorions were coated with clustered singular diatoms. The presence of algae resulted in a diurnal pattern of high dissolved oxygen levels coupled with elevated pH, coincident with peak daytime photosynthesis. Daily dissolved oxygen levels, measured at mid-day, are shown for clean gravel effluents and CB 1 g/kg doses in Figure 4-11. Daily pH values, measured at mid-day, are shown for clean gravel effluents and CB 1 g/kg doses in Figure 4-12.

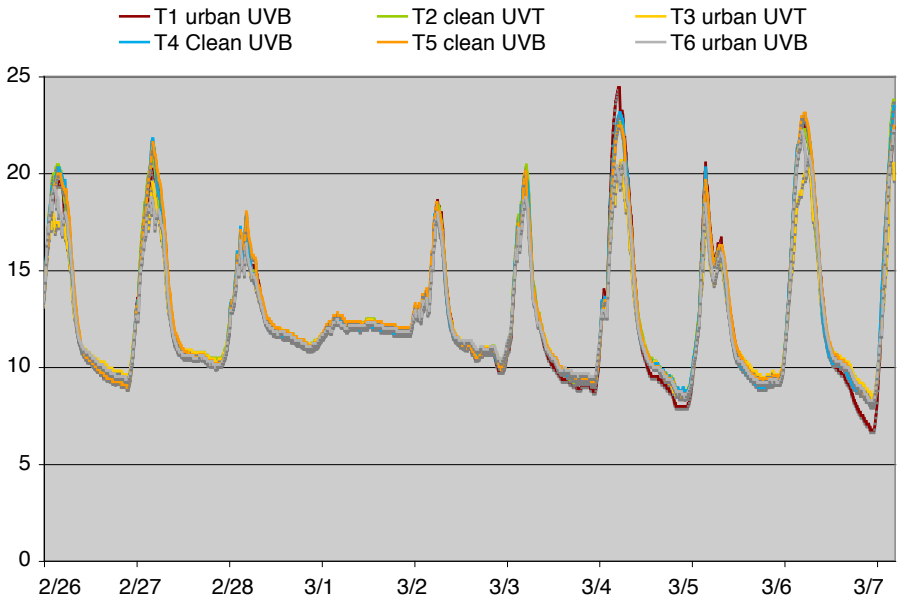


Figure 4-10: Trial 3 temperature logger data from control column effluents, recorded every 10 min.

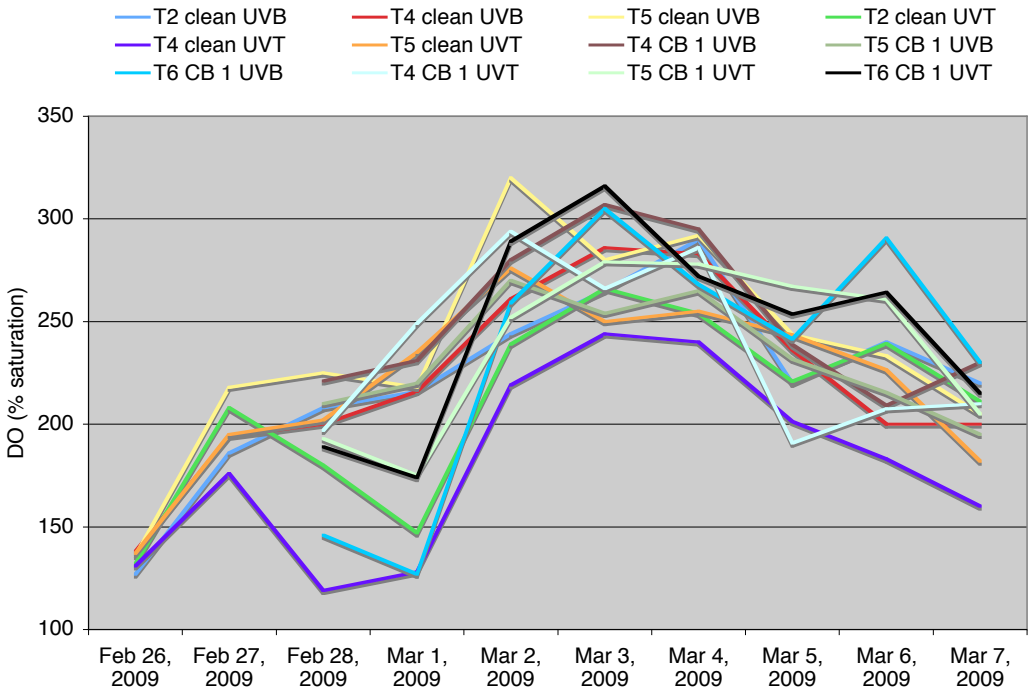


Figure 4-11: Trial 3 daily dissolved oxygen levels, clean gravel vs. CB 1 g/kg, UVB and UVT

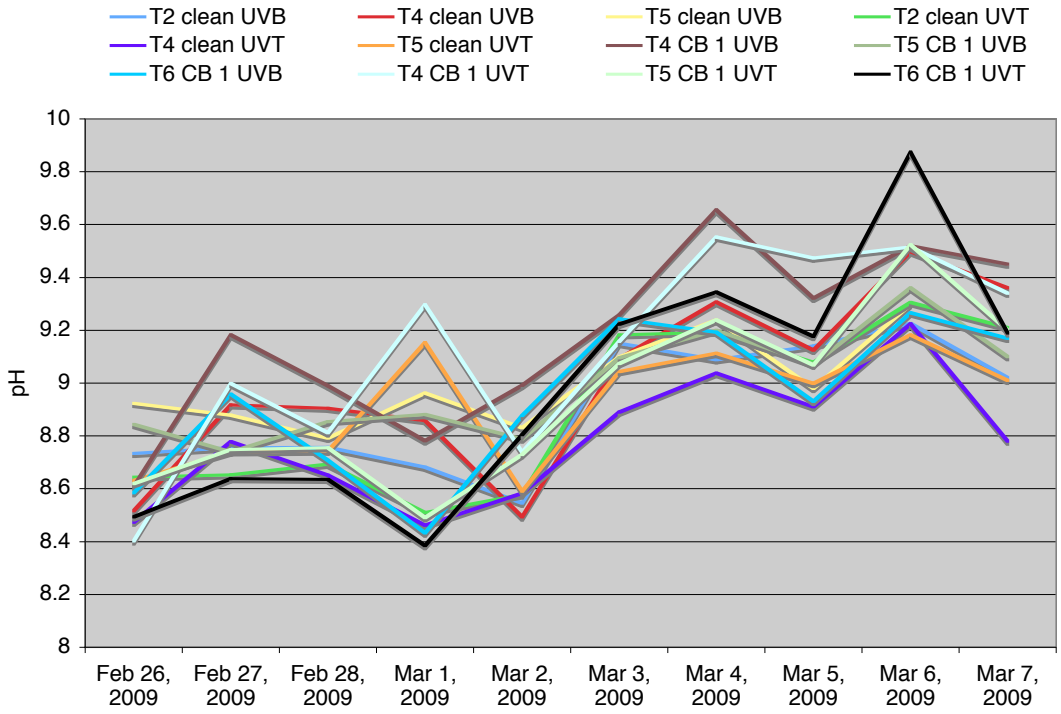


Figure 4-12: Trial 3 daily pH, clean gravel vs. CB 1 g/kg, UVB and UVT

Algal growth was assessed and described in a semi-quantitative manner for each column/incubation aquarium pair and noted. These data are shown in Table 4-1. Throughout this trial ammonia was undetected or at background levels.

Table 4-1: Characterization of algal growth in columns and incubator aquaria, Trial 3

Table	dose	plastic	column	aquarium	color
Table 1	ANS 0.1	UVB	slight	heavy	green
Table 3	ANS 0.1	UVB	heavy	slight/med	brown
Table 5	ANS 0.1	UVB	slight	medium	green
Table 1	ANS 0.1	UVT	slight	slight/med	green
Table 3	ANS 0.1	UVT	medium	heavy	green
Table 5	ANS 0.1	UVT	slight	slight	green
Table 1	ANS 0.3	UVB	slight	slight/med	green
Table 2	ANS 0.3	UVB	slight	heavy	green
Table 6	ANS 0.3	UVB	heavy	heavy	mixed
Table 1	ANS 0.3	UVT	slight	medium	green
Table 2	ANS 0.3	UVT	slight	slight	green
Table 6	ANS 0.3	UVT	slight	heavy	green
Table 3	ANS 1	UVB	slight	heavy	brown
Table 4	ANS 1	UVB	slight	slight	green
Table 5	ANS 1	UVB	slight	slight	green
Table 3	ANS 1	UVT	slight	slight	green
Table 4	ANS 1	UVT	slight	slight	green
Table 5	ANS 1	UVT	slight	slight	green
Table 2	CB 0.1	UVB	heavy	heavy	green
Table 4	CB 0.1	UVB	heavy	heavy	green
Table 6	CB 0.1	UVB	heavy	heavy	mixed
Table 2	CB 0.1	UVT	heavy	heavy	green
Table 4	CB 0.1	UVT	slight	slight/med	green
Table 6	CB 0.1	UVT	heavy	heavy	mixed
Table 1	CB 0.3	UVB	slight	medium	green
Table 2	CB 0.3	UVB	heavy	heavy	green
Table 3	CB 0.3	UVB	heavy	heavy	green
Table 1	CB 0.3	UVT	slight	slight/med	green
Table 2	CB 0.3	UVT	heavy	heavy	green
Table 3	CB 0.3	UVT	slight	slight	brown
Table 4	CB 1	UVB	slight	medium	green
Table 5	CB 1	UVB	slight	slight/med	green
Table 6	CB 1	UVB	slight	medium	green
Table 4	CB 1	UVT	slight	medium	green
Table 5	CB 1	UVT	heavy	medium	brown
Table 6	CB 1	UVT	medium	heavy	mixed
Table 2	clean	UVB	heavy	heavy	green
Table 4	clean	UVB	heavy	medium	green
Table 5	clean	UVB	heavy	heavy	green
Table 2	clean	UVT	slight	heavy	green
Table 4	clean	UVT	slight	slight	green
Table 5	clean	UVT	slight	medium	green
Table 1	Urban	UVB	slight	slight/med	green
Table 3	Urban	UVB	slight/med	medium	green
Table 6	Urban	UVB	heavy	heavy	mixed
Table 1	Urban	UVT	slight	medium	brown
Table 3	Urban	UVT	medium	slight	green
Table 6	Urban	UVT	medium	slight/med	green

4.4 Phototoxicity Trial 4, March 18-26, 2009. Due to the coating of eggs by diatoms and higher temperatures during Trial 3, some modifications were made for a fourth trial. Between the trials, all components were cleaned of algae. The gravel was rinsed with cold freshwater, and the lines, aquaria, and water baths were bleached and treated with thiosulfate. Flow of 25 ppt seawater was initiated 48 hr prior to embryo incubation. A major goal was to increase the water flow rate weathering the columns to help stabilize the temperatures. The size of the head tank limited the total volume at a higher rate, so some treatments had to be eliminated. The ANS columns and urban gravel negative control were eliminated, cutting the number of columns from 48 to 24. Flow rate was increased from 12 ml/min to 30 ml/min. In addition, to reduce the exponential growth of algae typically observed in the last few days of incubation, a 90% shade cloth was used to cover each bank of columns starting on day 5 of incubation. The shade cloth was removed for the last 24 hours of exposure.

The aqueous TPAH levels in this trial were similar to Trial 3, with the highest gravel loading producing TPAH in the 0.7-0.9 ppb range (Figure 4-13). Tissue TPAH levels were also similar to Trial 3, with the CB 1 g/kg treatments producing roughly 150-200 ppb by 8 dpf (Figure 4-14).

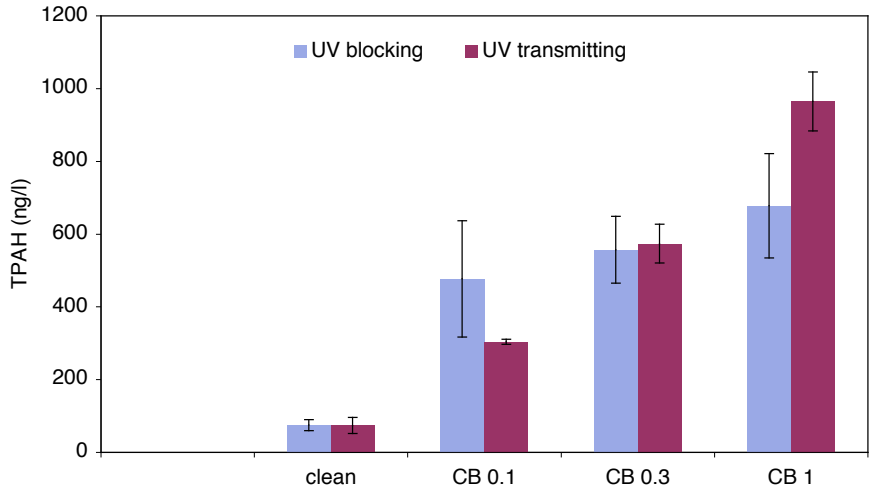


Figure 4-13: Total aqueous PAHs in column effluents (38 analytes). Values are mean ± SE for three replicate columns from samples taken at the start of incubation.

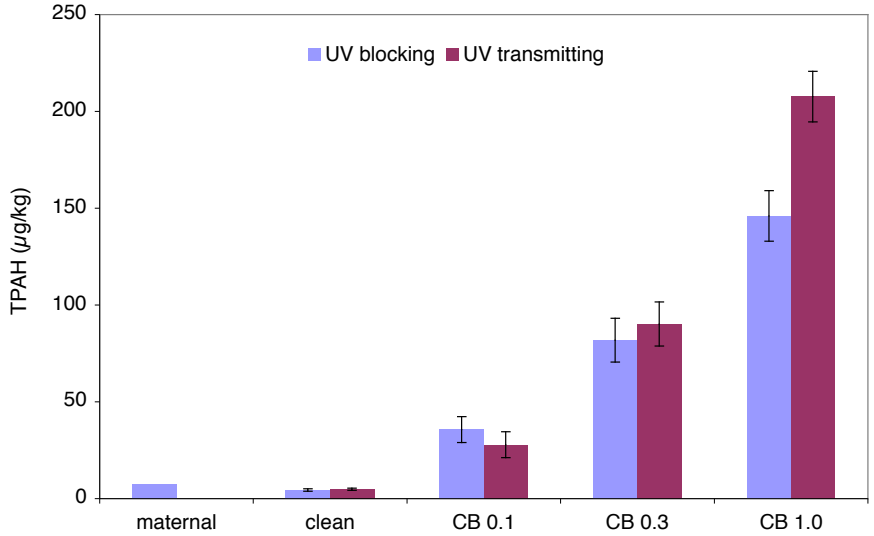


Figure 4-14: Total PAHs in embryos (38 analytes). Values are wet weight mean ± SE for three replicates.

As in previous trials, embryos were examined daily from 5 dpf and were found to progress into the eye pigmentation stage. By 8 dpf, large numbers of deteriorating eyed embryos were observed in the CB 1 g/kg UVT treatment. Accumulation of diatoms on the chorions was still heavy enough to require removal by

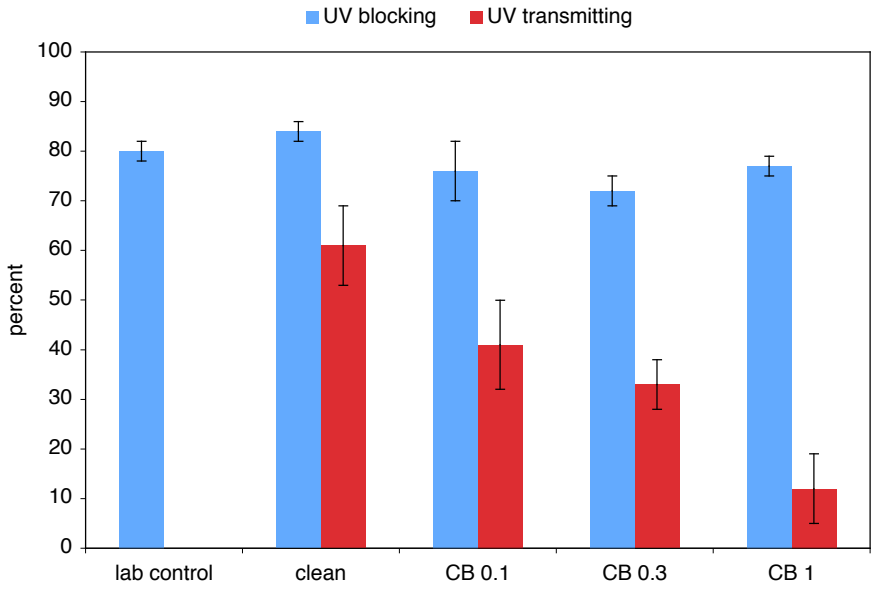


Figure 4-15: Viable eyed embryos, Trial 4. Mean and SE for n = 3. Denominator for percentage is total fertilized eggs per slide.

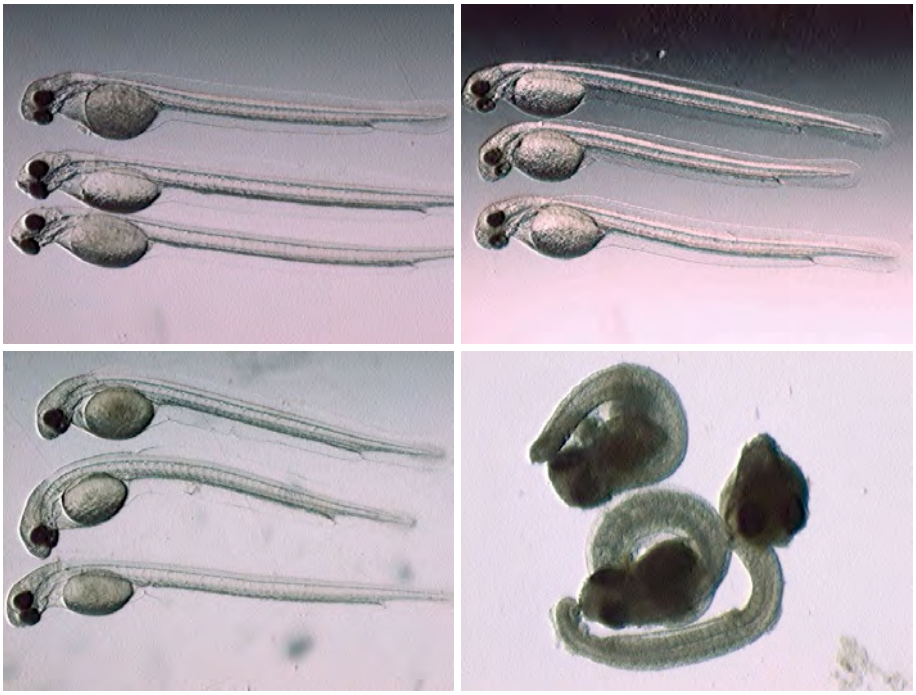


Figure 16: Lethal effects of CB 1 UVT treatment, Trial 4. Examples of viable dechorionated embryos shown for clean UVB and UVT treatments, and CB 1 g/kg UVB treatment, necrotic embryos from CB 1 g/kg UVT treatment.

careful scraping with forceps. To streamline the scoring process, in this trial the counts included total eggs on each slide, unfertilized eggs, and viable (i.e. non-necrotic) eyed embryos. There was not a count for embryos that died at early stages for each treatment, but random checks showed a background rate of

about 10% in laboratory controls and column specimens. The data for viable eyed embryos are shown in Figure 4-15. Examples of viable and necrotic embryos are shown in Figure 4-16.

The increased flow rate did lead to better temperature control, but there were still diurnal fluctuations

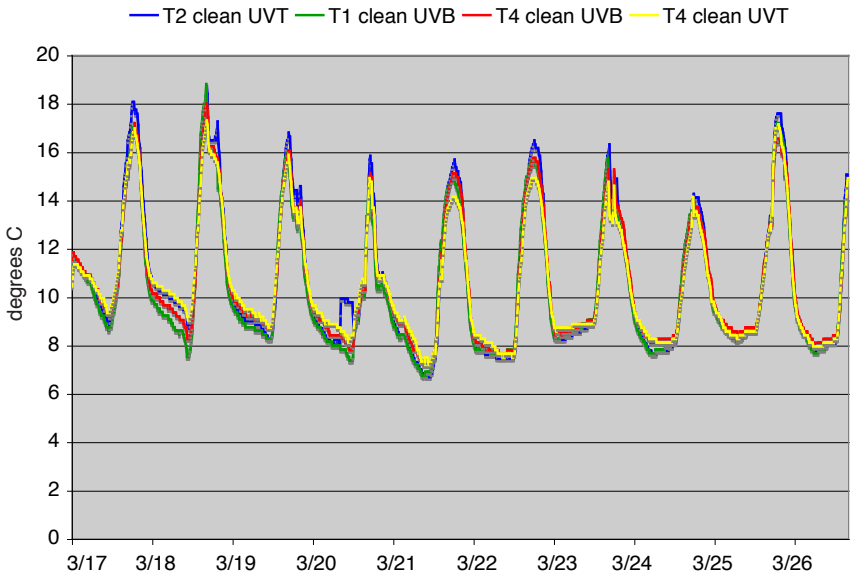


Figure 4-17: Trial 4 temperature logger data from control column effluents, recorded every 10 min.

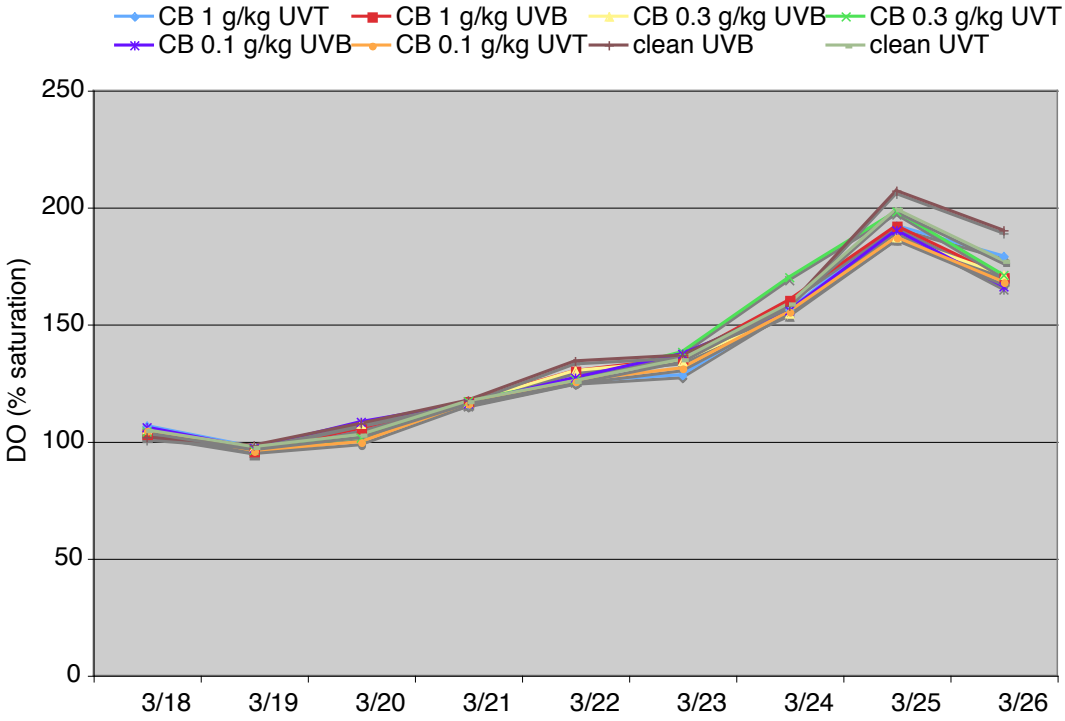


Figure 4-18: Trial 4 daily dissolved oxygen levels in one replicate of each treatment

with peaks around 18˚C at 12:00-14:00 on three days (Figure 4-17). Although growth of diatoms was reduced in this trial relative to Trial 3, there was still a gradual increase of algal growth throughout the incubation period, which resulted in a gradual increase in dissolved oxygen levels (Figure 4-18) and pH

(Figure 4-19). However, there was little variation among treatments with respect to temperature, dissolved oxygen, and pH. Throughout this trial ammonia was undetected or at background levels.

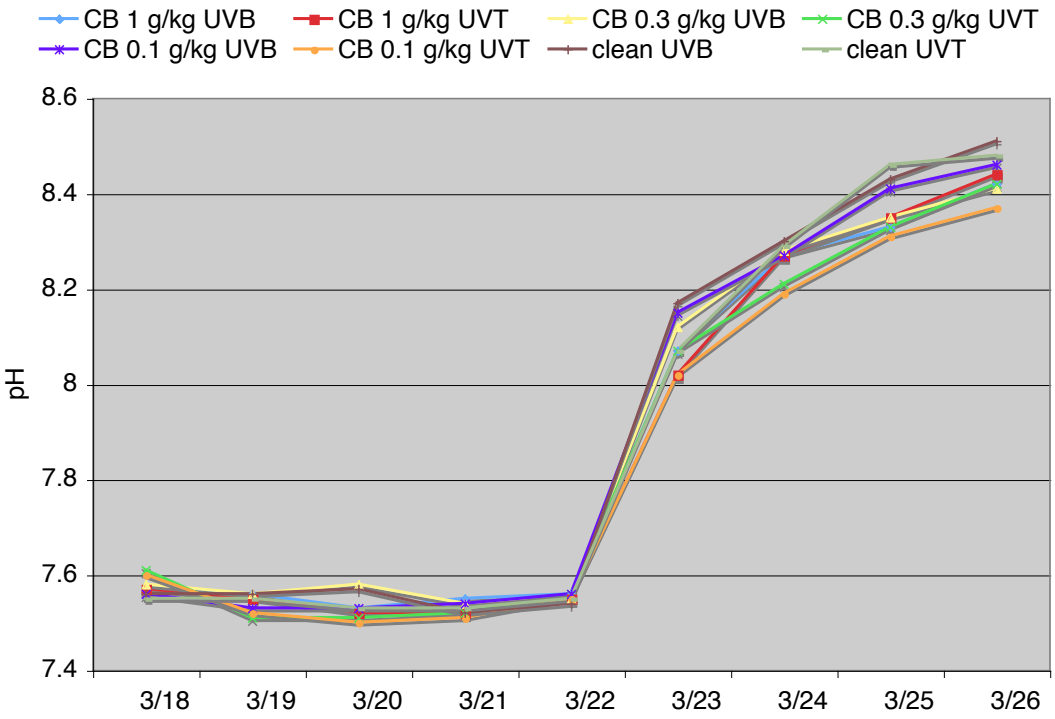
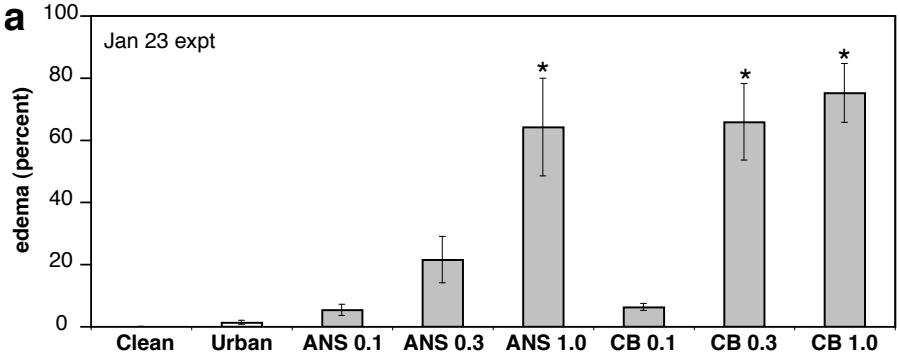
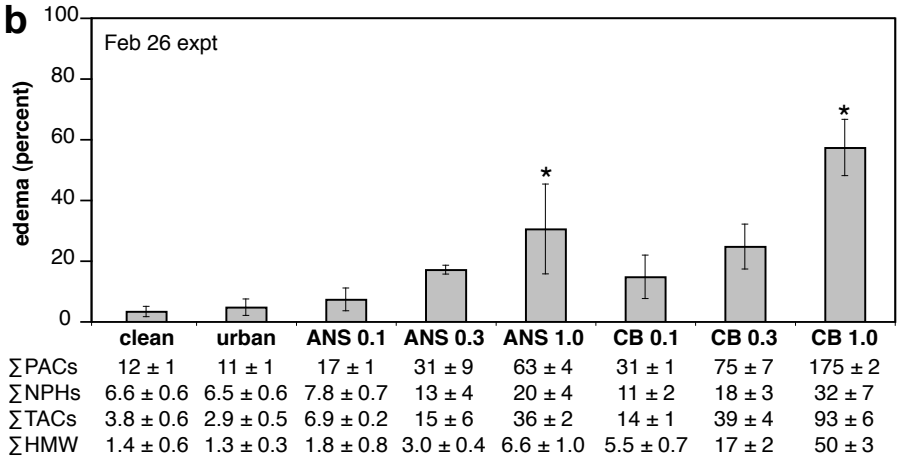


Figure 4-19: Trial 4 daily pH in one replicate of each treatment

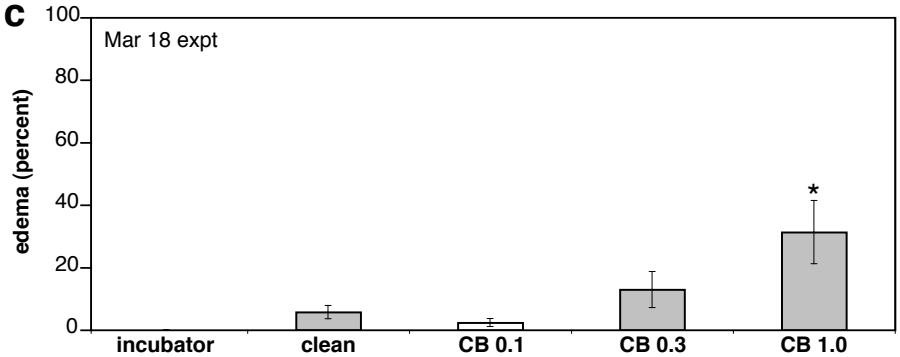
4.5 Cosco Busan bunker oil produced canonical petrogenic cardiotoxicity under UV-reducing conditions. In the first two experiments we compared exposure to equivalent oil mass loadings of ANSCO and CBBO. ANSCO served as a positive control to reproduce canonical crude oil toxicity, as a context for identifying possible novel effects from refined CBBO exposure. As expected from prior studies, dose-dependent pericardial edema was observed in hatched larvae following exposure to oiled gravel effluent under UV-reducing conditions (Figure 4-20). Incidence of edema generally correlated with declining water and tissue PAH concentrations. In the first experiment starting January 23, the incidence of larval edema was 64 ± 17% after exposure to ANSCO 1.0 g/kg gravel effluent, and 66 ± 12% and 75 ± 9% in larvae exposed to CBBO 0.3 g/kg and 1.0 g/kg gravel effluent, respectively (Figure 4-20a). Over the next month of weathering, aqueous PAH concentrations would be predicted to decline exponentially. Consistent with this, herring embryos from the exposure beginning Feb 26 showed a decline in edema to 30 ± 15% for exposure to ANSCO 1.0 g/kg, with an embryonic tissue ∑PAH concentration of 63 ± 4 ng/g (Figure 4-20b). Exposure to the equivalent mass dose of CBBO resulted in a higher incidence of edema (57 ± 9%) with a tissue ∑PAH concentration of 175 ± 2 ng/g. The CBBO 0.3 g/kg dose produced toxicity similar to the ANSCO 1.0 g/kg dose (25 ± 7% edema and ∑PAH 75 ± 7 ng/g). For CBBO exposures, the incidence of edema correlated closely ($P < 0.0001$, $R2 = 0.86$) with total tissue tricyclic aromatic compounds (∑TAC). Although remixing of the gravel and higher flow rates in the 18 March experiment resulted in higher ∑PAH, largely due to an increase in naphthalenes, tissue ∑TAC and concentrations of higher molecular weight compounds (∑HMW) at the highest CBBO dose were 82% (76 ± 8 ng/g) and 60% (30 ± 2 ng/g) lower, respectively, and the incidence of edema was significantly lower at 31 ± 10% (Figure 4-20c; t test, α =

0.05). These results indicate that under UV-blocking conditions, exposure to CBBO results in canonical petrogenic PAH toxicity represented by cardiogenic edema. The incidence of edema was higher for CBBO relative to an equivalent mass dose of ANSCO, consistent with the higher PAH fraction of the residual oil.





	clean	urban	ANS 0.1	ANS 0.3	ANS 1.0	CB 0.1	CB 0.3	CB 1.0
∑PACs	12 ± 1	11 ± 1	17 ± 1	31 ± 9	63 ± 4	31 ± 1	75 ± 7	175 ± 2
∑NPHs	6.6 ± 0.6	6.5 ± 0.6	7.8 ± 0.7	13 ± 4	20 ± 4	11 ± 2	18 ± 3	32 ± 7
∑TACs	3.8 ± 0.6	2.9 ± 0.5	6.9 ± 0.2	15 ± 6	36 ± 2	14 ± 1	39 ± 4	93 ± 6
∑HMW	1.4 ± 0.6	1.3 ± 0.3	1.8 ± 0.8	3.0 ± 0.4	6.6 ± 1.0	5.5 ± 0.7	17 ± 2	50 ± 3



	incubator	clean	CB 0.1	CB 0.3	CB 1.0
∑PACs	12 ± 1	4.5 ± 0.6	34 ± 6	75 ± 9	135 ± 12
∑NPHs	6.6 ± 0.6	2.9 ± 0.3	10 ± 1	18 ± 2	30 ± 2
∑TACs	3.8 ± 0.6	0.7 ± 0.1	19 ± 3	43 ± 6	76 ± 8
∑HMW	1.4 ± 0.6	0.7 ± 0.1	4.6 ± 1.1	14 ± 2	30 ± 2

Figure 4-20: Dose-dependent pericardial edema after oil exposure under UV-reducing plastic. Edema was quantified in live hatched larvae. Values represent the mean percent ± SEM from three replicates for each control or oil dose for the experiments starting 23 January (a), 26 February (b) and 18 March (c). Nominal oil loadings (0.1, 0.3, and 1.0 g/kg) are indicated for each oil (ANSCO, ANS; CBBO, CB). Tissue PAHs (ng/g wet weight) are shown for the 26 February and 18 March experiments as sum total (∑) PAHs, sum parent and alkylated naphthalenes (NPHs), sum parent and alkylated tricyclic compounds (TACs; fluorenes, dibenzothiophenes, phenanthrenes), and sum high molecular weight compounds (HMW; fluoranthene, pyrene, C1-fluoranthene/pyrenes, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[j]fluoranthene/benzo[k]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene/dibenz[a,c]anthracene, and benzo[ghi]perylene).

4.6 Trials 1-4, Assessment of hatching and larval morphology in the Laboratory Exposure and Phototoxicity Study. These data do not directly address the Specific Aims of the Laboratory Exposure and Phototoxicity Study as described in the Introduction. The primary focus of this study was to determine whether Cosco Busan bunker oil could produce lethal embryonic necrosis. Data on larval hatching rates and morphology are provided as supplemental information in Appendix 2 of this report.

4.7 Laboratory Salinity Study. This study tested alternative hypotheses whether (1) high salinity could produce embryonic lethality such as that observed at intertidal zones of oiled sites in the 2007-08 spawning season, and (2) whether embryos fertilized and incubated at high salinity would show pericardial edema when dechorionated at low salinity (i.e. 16 ppt), as a potential explanation for signs of edema in subtidal caged embryos at oiled sites in 2007-08.

Fertilizations were carried out at the Bodega Marine Lab on March 5, 2009 according to the study plan. Duplicate slides were received at NWFSC from BML on 3/13/09 (8 dpf), along with BML 16 ppt seawater for processing. All slides were examined upon receipt and showed similar numbers of viable eyed embryos. Due to time constraints, the most relevant treatments were selected for dechorionation, i.e. optimal laboratory salinity regimen (fertilized at 16 ppt, incubated at 16 ppt) and high salinity regimen (fertilized at 30 ppt, incubated at 30 ppt). For dechorionation, slides were transferred to 16 ppt seawater and held at 12 °C on a cooling stage. At least 20 embryos were dechorionated at 16 ppt from a single slide for each treatment, and held at 16 ppt for imaging.

At BML after 7 days of incubation, the remaining slides were transferred to 16 ppt salinity and incubated at 12^0C with daily water changes of 16 ppt seawater. At commencement of hatching, the remaining embryos and larvae were evaluated as described previously.

Embryo Results:

A. Fertilized at 16 ppt, incubated at 16 ppt, dechorionated and imaged at 16 ppt
23/23 embryos between Hill and Johnston (1997) stages *o* and *p*. Edema present in 0/23 embryos. Body axis defects present in 0/23 embryos. Pericardial sPAHe larger than embryos from high salinity regimen (see below), but this is due to more advanced head rotation. Representative samples shown in Figure 4-21, top.

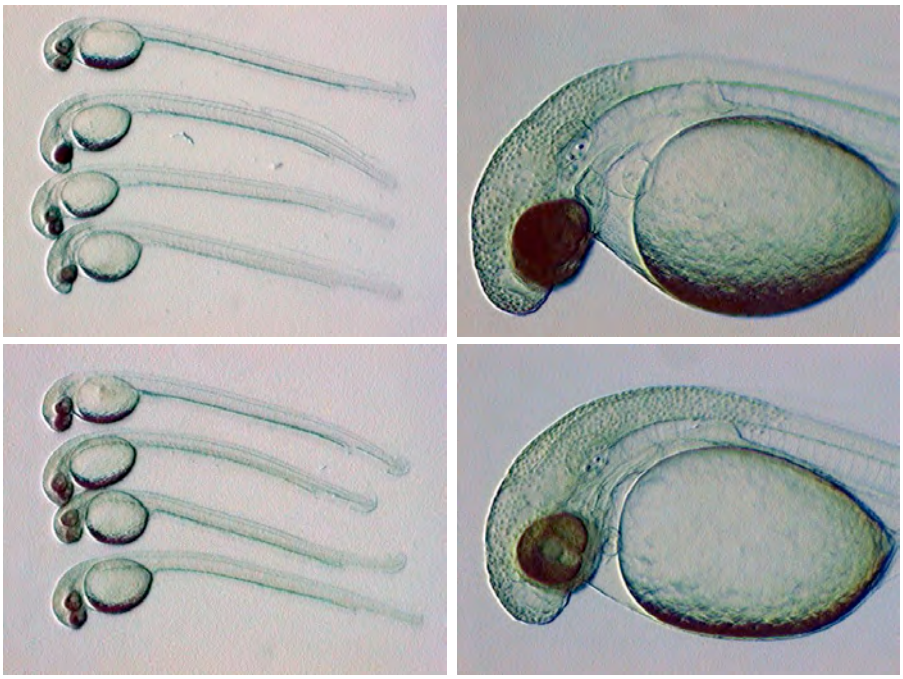


Figure 4-21: Morphology of embryos exposed to optimal salinity (16 ppt, top) or high salinity (32 ppt, bottom).

B. Fertilized at 30 ppt, incubated at 30 ppt, dechorionated and imaged at 16 ppt.
26/26 embryos between stages *o* and *p*, but slightly delayed relative to optimal regimen. This was evident in the degree of head rotation, eye pigmentation, and length of tail bud. Edema present in 0/26 embryos. Body axis defects present in 0/26 embryos. Representative samples shown in Figure 4-21Figure, bottom.

Hatching Results:
Cumulative daily hatch rates were collected for all tested salinity regimens: Fertilized at 16 ppt and incubated at 16 ppt (16-16), fertilized at 16 ppt and incubated at 22 ppt (16-22), fertilized at 22 ppt and incubated at 22 ppt (22-22), fertilized at 16 ppt and incubated at 30 ppt (16-30), and fertilized at 30 ppt and incubated at 30 ppt (30-30). Data (Figure 4-22) are reported as percent normal hatch, that is the number of hatched larvae with normal morphology divided by the total number of embryos/larvae (unhatched embryos, dead hatched and live hatched). Total and normal hatching rates were observed to have the following trend: total hatching rates for embryos fertilized and incubated in 16 ppt > embryos fertilized in 16 ppt and incubated in 22 ppt > embryos fertilized in 22 ppt and incubated in 22 ppt > embryos fertilized in 30 ppt and incubated in 30 ppt > embryos fertilized in 16 ppt and incubated in 30 ppt (Figure 4-22). The same trend was obserbed for normal hatching, except that the hatching rate for embryos fertilized in 16 ppt and incubated in 30 ppt was greater than that for embryos fertilized in 30 ppt and incubated in 30 ppt.

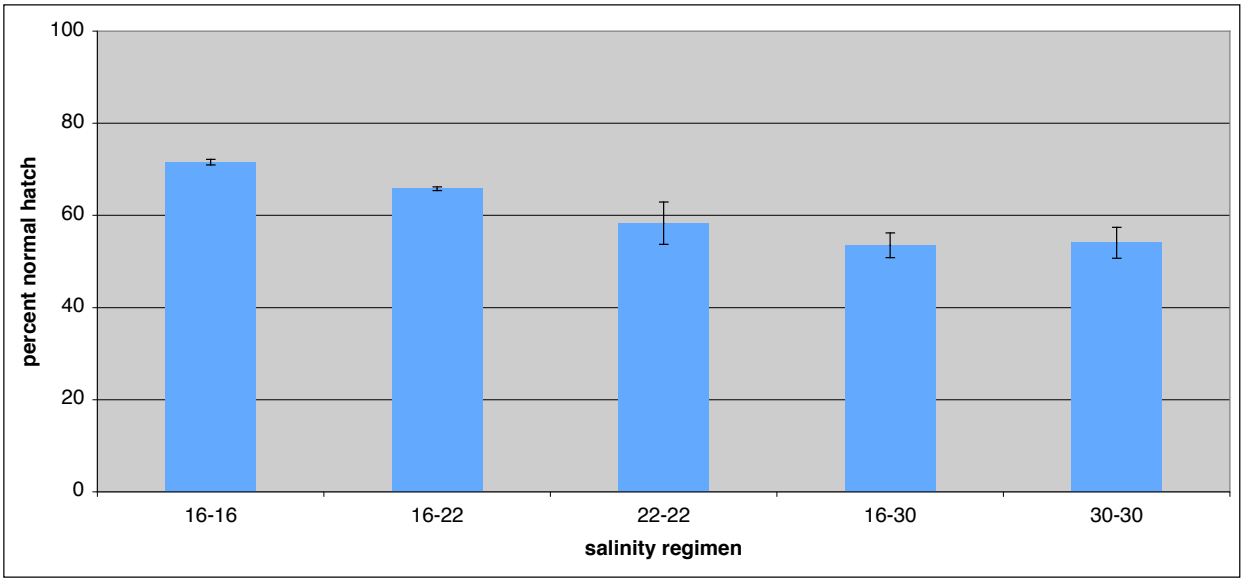


Figure 4-22: Normal hatch rates after fertilization and incubation under various salinity regimens. Data are mean ± SE for two replicates.

Morphological abnormalities were observed in all treatments (Figure 4-23). The incidence of edema was highest for embryos fertilized and incubated in 16 ppt seawater (3.08 +/- 2.57), and lowest for embryos fertilized and incubated in 22 ppt (0.97 +/- 0.31). In general, the incidence of other morphological abnormalities observed (opaque yolk sac, bent heads, scoliosis, and jaw abnormalities) was similar for all treatments except for embryos fertilized and incubated in 30 ppt. Yolk opacity, bent heads, and scoliosis were higher in this treatment. Jaw abnormalities were highest in the embryos fertilized in 16 ppt and incubated at 22 ppt, and were lowest in the 16-30 and 30-30 treatment groups.

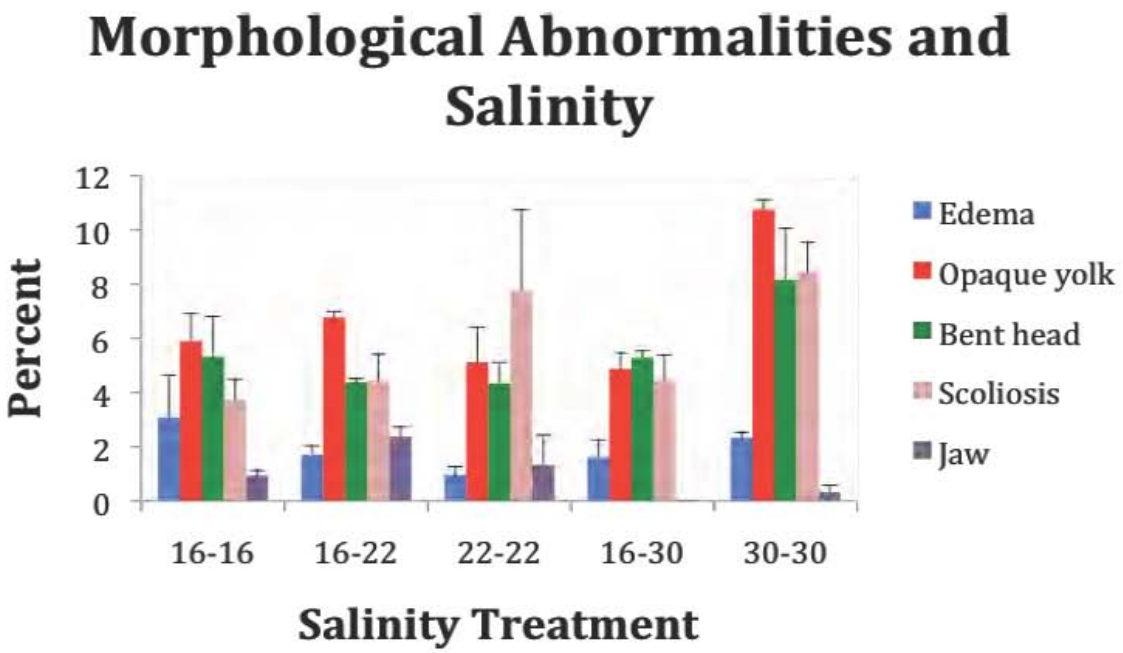


Figure 4-23: Morphological abnormalities in herring larvae fertilized at 16, 22, or 30 ppt and incubated at 16, 22 or 30 ppt. (Means +/- SE)

Section 5: Discussion

5.1 Field studies. Unlike *Exxon Valdez*, the *Cosco Busan* oil spill occurred within a highly urbanized estuary with multiple inputs of petroleum hydrocarbons. This posed the difficult challenge of assessing the ecological impacts of the spill against a backdrop of pollution in San Francisco Bay. Based on years of oil toxicity research in the years since *Exxon Valdez*, we anticipated lingering oil toxicity, if any, would be evident as a small increase in the detection of sublethal cardiac effects (arrhythmia, edema) in herring embryos incubated near oiled shorelines. While significant increases in bradycardia and pericardial edema were observed in caged embryos from oiled sites relative to unoiled locations, natural spawn from oiled intertidal zones revealed an unexpectedly severe (i.e., lethal) form of developmental toxicity.

The high rate of natural spawn mortality at oiled sites in 2008 does not appear attributable to natural causes or anthropogenic causes unrelated to the spill. The spawning layers were not dense enough (< 4 layers at all sites) to cause the density-dependent hypoxia that occurs when eggs are deposited in layers of greater than eight eggs thick [18-22]. Euryhaline Pacific herring embryos develop normally at salinities of 8-28‰ [12,16], and even suboptimal salinities would not be expected to cause acute mortality late in development. Natural spawn did not show evidence of accelerated development, as would be expected following exposures to high, potentially lethal temperatures [16, 83]. Coating of herring eggs with fine sediments does not produce the late developmental mortality we observed here [30-34, 84]. Two sewage spills occurred during the 2008 spawning season; on 13 January in Richardson Bay, near oiled sites SA and PP, and 14 February 2008 offshore of San Quentin Prison, near reference site PSQ. However, the available evidence indicates that sewage (i.e., concentrated sludge) is not acutely lethal to herring embryos [35]. Finally, background PCB and DDT levels in ovaries and embryos from San Francisco Bay are not expected to cause the observed mortalities, as the levels were much lower than that associated with reduced hatching success in Baltic herring [85].

Our chemical analyses of PAHs in embryos and PEMDs support the conclusion that embryos from oiled sites were exposed to oil, particularly at Keil Cove (KC), even though a PAH "fingerprint" of *Cosco Busan* oil was not discernable against the background of urban PAH inputs. On the other hand, the PEMD data indicate unique patterns of PAH inputs across sites, consistent with the diversity of proximal land use patterns and vessel activities. However, embryonic phenotype did not follow this pattern of site-specific chemical variation. In contrast, the effects observed in natural spawn from each of the three oiled sites (SA, PP, KC) were indistinguishable, and pericardial edema was observed only in larvae that were incubated subtidally at oiled sites. The only common feature linking these sites was a shoreline presence of visible oil in the weeks following the spill. The absence of these effects at reference sites and the marked recovery at oiled sites by 2010 indicate that background urban inputs of contaminants (e.g. via stormwater) are not likely causal. The most parsimonious explanation is that the 2008 herring embryo mortality in San Francisco Bay was caused by exposure to *Cosco Busan* oil.

PAHs in the tissues of embryos collected from oiled intertidal sites in 2008 were below levels that would be expected to cause acute lethality based on laboratory studies [38,62]. This, together with the dramatic difference in survival between intertidal spawn and embryos in nearby subtidal cages, implicates natural sunlight as a contributing factor in the observed embryolarval toxicity. Sequential exposures to crude oil and sunlight in the laboratory are acutely lethal in herring larvae [93]. This presumably occurs via activation of PAHs or other oil components by ultraviolet radiation ("phototoxicity"), thereby generating reactive oxygen species that cause membrane damage [87]. Recent work using zebrafish has further shown that bunker oils have a much higher phototoxic potential than crude oil and cause an acutely lethal cellular necrosis when embryos are exposed sequentially to oil and sunlight [97]. Lastly, the

2009 laboratory studies to investigate the potential role of sunlight in the observed 2008 natural spawn mortality showed that *Cosco Busan* bunker oil contains a phototoxic activity that (1) produces late embryonic mortality in herring embryos characterized by a loss of tissue integrity similar to that observed in the 2008 field-collected samples, (2) is resistant to weathering, and (3) is unexplained by tissue PAH concentrations, thus suggesting a causal role for one or more unmeasured compounds. Because modern bunker fuels contain the concentrated residuum of the crude oil refining process, they have much higher relative levels of many compounds, including the uncharacterized polar compounds that make up an "unresolved complex mixture" [86]. The most parsimonious explanation for our collective findings is that an uncharacterized and slowly weathering component of *Cosco Busan* bunker oil accumulated in natural spawn and then interacted with sunlight during low tides to produce lethal phototoxicity. Embryos in nearby cages, submerged beneath ~ 1 m or more of highly turbid San Francisco Bay water, exhibited canonical oil toxicity (i.e. bradycardia and pericardial edema) with no indication of a sunlight interaction.

Research following the Exxon Valdez oil spill established a new paradigm for oil toxicity to fish at early life stages, with a central role for PAHs. Our ecological assessments of the *Cosco Busan* spill have extended this and reinforced 1) the importance of oil composition (i.e., crude vs. bunker), 2) the significance of combinatorial stressors (i.e., oil and sunlight), 3) the current limitations of tissue PAH chemistry as a predictor of embryo toxicity, 4) the need to toxicologically characterize the non-PAH components of refined fuels, and 5) the exceptional vulnerability of fish early life stages to spilled oil.

5.2 Laboratory studies. Based on the results of the oiled gravel column phototoxicity studies, we conclude that below a certain dose of UV radiation, *Cosco Busan* bunker oil produces canonical and sublethal petrogenic PAH cardiotoxicity. These effects were significant at aqueous total PAH concentrations of 0.5 ppb, from very lightly oiled gravel (0.3 g/kg) – i.e., unlikely to be characterized as visibly oiled in a post-spill shoreline survey. Thus, in the absence of sunlight, *Cosco Busan* bunker oil toxicity resembles previous observations of crude oil toxicity that is largely attributable to the tricyclic PAHs such as the phenanthrenes. Consistent with this, ANSCO exposure produced a lower incidence of pericardial edema than a *Cosco Busan* bunker oil exposure that, while mass equivalent, contained a 2.3-fold higher tricyclic PAH content. This supports an interpretation of findings of petrogenic cardiotoxic bradycardia and edema in caged embryos incubated in the turbid subtidal zones at oiled sites. In sharp contrast, in the presence of natural sunlight, *Cosco Busan* bunker oil produced a novel form of lethal toxicity that is not predictable based on the known toxicity of an equivalent mass loading of unrefined crude oil.

Exposures to UV in natural sunlight were both necessary and sufficient to activate a phototoxic potential in CB *Cosco Busan* bunker oil BO and cause abrupt late-embryonic mortality in early life stage herring. This toxic etiology is very similar to that recently reported for zebrafish embryos exposed to bunker oils [97], and it persisted relatively unabated after two months of column weathering. These findings, together with the strikingly similar condition of naturally-spawned herring embryos sampled four months following the *Cosco Busan* spill, support the conclusion that embryos from oiled intertidal locations in San Francisco Bay succumbed to *Cosco Busan* bunker oil-induced phototoxicity.

5.3 Alternative hypotheses. Several other hypotheses have been discussed above, i.e. hypoxia, suboptimal salinity, sewage spills, suspended sediment. The only other alternative hypothesis remaining is that poor or immature maternal condition resulted in low quality eggs that, coupled with suboptimal salinity, resulted in high mortality at oiled sites in 2008. Several lines of evidence discredit this hypothesis. First, the natural spawn deposition that occurred in 2008 was a continuous "wave" that started at reference site SRB and propagated west continuously over the next several days. It is highly unlikely that there was a distinct

subpopulation of females in poor condition that spawned only at the oiled sites and not at the reference site. More importantly, there is not a plausible biological mechanism that links poor maternal condition (or suboptimal salinity) to the type of acute, necrotic mortality observed at oiled sites. These embryos apparently developed normally up to the hatching stage, then succumbed to an acute insult. While not studied directly in herring, poor maternal condition in fish generally leads to smaller eggs that produce smaller but morphologically normal larvae. This is well established in reef fish that deposit demersal eggs, similar to herring, and in ovoviviparous rockfish [98-101]. In other situations, poor egg quality has been shown to result in very early embryonic defects, e.g. abnormal cleavage or gastrulation [102,103]. This would result in pre-hatch embryos with true developmental defects (e.g. abnormal patterning of tissues), rather than sudden acute mortality of normal formed embryos.

The combination of air, sunlight and elevated temperature has also been suggested as a possible etiology for embryo lethality observed in 2008 natural spawn from the intertidal zone. However, several lines of evidence are also inconsistent with this hypothesis. The tidal and weather conditions during the 2008 spawning events and field sampling do not support the likelihood of high temperature shock to intertidal spawn. The low tides during the incubation period (Feb 20-26, 2008) were in the morning and late afternoon, and lowest tide was +1 ft, indicating that the eggs would not have been exposed to peak daytime air temperatures. Low tides during collection (Feb 27-29, 2008), were late morning to mid-day, but all samples were collected from below the water surface, and none were exposed to air. In San Francisco the highest recorded daytime air temperature during the incubation period was 19°C and average temperatures were in the range of 12-13°C. These conditions were actually matched closely during some of the oiled gravel column studies, which demonstrated that temperature elevation alone could not reproduce late embryonic lethality. In contrast, the 2009 laboratory studies demonstrated a clear mechanism by which *Cosco Busan* bunker oil produces acute, late stage mortality through phototoxicity.

Section 6: Summary and Conclusions

- Incubation of caged herring embryos in the subtidal zone at oiled sites 3 months following the spill resulted in signs of canonical petrogenic PAH sublethal toxicity, characterized by reduced heart rate and pericardial edema.
- Natural spawn deposited in the intertidal zones of oiled sites 3 months after the spill showed near complete mortality, characterized by acute necrosis of late-stage embryos (near hatching).
- Signs of sublethal oil cardiotoxicity and acute late-stage mortality were absent at reference sites 3 months following the spill and at urban reference sites 15 month and 27 months following the spill.
- Acute late-stage necrotic mortality was absent in natural spawn at re-sampled oiled site intertidal zones 27 months following the spill.
- Forensic analytical chemistry focusing on PAHs showed very low levels (near detection limits) in both caged embryos (17-52 ppb) and natural spawn (18-81 ppb) from all sites.
- Low levels coupled with high variability weakened standard comparative statistics for PAH data, but other methods support the presence of a petrogenic signal in embryos and PEMDS above background at oiled sites.
- Increased petrogenic input at the most heavily oiled site, Keil Cove, was also supported by PEMD data.
- Consistent with the persistent elevation of pericardial edema at Keil Cove, a petrogenic signal remained elevated in embryos from this heavily oiled site 27 months after the spill.
- Under conditions of reduced UV exposure, Cosco Busan bunker oil produced canonical oil cardiotoxicity with a lowest effective tissue concentration in the range of 30-75 ppb.
- Under conditions of normal UV exposure (i.e. unblocked), Cosco Busan bunker oil is both necessary and sufficient to cause an acute phototoxic response characterized by loss of tissue integrity (necrosis) in late stage herring embryos.
- Cosco Busan bunker oil remained highly phototoxic even after 2 months of weathering in oiled gravel columns.

These findings support the following conclusions: First, biological indicators such as herring embryos appear to be more sensitive for detecting oil-related adverse affects than current methods in analytical chemistry used for quantification of PAHs. Thus, an increased incidence of pericardial edema was detected in embryos incubated in the subtidal zone at oiled sites, despite lack of a clear chemical measure of exposure. The laboratory studies indicate that oil-induced pericardial edema occurs near and below the detection limits for tissue PAHs. Despite the inability to "fingerprint" Cosco Busan oil in embryos or PEMDs at all oiled sites, the highly consistent lethal phenotype observed in natural spawn indicates a common exposure at all oiled sites. The PEMD data indicate that each site has unique urban/maritime inputs of PAHs, therefore, an urban source cannot be the common exposure. Similarly, differences in salinity or temperature, potential exposure to sewage effluent, and other factors were not consistent among all the oiled sites. The one common factor to these sites was the presence of Cosco Busan oil detected by SCAT surveys. Therefore, the most parsimonious explanation for the collective findings is that an uncharacterized (i.e. non-PAH) and slowly weathering component of Cosco Busan bunker oil accumulated in natural spawn and then interacted with sunlight during low tides to produce lethal phototoxicity. Embryos in nearby cages, shielded by highly turbid San Francisco Bay water, exhibited canonical oil toxicity (i.e. bradycardia and pericardial edema) with no indication of a sunlight interaction. Recovery at oiled sites evident in 2010 natural spawn sampling are consistent with eventual loss of Cosco Busan oil toxicity with prolonged weathering, and indicate that other continuous urban background stressors were not the cause of sublethal or lethal toxicity in 2008.

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Section 7: Attachments

7.1 Summary of samples collected and data files

2008

Caged embryo outplant 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.

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.

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.

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.

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.

2009

Raw data files:

Laboratory studies:

Trial 1 (Preliminary)

Sample Code Key: N/A
Egg Source Information: CBOS09 fem wts.xls**,** fertilization_tests.pdf (in folder “Data files and lab notes”)
Experimental Conditions: column temp log Jan-Mar09.xls, ColumnWQJan14toFeb23.pdf (in folder “Data files and lab notes”)
Embryo Images: in folders “Data files and lab notes”>”Trial 1”> “5 dpf”, “6 dpf”, and “8 dpf”

Trial 2 (Aborted)

Sample Code Key: N/A
Egg Source Information: CBOS09 fem wts.xls**,** fertilization_tests.pdf (in folder "Data files and lab notes")
Experimental Conditions: column temp log Jan-Mar09.xls, ColumnWQJan14toFeb23.pdf (in folder "Data files and lab notes")
Embryo Images and scoring: in folders "Data files and lab notes">"Trial 2"; "Data files and lab notes"> Embryo Scores Final.xls (Trial 2 tab)

Trial 3

Sample Code Key: "Data files and lab notes">"Trial 3">Trial 3 key to egg chemistry.doc
Egg Source Information: CBOS09 fem wts.xls**,** fertilization_tests.pdf (in folder "Data files and lab notes")
Experimental Conditions: column temp log Jan-Mar09.xls, WaterQuality022609.xls (in folder "Data files and lab notes")
Embryo Images and Scoring Results: in folders "Data files and lab notes">"Trial 3"; "Data files and lab notes"> Embryo Scores Final.xls (Trial 3 tab)
Water Sample PAH Analysis: in folders "Data files and lab notes">"Trial 3">Trial 3 water PAH.xls
Eggs Sample PAH Analysis: in folders "Data files and lab notes">"Trial 3">Trial 3 tissue PAH.xls

Trial 4

Sample Code Key: "Data files and lab notes">"Trial 4">Trial 4 key to egg chemistry.doc
Egg Source Information: CBOS09 fem wts.xls**,** fertilization_tests.pdf (in folder "Data files and lab notes")
Experimental Conditions: column temp log 031809.xls, WaterQuality031809.xls (in folder "Data files and lab notes")
Embryo Images and Scoring Results: in folders "Data files and lab notes">"Trial 4"; "Data files and lab notes"> Embryo Scores Final.xls (Trial 4 tab)
Water Sample PAH Analysis: in folders "Data files and lab notes">"Trial 4">Trial 4 water PAH.xls
Eggs Sample PAH Analysis: in folders "Data files and lab notes">"Trial 4">Trial 4 tissue PAH.xls

Laboratory salinity study:

Egg Source Information: CBOS09 fem wts.xls**,** fertilization_tests.pdf (in folder "Data files and lab notes")
Experimental Conditions: in folder "Work plans and SOPs">CBOS Salinity Experiments.doc
Embryo Images and Scoring Results: in folders "2008-09 Salinity study">"16-16-16 ppt" and "30-30-16 ppt", data files CBOS Salinity Study embryo results.doc and CBOS salinity hatch data 09.xls

Natural spawn sampling:

Single site sampled, Paradise Cove
A. Image and video datasets for 50 embryos each for 8 sub-transects.
B. Fixed embryos from all imaged specimens for all sites (for CYP1A immunofluorescence).

C. Frozen 3 g samples of embryos for PAH quantification from each sub-transect.
Data files:
•CBOS external drive: Hatch data:2009:MRS-003b-FEM2 natural spawn 09.xlsx
•New MRS-003b-FEM2 natural spawn 09_v2.xls
•CBOS external drive:CBOS:CBOS images:Paradise natural spawn 1-29-09

2010
Natural spawn samples:

A. Image and video datasets for 3 random subsamples from each of 8 sub-transects of natural spawn on algae substrate. Sausalito site had spawn at only 3 of the 8 original coordinates (27 datasets total, Keil Cove, Peninsula Point, Paradise Cove, 8 each).
B. Frozen 3 g samples of embryos for PAH quantification from 27 samples.
C. Larval hatch data for 27 sub-samples of each natural spawn collection