

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

Draft Proposal
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Introduction:

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

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

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

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

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

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

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

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

Background:

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

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

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

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

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

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

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

Specific Aims:

We propose the following Specific Aims:

1. Monitor the in situ exposure of herring embryos to PAHs at sites with varying degrees of oiling.
2. Assess the toxicological response of herring embryos over a range of PAH exposures in the field.
3. Characterize the toxicity of *Cosco Busan* oil to herring embryos under controlled laboratory exposure conditions.
4. Estimate the loss of individual herring from the San Francisco Bay population due to both lethal and sublethal impacts of oil.

Injury Assessment Study Design:

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

Rationale:

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

Approach:

1.1 Field collection of naturally spawned herring eggs.

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

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

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

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

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

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

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

1.3 In situ deployment of passive sampling devices

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

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

1.4 Collection of sediments

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

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

Should herring spawning sites occur at depths below 20 m, sediments will be collected from boats using a Van Veen grab sampler (0.1m²), or a smaller Ponar-type sediment grab, with three grab events occurring at this depth. The top 2 cm of sediment will be collected from the surface of the grab into a pre-rinsed stainless steel bowl, using a pre-rinsed stainless steel spoon, the contents homogenized for 1 min, and then a portion of this total placed into a single pre-rinsed and pre-labelled 250 ml glass jar (as in an IChem jar). All sediment samples will be frozen within 3 hrs. of collection for storage until analysis.

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

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

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

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

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

1.6 Analyses of CYP1A induction in herring embryos.

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

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

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

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

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

Rationale:

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

Approach:

2.1 Field collection of naturally spawned herring eggs.

Naturally spawned eggs will be collected and transported as described above in Aim 1.1. Studies of Atlantic herring [30] and Pacific herring [14] indicate that the first cardiac contractions begin in the range of 40-48 accumulated thermal units (days post fertilization x temp °C).

Temperatures in the San Francisco Bay during Dec-Jan are in the range of 12°C [http://sfbay.wr.usgs.gov/sediment/cont_monitoring/index.html], so cardiac contraction in naturally spawned embryos is anticipated to begin as early as three days post-fertilization. Spawning sites will be monitored daily for new spawn, and embryonic staging will be assessed in the field by observation of embryos fixed with Stockard's solution. Sites with cleavage-stage embryos will be resampled daily, and once embryos reach the organogenesis stages with a functional heart, subsamples will be transported daily through hatching stages (estimated 10 dpf) for laboratory assessment (see below).

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

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

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

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

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

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

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

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

Rationale:

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

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

Approach:

3.1 Construction of oiled gravel columns.

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

3.2 Exposure of herring embryos to oiled rock column effluent.

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

3.3 Chemical analyses of PAHs in oiled rock column effluent.

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

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

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

Budget:

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

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

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

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

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

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

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

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

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

References

- ¹ Kocan, R.M., Hose, J. E., Brown, E.D., and Baker, T.T., Pacific herring (*Clupea pallasii*) embryo sensitivity to Prudhoe Bay petroleum hydrocarbons: Laboratory evaluation and in situ exposure at oiled and unoled sites in Prince William Sound. *Can. J. Fish. Aquat. Sci.* **53**, 2366-2387 (1996).
- ² Marty, G. D., Short, J. W., Dambach, D. M., Willits, N. H., Heintz, R. A., Rice, S. D., Stegeman, J. J., and Hinton, D. E., Ascites, premature emergence, increased gonadal cell apoptosis, and cytochrome P4501A induction in pink salmon larvae continuously exposed to oil-contaminated gravel during development. *Can. J. Zool.-Rev. Can. Zool.* **75** (6), 989-1007 (1997).
- ³ Carls, M. G., Rice, S. D., and Hose, J. E., Sensitivity of fish embryos to weathered crude oil: Part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasii*). *Environ. Toxicol. Chem.* **18** (3), 481-493 (1999).
- ⁴ Heintz, R. A., Short, J. W., and Rice, S. D., Sensitivity of fish embryos to weathered crude oil: Part II. Increased mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos incubating downstream from weathered Exxon Valdez crude oil. *Environ. Toxicol. Chem.* **18** (3), 494-503 (1999).
- ⁵ Couillard, C. M., A microscale test to measure petroleum oil toxicity to mummichog embryos. *Environ. Toxicol.* **17** (3), 195-202 (2002).
- ⁶ Pollino, C. A. and Holdway, D. A., Toxicity testing of crude oil and related compounds using early life stages of the crimson-spotted rainbowfish (*Melanotaenia fluviatilis*). *Ecotox. Environ. Safe.* **52** (3), 180-189 (2002).
- ⁷ Rhodes, S., Farwell, A., Hewitt, L. M., MacKinnon, M., and Dixon, D. G., The effects of dimethylated and alkylated polycyclic aromatic hydrocarbons on the embryonic development of the Japanese medaka. *Ecotox. Environ. Safe.* **60** (3), 247-258 (2005).
- ⁸ Vines, C. A., Robbins, T., Griffin, F. J., and Cherr, G. N., The effects of diffusible creosote-derived compounds on development in Pacific herring (*Clupea pallasii*). *Aquat. Toxicol.* **51** (2), 225-239 (2000).
- ⁹ Heintz, R. A., Rice, S. D., Wertheimer, A. C., Bradshaw, R. F., Thrower, F. P., Joyce, J. E., and Short, J. W., Delayed effects on growth and marine survival of pink salmon *Oncorhynchus gorbuscha* after exposure to crude oil during embryonic development. *Mar. Ecol. Prog. Ser.* **208**, 205-216 (2000).
- ¹⁰ Incardona, J. P., Carls, M. G., Teraoka, H., Sloan, C. A., Collier, T. K., and Scholz, N. L., Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development. *Environ. Health Perspect.* **113**, 1755-1762 (2005).

- 11 Incardona, J. P., Collier, T. K., and Scholz, N. L., Defects in cardiac function precede
morphological abnormalities in fish embryos exposed to polycyclic aromatic
hydrocarbons. *Toxicol. Appl. Pharmacol.* **196** (2), 191-205 (2004).
- 12 Incardona, J. P., Day, H. L., Collier, T. K., and Scholz, N. L., Developmental toxicity of
4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH
receptor isoforms and hepatic cytochrome P450 1A metabolism. *Toxicol. Appl.*
Pharmacol. **217**, 308-321 (2006).
- 13 Khan, C. W., Saravanabhavan, G., Clarke, L. M. J., Hollebone, B. P., Brown, R. S.,
Wang, Z., Short, J., Lee, K., King, T., and Hodson, P. V., in *SETAC North America 28th*
Annual Meeting (Milwaukee, WI, 2007).
- 14 Incardona, J. P., Mechanisms of petroleum hydrocarbon toxicity in fish early life history
stages. Final Report FY2006 for grant from Oiled Wildlife Care Network/CA Dept of
Fish and Game (2007).
- 15 Carls, M. G., Heintz, R. A., Marty, G. D., and Rice, S. D., Cytochrome P4501A induction
in oil-exposed pink salmon *Oncorhynchus gorbuscha* embryos predicts reduced survival
potential. *Mar. Ecol. Prog. Ser.* **301**, 253-265 (2005).
- 16 Aas, E., Baussant, T., Balk, L., Liewenborg, B., and Andersen, O. K., PAH metabolites in
bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic
oil exposure: a laboratory experiment with Atlantic cod. *Aquat. Toxicol.* **51** (2), 241-258
(2000).
- 17 Wassenberg, D. M., Swails, E. E., and Di Giulio, Richard T., Effects of single and
combined exposures to benzo(a)pyrene and 3,3',4,4',5-pentachlorobiphenyl on EROD
activity and development in *Fundulus heteroclitus*. *Mar. Environ. Res.* **54** (3-5), 279-283
(2002).
- 18 Zabel, E. W., Cook, P. M., and Peterson, R. d E., Potency of 3,3',4,4',5-
pentachlorobiphenyl (PCB 126), alone and in combination with 2,3,7,8-
tetrachlorodibenzo-p-dioxin (TCDD), to produce lake trout early life-stage mortality.
Environ. Toxicol. Chem. **14** (12), 2175-2179 (1995).
- 19 Oros, D. R., Ross, J. R. M., Spies, R. B., and Mumley, T., Polycyclic aromatic
hydrocarbon (PAH) contamination in San Francisco Bay: A 10-year retrospective of
monitoring in an urbanized estuary. *Environ. Res.* **105** (1), 101-118 (2007).
- 20 Oros, D. R. and Ross, J. R. M., Polycyclic aromatic hydrocarbons in San Francisco
Estuary sediments. *Marine Chemistry* **86** (3-4), 169-184 (2004).
- 21 Ross, J. R. M. and Oros, D. R., Polycyclic aromatic hydrocarbons in the San Francisco
Estuary water column: Sources, spatial distributions, and temporal trends (1993-2001).
Chemosphere **57** (8), 909-920 (2004).
- 22 Hershberger, P. K., Elder, N. E., Wittouck, J., Stick, K., and Kocan, R. M., Abnormalities
in larvae from the once-largest Pacific herring population in Washington State result
primarily from factors independent of spawning location. *Trans. Am. Fish. Soc.* **134** (2),
326-337 (2005).
- 23 Carls, M. G., Holland, L. G., Short, J. W., Heintz, R. A., and Rice, S. D., Monitoring
polynuclear aromatic hydrocarbons in aqueous environments with passive low-density
polyethylene membrane devices. *Environ. Toxicol. Chem.* **23** (6), 1416-1424 (2004).
- 24 Moles, A., Holland, L., and Andersson, O., Assessment of the significance of direct and
indirect pollution inputs to a major salmon-producing river using polyethylene membrane
devices. *Environ. Toxicol. Chem.* **25** (8), 2011-2017 (2006).

- 25 Sloan, C. A., Brown, D. W., Pearce, R. W., Boyer, R. H., Bolton, J. L., Burrows, D. G.,
Herman, D. P., and Krahn, M. M., in *Techniques in Aquatic Toxicology*, edited by G. K.
Ostrander (CRC Press, Boca Raton, FL, 2005), Vol. 2, pp. 631-651.
- 26 Ylitalo, G. M., Yanagida, G. K., Hufnagle, L., and Krahn, M. M., in *Techniques in*
Aquatic Toxicology, edited by G. K. Ostrander (CRC Press, Boca Raton, FL, 2005), Vol.
2, pp. 227-237.
- 27 Sloan, C. A., Brown, D. W., Pearce, R. W., Boyer, R. H., Bolton, J. L., Burrows, D. G.,
P., Herman D., and Krahn, M. M., Report No. NMFS-NWFSC-59, 2004.
- 28 Carls, M. G., Hose, J. E., Thomas, R. E., and Rice, S. D., Exposure of Pacific herring to
weathered crude oil: Assessing effects on ova. *Environ. Toxicol. Chem.* **19** (6), 1649-
1659 (2000).
- 29 Krahn, M. M., Burrows, D. G., Ylitalo, G. M., Brown, D. W., Wigren, C. A., Collier, T.
K., Chan, S. L., and Varanasi, U., Mass Spectrometric Analysis For Aromatic
Compounds in Bile of Fish Sampled After the Exxon Valdez Oil Spill. *Environ. Sci.*
Technol. **26** (1), 116-126 (1992).
- 30 Hill, J. and Johnston, I. A., Photomicrographic atlas of Atlantic herring embryonic
development. *Journal of Fish Biology* **51** (5), 960-977 (1997).
- 31 Marty, G. D., Hose, J. E., McGurk, M. D., Brown, E. D., and Hinton, D. E.,
Histopathology and cytogenetic evaluation of Pacific herring larvae exposed to petroleum
hydrocarbons in the laboratory or in Prince William Sound, Alaska, after the Exxon
Valdez oil spill. *Can. J. Fish. Aquat. Sci.* **54** (8), 1846-1857 (1997).
- 32 Brannon, E. L., Collins, K. M., Brown, J. S., Neff, J. M., Parker, K. R., and Stubblefield,
W. A., Toxicity of weathered Exxon Valdez crude oil to pink salmon embryos. *Environ.*
Toxicol. Chem. **25** (4), 962-972 (2006).