Framework for Assessment of Causal Relationships between Early Life Stage Developmental Anomalies of *Clupea pallasi* and *Cosco Busan* Oil

AN INTERPRETIVE SUMMARY OF 2007, 2008, 2009, AND 2010 DATASETS

JACK Q WORD, LUCINDA S WORD, SUSAN D WATTS, MEG R PINZA, TRACY L SCHUH

Submitted to:

Walter Pearson
Peapod Research

and

Gary Mauseth
Polaris Applied Sciences, Inc.
12525 - 131st Court NE
Kirkland, WA 98034

Submitted by:

NewFields
4729 NE View Drive
Port Gamble, WA 98364

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EXECUTIVE SUMMARY

A number of research and damage assessment studies were initiated following the Cosco Busan Oil spill (CBOS) in San Francisco Bay on 7 November 2007 culminating in a 2010 laboratory assessment of the effects of non-contaminant stressors. The integrated results from analytical analyses demonstrated a chemical signature of Cosco Busan oil (CBO) and also demonstrated that the signature was present in two of the water samples collected adjacent to areas that showed shoreline impact. The water samples were collected in November 2007; subsequently the beaches were cleaned of oil prior to the Pacific herring spawn in January – March 2008. Results from assessments of Pacific herring embryo development at sites potentially impacted by CBO showed that significant developmental abnormalities occurred in eggs spawned in the intertidal zone in 2008, but in the absence of a CBO chemical signature in the eggs. Following those initial assessments, follow-on work was performed to experimentally demonstrate the presence or absence of a CBO signature in developing herring eggs, specifically exposed to CBO, another source of oil (Exxon Valdez Oil Spill - EVOS), urban background petroleum concentrations and controls. Laboratory artifacts and field or laboratory variables were noted during review of data from 2008 field studies and the 2009 experimental study; these were primarily environmental stressors that appeared to be at least as significantly associated with the developmental abnormalities as CBO exposure. In 2010, these environmental variables were evaluated under experimental conditions designed to mimic the 2008 field conditions. This document draws on results from all CB studies to examine causal relationships between the CBOS in 2007 and its potential to exert adverse impact to early development stages in herring. The assessment framework (from Fox 1991; Tillitt et al. 2008) addresses six primary lines of evidence to determine the probable cause(s) of effects observed in 2008. Those lines of evidence are:

- Probability and Time Order – Did the oil spill occur prior to the spawning events and was there sufficient ability to identify CBO and to document its presence during the spawn events?

- Strength of Association – Is the CBO signature in the oil and water distinct from other sources of petroleum; is it also a distinct signature in eggs known to be exposed to CBO?

- Specificity - Are each of the biological responses solely associated with CBO or can other factors result in similar response patterns?

- Consistency of Association – Are similar effects observed in the absence of CBO?

- Predictive Performance – Are the observed biological responses predictable based on results from the scientific literature?

- Coherence – Were CBO chemical signatures observed in 2008? Were the effects predictable based on concentrations of CBO contaminants? Were biological effects observed in 2008 demonstrative of petroleum exposure or to other factors?

This interpretive report uses this causal inference framework to combine scientific data collected between 2007 through 2010 with effects-based literature summaries to arrive at the following conclusions:

- The Pacific herring eggs spawned in 2008 and collected from the intertidal areas characterized as being exposed to CBO (in 2007) were not exposed to CBO during their development. The chemical exposure signature in the developing eggs was consistent with urban background and burned wood or creosote and not with CBO. The developing eggs were instead exposed to urban San Francisco Bay PAH contamination (Peninsula Point and San Rafael) and/or urban Bay PAH contamination augmented by sources enriched with fluoranthene and pyrene that may have come from burned wood or creosote (Keil Cove and Sausalito).
- Experiments conducted with known CBO exposure in 2009 produced a diagnostic chemical signature of CBO which is distinctly different from ANS and urban sources of PAH exposure under either transmitted or blocked UV light and at all concentrations of CBO. The absence of the CBO chemical exposure signature in 2008 means that the developing eggs were not exposed to CBO and as a result were also not exposed to any of the measured or unmeasured chemical components contained in CBO.
- A distinct biological signature was developed during the 2009 and 2010 studies that separates the effects of petroleum contamination from other stressors. The biological signature associated with petroleum related effects is the incidence and severity of pericardial edema. Yolk sac edema incidence was demonstrated to occur with temperature and salinity stresses in the 2010 study at similar rates as observed in 2008. Pericardial edema was not present except in organisms that had extensive body axis defects in both the 2008 and 2010 studies. The presence of yolk sac edema, extreme body axis defects and early mortality in developing eggs observed in 2010 in the absence of petroleum was also observed in natural spawn events of 2008 and 2009. The absence of the petroleum related pericardial edema response combined with the incidence and intensity of yolk sac edema, extreme body axis defects and early mortality of developing eggs are all consistent with effects being created by factors other than CBO exposure.

This body of data demonstrates that CBO spilled in November 2007 did not create adverse effects on the development of Pacific herring embryos that were spawned three to five months later in the intertidal environment of central San Francisco Bay.

1 INTRODUCTION

The Cosco Busan struck the San Francisco Bay Bridge on 7 November 2007 and spilled approximately 54,000 gallons of bunker fuel oil into San Francisco Bay. The resource agencies responsible for determining the fate and effects of the spill for Natural Resource Damage Assessment (NRDA) evaluated many potential candidate species as surrogates within the central portion of San Francisco Bay. The principal agencies, NOAA and California Department of Fish and Game, selected Clupea pallasi (Pacific herring) as an ideal surrogate species for NRDA assessment. Herring occupy critical positions within regional food webs, have high potential for exposure to petroleum during their intertidal and shallow subtidal spawning and egg development processes, and have a high commercial value. Additionally, herring have been the subject of numerous detailed biological investigations performed with oil (Linden et al. 1978, 1980; Smith and Cameron 1979; Pearson et al. 1985, 1995, 1999; Carls et al. 1999, 2002). Since the spill event significant efforts have been expended to demonstrate that adverse effects of that spill disrupted the normal development of Pacific herring eggs during the spawning season following the spill. The primary studies evaluated in this document include the oil and water chemistry sampling and analyses, and the Shoreline Cleanup Assessment Team (SCAT) observations in 2007 after the spill event, the intertidal natural spawning assessments and the experimental subtidal programs conducted in 2008 by NOAA and BML (NOAA/BML 2008), the intertidal natural spawning assessment of Paradise Cove in 2009, the oil generation experiments conducted in 2009 at BML (Incardona and Vines 2009), the assessments performed by NewFields on data and images provided by NOAA and BML (NewFields 2009a, 2009b; 2010a), and the experimental testing program conducted at NewFields to evaluate various non-contaminant influences to early development of Pacific herring (NewFields 2010b). The objective of this report is to demonstrate the probable cause(s) of anomalies reported for naturally spawned herring larvae by identifying the corresponding unique chemical exposure (Section 2) and biological response (Section 3) signatures. We then use an ecoepidemiological framework based on criteria which are used to determine strength of evidence for causation of an effect by a putative causal agent (Fox 1991; Tillitt et al. 2008).

1.1 SUMMARY OF COSCO BUSAN OIL SPILL RELATED STUDIES

1.1.1 POST SPILL SAMPLING – 2007

SCAT observations immediately after the spill identified areas of oiled shoreline with qualitative assessments of the degree of oiling on each shoreline segment (shown in NOAA/BML 2008; Table 3-1). Approximately three weeks after the spill, water samples were collected from sites in the north central San Francisco Bay that had been identified with various degrees of oiling; Keil Cove, Sausalito, and Horseshoe Cove (Figure 1-1). Subsequent to the collection of water samples, beaches with observed oil were cleaned prior to the herring spawn period (NOAA/BML 2008; Table 3-1).

1.1.2 FIELD STUDY BY NOAA AND BML – 2008

Studies of the potential biological effects of the COSCO BUSAN oil spill on San Francisco Bay herring began in January 2008 and included examinations of embryos at oiled and reference areas as well as chemical analyses of herring eggs, PolyEthylene Membrane Devices (PEMDs), and sediments (NOAA/BML 2008).

The first segment of the 2008 studies placed artificially spawned herring eggs into cages that were then deployed in the shallow subtidal zones at 6 sites, two reference (SRB, PSQ, Figure 1-1) and 4 oiled (KC, PP, SA, HC, Figure 1-1). PEMDs were deployed with the cages. The PEMDs and sediments near the cages were collected for analyses of PAHs and persistent organic pollutants (POPs). After about 7 days of incubation and before hatching, the cages were recovered, and the eggs were subdivided to provide eggs for three efforts. First, artificially spawned herring eggs were analyzed for PAHs and POPs. Second, artificially spawned herring embryos were dechorionated for determination of heart beat, arrhythmia, and frequencies of morphological abnormalities. A third set of eggs recovered from the cages were incubated at 12°C and half-strength sea water (about 14 to 16 practical salinity units) for about 5 days until the eggs hatched. Hatched larvae were examined to measure the rate of hatching and the frequency of live normal larvae at hatch. The live normal hatch from all sites for the artificially spawned eggs was excellent and no adverse effects could be attributed to the CBO to eggs in the subtidal areas. The sediment from the areas adjacent to these subtidal cages did not have PAH distributions characteristic of CBO.

Figure 1-1. Locations of Study Sites within San Francisco Bay.

The second segment of the 2008 studies collected naturally spawned herring eggs on macro-algae (e.g., Fucus spp, Gracilaria spp) from intertidal areas (0 to 1 ft MLLW) and then incubated the eggs in the laboratory (SRB, KC, PP, SA, Figure 1-1). All sites had received herring spawn in recent years. The collected eggs were treated similarly to those from the artificially spawned larvae and divided for chemical analysis, examination via dechorionation of the embryo, and incubated until hatch under laboratory conditions.

There were two additional analyses subsequent to NOAA/BML (2008). The first performed QA assessment, fingerprint assessment, and statistical analysis of the chemistry data associated with the NOAA/BML study (Douglas 2009a, 2009b). The second examined a subset of digital imagery taken during the NOAA/BML study of artificially spawned and naturally spawned embryos (NewFields 2009a).

1.1.3 Laboratory Study by NOAA and BML – 2009

A series of laboratory experiments was conducted by NOAA and BML in 2009 to demonstrate that the effects reported in 2008 associated with relatively low PAH concentrations could be replicated under controlled laboratory conditions. The objective of the 2009 experiments was to demonstrate that CBO petroleum exposure to early life stages of herring would produce a similar pattern of effects as those reported by NOAA/BML from the 2008 studies. Additionally, the water and tissue samples exposed to

various oil treatments were chemically analyzed to evaluate whether exposure to CBO could be demonstrated.

The experimental procedures used for the 2009 experiments were adapted from studies using oil generator columns to investigate the effects of stranded oil on Pacific herring and pink salmon during the Exxon Valdez assessments (Marty et al. 1997; Carls et al. 1999; Heintz et al. 1999). The CBO treatments were created using a generator column packed with clean gravel and spiked with nominal concentrations of oil (0.1 g/kg, 0.3 g/kg and 1.0 g/kg). In addition, weathered Alaska North Slope petroleum (ANS) was included to provide a basis of comparison (positive control) to other published studies. Inclusion of ANS in the study design afforded an additional quality assurance step because any effects observed from exposure to ANS should be similar to effects reported for this oil by other researchers. In addition to the clean gravel control samples, gravel from an urban area of San Francisco Bay was also included in the study design.

The experimental design included two light conditions; one set of treatments was covered by Plexiglas™ that transmitted ultraviolet light (UVT), the other set was covered by Plexiglas™ that blocked ultraviolet light (UVB). NOAA focused on a subset of dechorionated embryos and evaluated viable eyed embryos and larval abnormalities such as body axis defects, and edema. BML focused on a larger data set and evaluated effects on post hatched larvae such as percent normal hatch, body axis defects, and edema.

NewFields conducted an independent review of the 2009 experiments. The data records produced by NOAA and BML and images for a subset of both eggs and larvae collected during the laboratory studies were delivered to NewFields for additional assessment (NewFields 2009b, 2010a).

1.1.4 Laboratory Study by NewFields – 2010

The results of the 2009 laboratory experiments demonstrated that CBO oil exposure provides an identifiable chemical signature in both water and egg tissues at all exposure concentrations even though the biological responses were not clearly related to CBO exposure in all cases; other identified factors and laboratory artifacts contributed to this discordance of results (NewFields 2010b). After an assessment of causal relationships suggested by the 2009 dataset, several potential causes to the observed 2008 effects were discounted (Pearson 2009), and only two hypotheses remained:

1) An unmeasured CBO component combined with ultraviolet (UV) radiation may have caused the decreased hatching success in 2008, and

2) A combination of environmental stressors including variable salinity, temperature, UV transmittance, and donor fish condition may have contributed to the effects.

The first hypothesis was also discounted during review of the 2009 experimental program which demonstrated that CBO exposure resulted in a distinct PAH chemical signature at all exposure concentrations and that this chemical signature was not present in any of the 2008 field collected organisms, the sediments, or the PEMDs. Without the demonstrated CBO chemical signature, unmeasured components of CBO cannot be present and cannot elicit a biological response. Subsequent to that review a laboratory study was designed to test the second hypothesis. To evaluate the potential contribution of environmental factors on disruptions to normal development of Pacific herring embryos, laboratory experiments were set up to mimic the actual fluctuating field conditions encountered during the 2008 spawning season. Two locations were selected within San Francisco Bay: Point San Quentin was chosen to represent average conditions in the north central part of the San Francisco Bay, and Peninsula Point was selected to represent conditions in the south central part of the bay. The experiment was conducted at the NewFields Environmental Laboratory at Port Gamble, WA. This facility was selected because it can provide clean seawater for continuous flow experiments and has the

technical capability to precisely achieve the variations in temperature and salinity conditions required for this experiment. Study results were reported separately (NewFields 2010b). An additional environmental stressor of suspended sediment identified by researchers at BML (Griffin et al. 2009) was not included in the experiment, but may also have contributed to abnormalities observed in 2008.

1.2 Establishing Chemical and Biological Signatures

When making a case for a NRDA assessment, it is necessary to work with retrospective risk assessment tools and an inverted logic tree of hypotheses rather than with the more straightforward statistical rigor built into well-controlled experimental studies. San Francisco Bay is an urban influenced marine environment with a history of environmental problems and a multiplicity of anthropogenic and environmental stressors to consider. Demonstrating that contaminants from a single source were related to observed biological effects requires the validation of these premises: 1) the source of exposure can be identified with unique chemical profiling, i.e. there is a chemical signature; and 2) a pattern of anomalies has a distinct feature, or biological response signature.

Specialized studies in 2009 (experimental oil exposures) and 2010 (nonchemical stressors) were implemented to directly address the relative influence of these potential stressors. Examination of the test results from each of these studies forms the basis of establishing possible cause and effect relationships to developing herring from the 2008 natural spawn. The review of these datasets provided information for the identification of chemical and biological signatures for known sources of PAH contamination (2009 experiment) and also in the absence of PAH contamination (2010 experiment) to demonstrate whether or not 2008 herring eggs were affected by CBO PAH exposure. A key component to this approach is the ability to distinguish a CBO PAH chemical signature that is distinct from other sources of petroleum including EVOS crude, urban background, and from clean sediment. A biological response signature for exposure to petroleum distinct from other stressors was also identified on the basis of information from the 2009 and 2010 experimental studies. These two sets of experiments provide the information necessary to distinguish biological responses associated with CBO and environmental stressors that were present in 2008 and/or were introduced as laboratory artifacts during the 2009 experiments.

1.3 Defining Probable Cause

A causal framework was used to examine the hypothesis central to establishing the link between CBO exposure and diminished health of the intertidal herring spawn in 2008; the hypothesis under examination is that the adverse effects to naturally spawned herring eggs collected in 2008 from the intertidal zone were caused by Cosco Busan oil. The criteria to be met fall into the following hierarchical arguments based on Tillitt et al. (2008) and Fox (1991):

- Probability and Time-Order: Did the aftermath of the 2007 CBO spill result in identifiable fuel oil concentrations at herring spawning locations in central San Francisco Bay in 2008?
- Strength of Association: Has it been shown that CBO fuel oil can produce a recognizable chemical signature and also a consistent pattern of adverse biological effects?
- Specificity: Is CBO the only causative agent to create this pattern of effects?
- Consistency of Association: Have the same cause-and-effect relationships for CBO occurred under different conditions?
- Predictive Performance: Does experimental evidence support the cause-and-effect relationship; is there a dose-relationship?
- Coherence: Were CBO concentrations in 2008 above known toxic thresholds?

The assessment framework is presented in Figure 1-2 and forms the basis of the discussion section (Section 4).

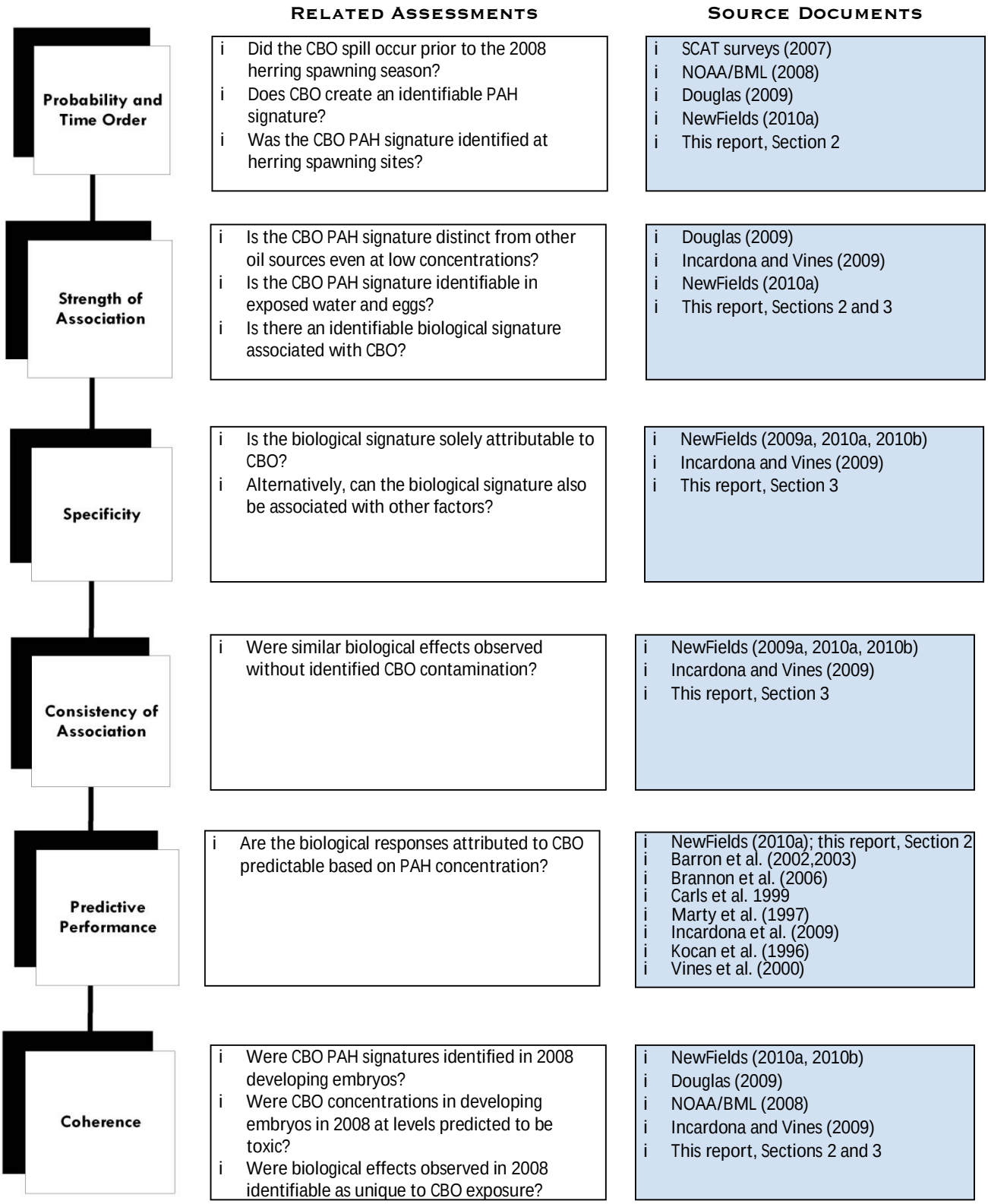


Figure 1-2. Assessment Framework for Establishing Cause-and-Effect Relationships for CBO Exposure.

2 DETERMINATION OF THE CBO CHEMICAL SIGNATURE AND DOSE RELATED ADVERSE EFFECTS

Demonstration of effects related to exposure to contaminants requires validation that the organisms were actually exposed to the contaminants of concern.

1) Matching contaminant fingerprints of biologically available components in both the petroleum source and the affected organisms, and
2) Contaminant concentrations comparable to known petroleum dose response relationships.

Responses exceeding known dose response relationships are due either to unknown compounds in the source or to other factors not associated with the source.

2.1 PETROLEUM EXPOSURE SIGNATURES

Exposure to complex mixtures of chemicals and the subsequent uptake into an organism's tissues provides evidence that an organism has been exposed to that complex mixture. Matching the relative concentrations of chemicals contained in the mixture that are transportable into water and subsequently into the tissues is an excellent method to demonstrate biological exposure to that mixture. In the course of the studies following the CBO spill, the relative concentrations of 39 PAHs and their alkylated homologs were measured in a number of environmental and experimental samples. The results of these analyses can be used to demonstrate whether or not the CBO from the spill did persist in San Francisco Bay and whether the herring spawn in 2008 were adversely affected by CBO. These samples and analyses are discussed in the following sections.

2.1.1 KNOWN PETROLEUM SOURCES: CBO AND ANS

CBO collected from Cosco Busan in 2007 and ANS (EVOS) oil archived from the Exxon Valdez were analyzed for PAHs and alkylated homologs by Alpha Analytical in Mansfield, Massachusetts. The PAH signature of the CBO mixture establishes the baseline for the demonstration of exposure and comparison of the CBO signature to the PAH signature of the ANS mixture demonstrates that two known source types can be distinguished. As demonstrated in Figure 2-1, CBO concentrations of total PAH were higher by approximately 3-fold than a sum of the same compounds in the ANS crude (27,700 compared to 9,100 mg PAH/kg oil). An additional diagnostic characteristic is the relative concentrations of the naphthalene and alkylated naphthalene homolog compounds compared to the chrysene homologs. ANS had more naphthalene than chrysene homologs when compared with the CBO (naphthalene/chrysene homolog ratios of 13 for ANS and 3 for CBO or a ~4-fold enrichment).

The relative concentrations of PAH in these two oil types show a distinctive chemical signature for each of these petroleum sources providing a point of comparison to environmental and biological samples collected during studies following the oil spill.

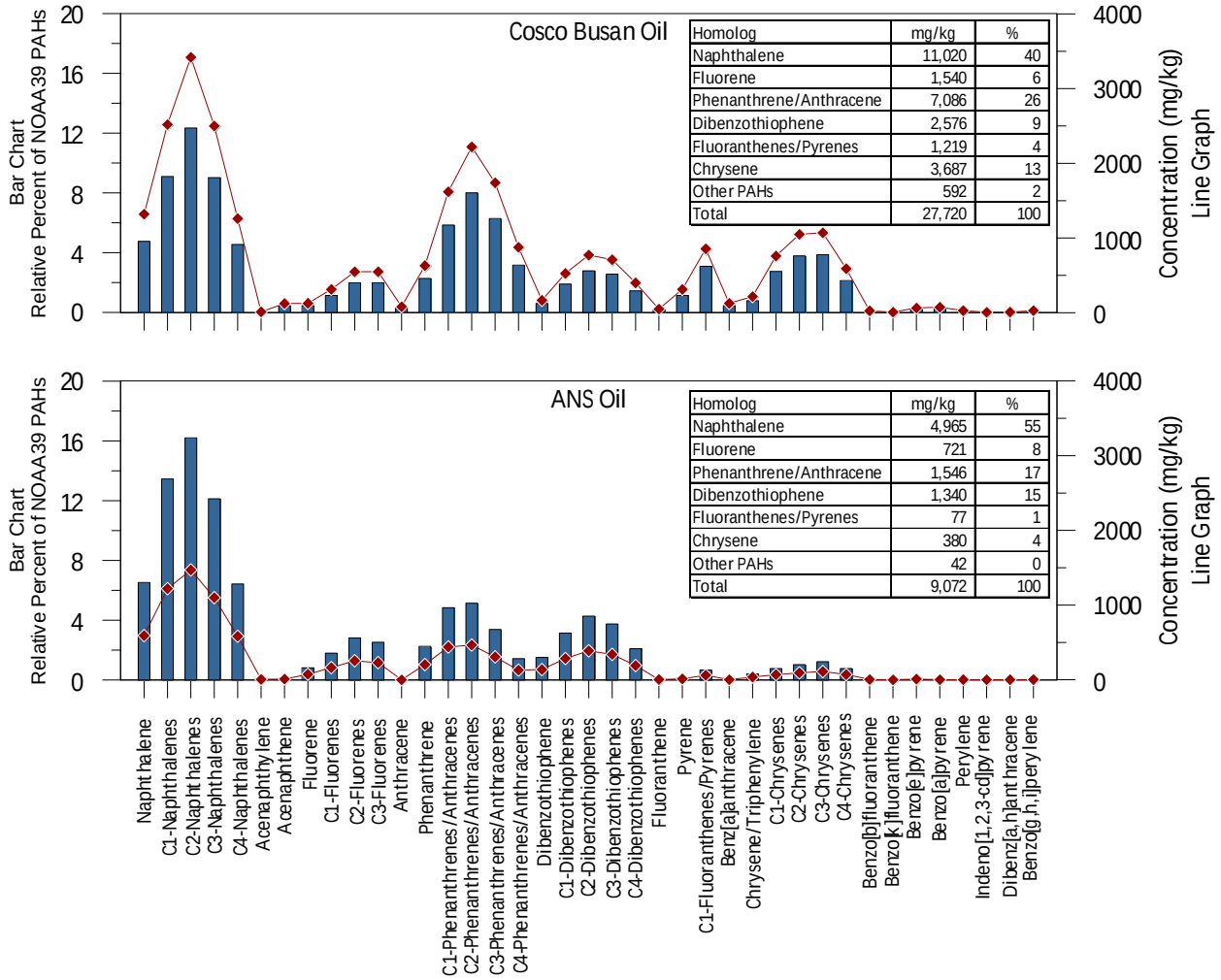


Figure 2-1. Relative and Actual Concentrations in Cosco Busan and ANS Oil.

2.1.2 Environmental Samples Collected After Oil Spill

Water samples collected subsequent to the CBO spill in 2007 but prior to the 2008 herring spawning events in were suspected to contain CBO oil mixtures. Samples were collected in the north central area of San Francisco Bay in areas where herring had previously spawned. These samples show that the chemical signature of PAH in some of the water samples collected after the spill event was similar to that of CBO, the signature matched most closely at Keil Cove (Figure 2-2). Sediment samples were collected in March 2008 adjacent to the caged samples of the 2008 field study. The sediment samples from the reference areas in San Rafael Bay and Point San Quentin were similar to the one from Horseshoe Cove, a site noted to have moderate shoreline oiling (NOAA/BML 2008); none of the sediment samples show the Cosco Busan signature as shown in Figure 2-3.

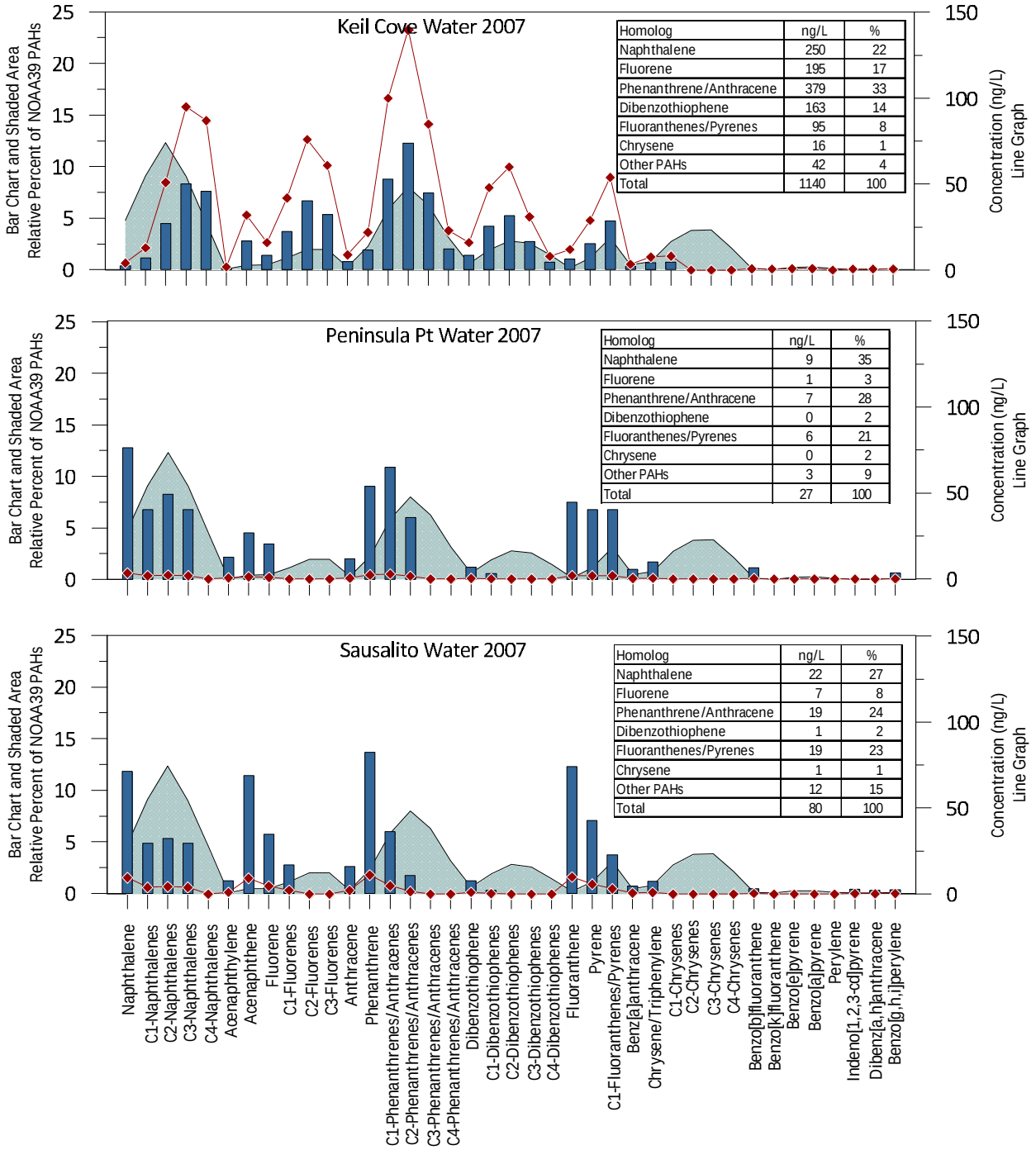


Figure 2-2. Relative and Actual Concentrations of PAHs in Water Samples Collected in 2007. Shaded areas show CBO signature.

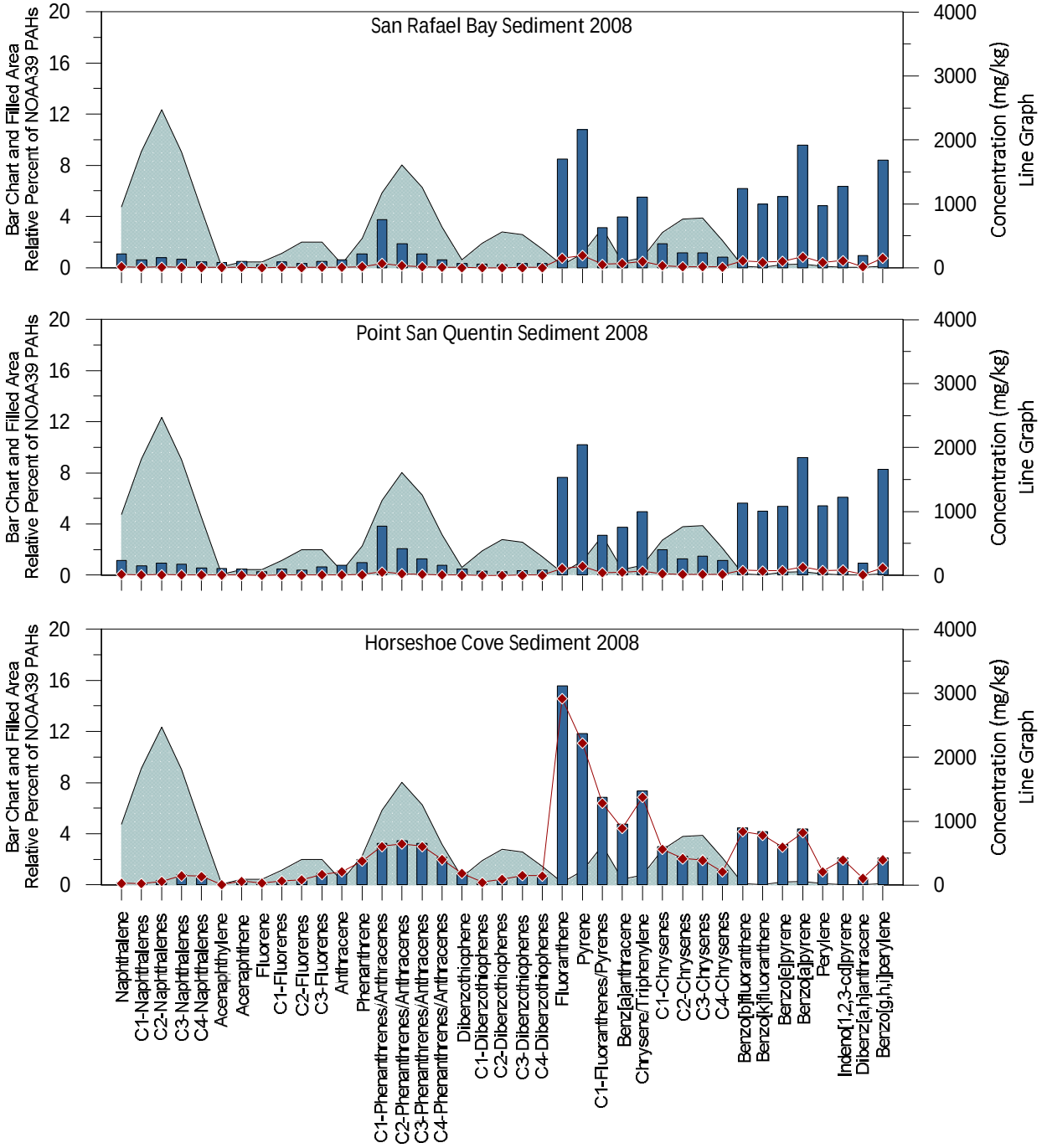


Figure 2-3. Relative and Actual Concentrations of PAHs in Sediment Samples.
Shaded area shows the CBO signature.

2.1.3 Biological Uptake by Known Sources of PAH Contaminants

The 2009 NOAA/BML laboratory study used oil from known sources for herring embryo exposure. CBO and ANS petroleum were spiked into columns of clean gravel at different concentrations and San Francisco Bay urban gravel was collected from intertidal sediment in north San Pablo Bay. These columns generated mixtures of PAH in the incubation water for Pacific herring eggs. PAH analyses were

made on water from the various spiked concentrations and on the eggs exposed to the oil via the incubation water. Comparing the results of these analyses demonstrates known sources of contamination produce unique PAH signatures into the water and those signatures are retained in eggs exposed to that contaminant source as shown in (Figure 2-4), regardless of the amount of UV exposure. CBO, ANS, Urban, and Clean PAH distributions in these experimental chambers are uniquely identified and demonstrate that exposure to CBO can be discerned under controlled experiments. Additionally, the signature of the oil in both the water and the egg tissue was evident in all three of the nominal doses of CBO (1.0 g/kg, 0.3 g/kg, and 0.1 g/kg). Figure 2-5 demonstrates that CBO exposure, even at a low dose, will absorb into developing eggs.

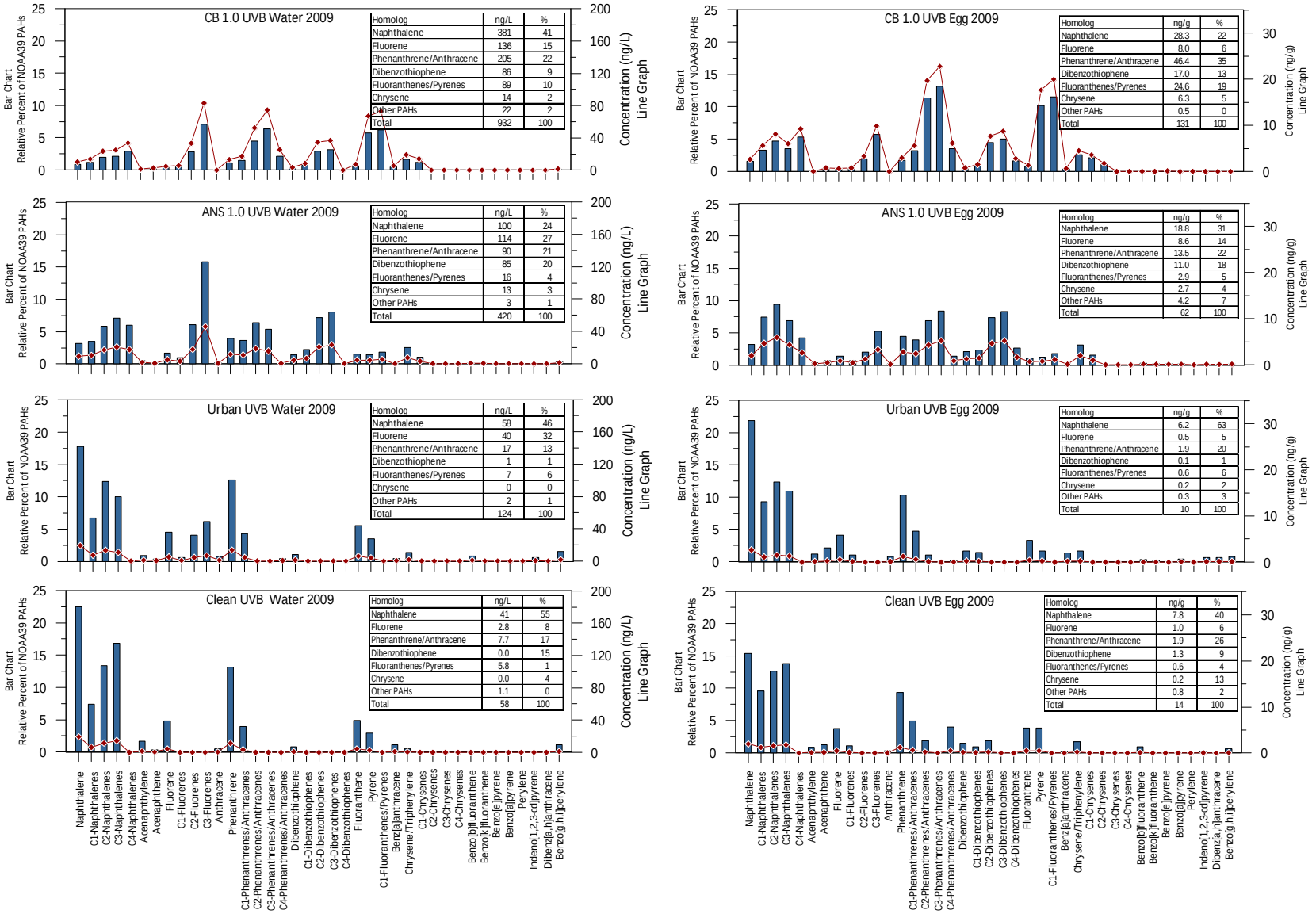


Figure 2-4. Relative and Actual Concentrations of PAHs in Water and Egg Samples, 2009 Study.

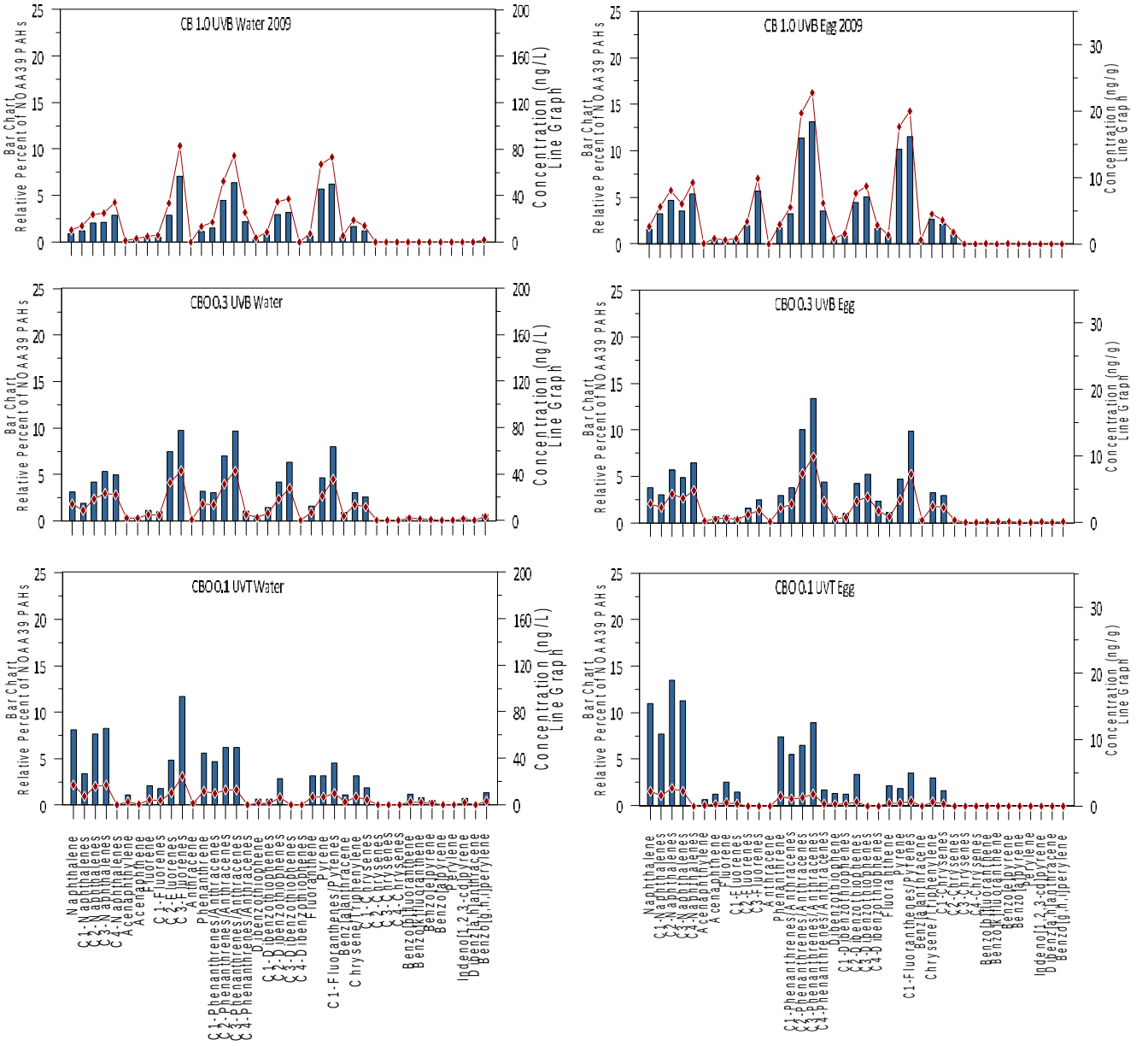


Figure 2-5. Relative and Actual Concentrations of CBO in Three Experimental Doses.

2.1.4 TISSUES OF ADULT HERRING COLLECTED JANUARY 2008

The concentrations of PAH in the tissues of fish collected in January 2008 in Richardson Bay were assessed just prior to the natural spawn in the 2008 study at nearby sites. The concentrations of PAHs in these fish and the ovaries provide a pre-exposure assessment indicating the absence of exposure to CBO by the adult fish prior to their spawning in 2008. As demonstrated in Figure 2-6 , PAH concentrations were low in these fish and relative PAH concentration patterns look like those shown above in Figure 2-4 for the urban and clean samples. Therefore, the adults that spawned in 2008 show no exposure to CBO.

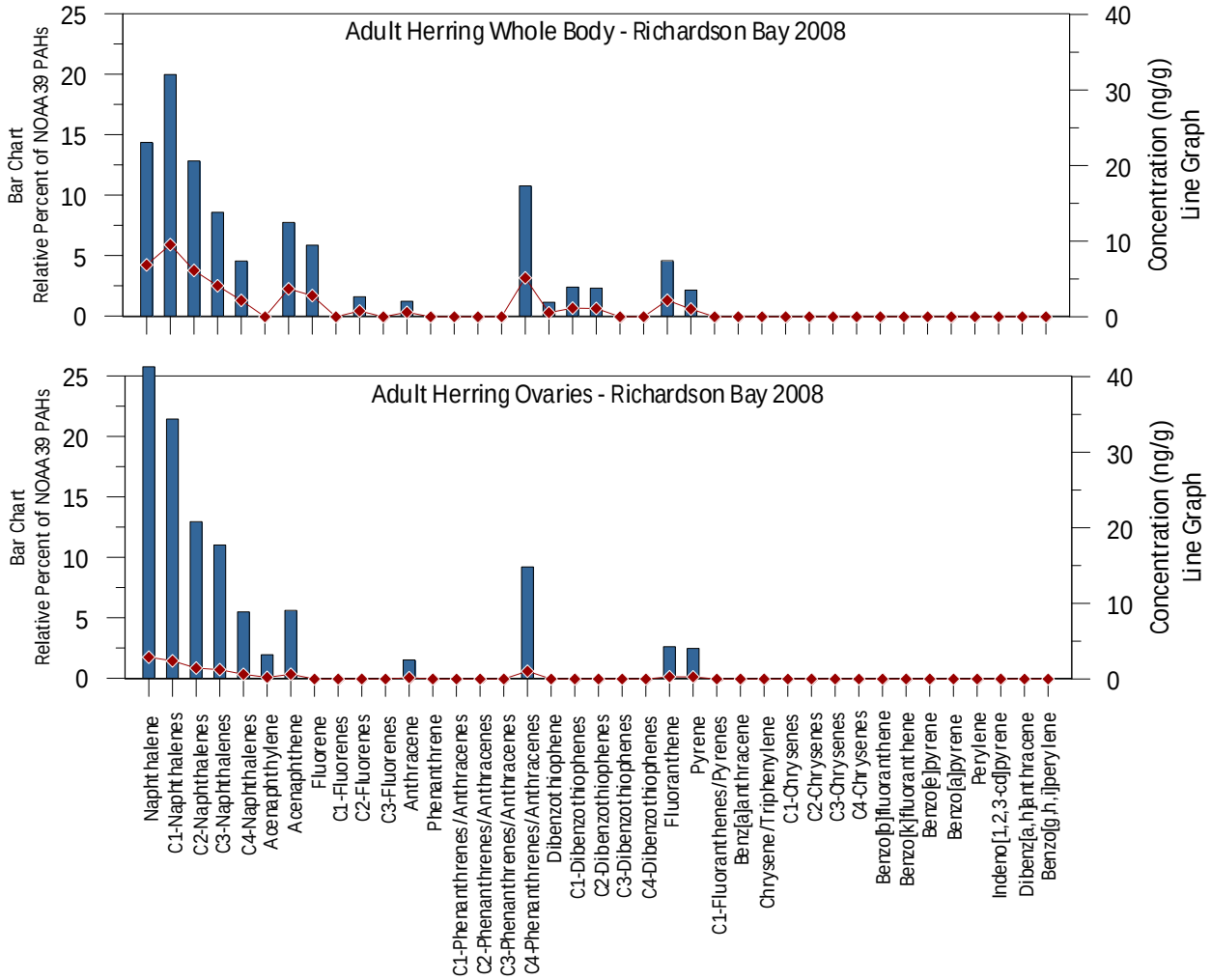


Figure 2-6. Relative and Actual PAH Concentrations in Adult Herring Collected in 2008.

2.1.5 NATURALLY SPAWNED HERRING EGGS COLLECTED IN 2008

The 2008 field study conducted by NOAA/BML collected naturally spawned eggs from sites that had been observed with oil contamination following the 2007 spill (NOAA/BML 2008). The PAH distributions in these naturally spawned eggs carry a signature that is indicative of the exposure history. Figure 2-7 shows the relative concentrations (bars) of PAHs at each of the sites overlaid on the signature produced by the known exposure to CBO in the 2009 NOAA/BML laboratory study (left side of figure). None of the study sites demonstrate a strong correspondence between their signature and that of known CBO exposure. The right side of the figure compares the relative concentrations to the egg signature

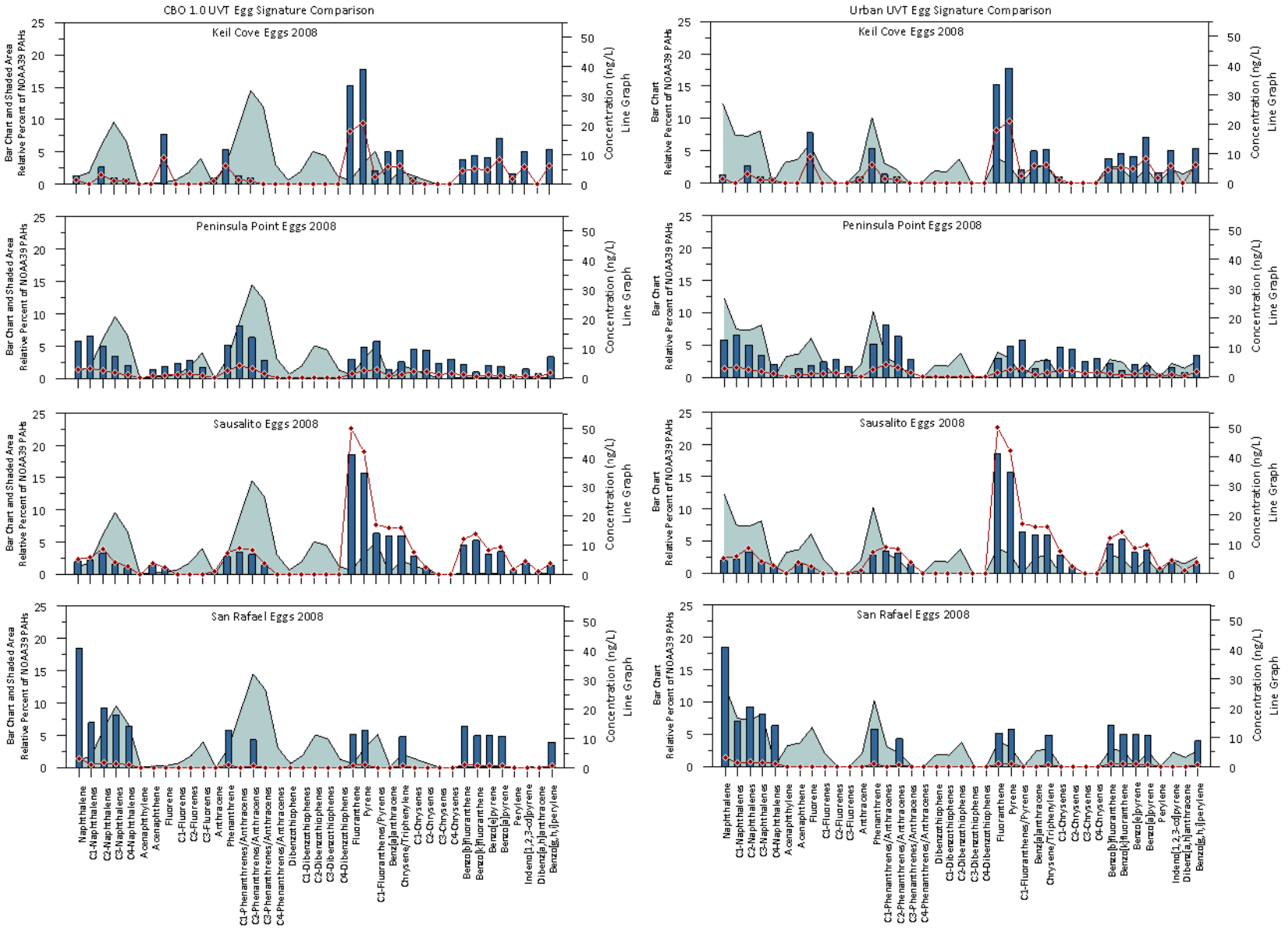


Figure 2-7. Relative and Actual PAH Concentrations in Naturally Spawned Eggs in 2008 Compared to 2009 Study CBO and Urban Signatures in Eggs.

produced by the urban gravel UVT treatment from the 2009 laboratory study. The signatures for eggs from Peninsula Point and from San Rafael match the urban signature quite well. The signatures for eggs from Keil Cove and Sausalito are more similar to the urban signature than to that from CBO; however both sites are dominated by fluoranthene and pyrene concentrations that are not indicative of either the CBO or urban signature. This indicates another source of PAHs at those locations.

2.1.6 DONOR FISH FOR 2010 LABORATORY STUDY

PAH analyses were performed on the donor fish used in the 2010 laboratory study to determine whether any parental oil exposure was a factor in the study. The smaller fish used in the study are presumed to be two year-old fish that hatched in early 2008 following the oil spill. Results of these analyses are shown in Figure 2-8, all samples indicate the same urban PAH signature seen previously in Figure 2-7.

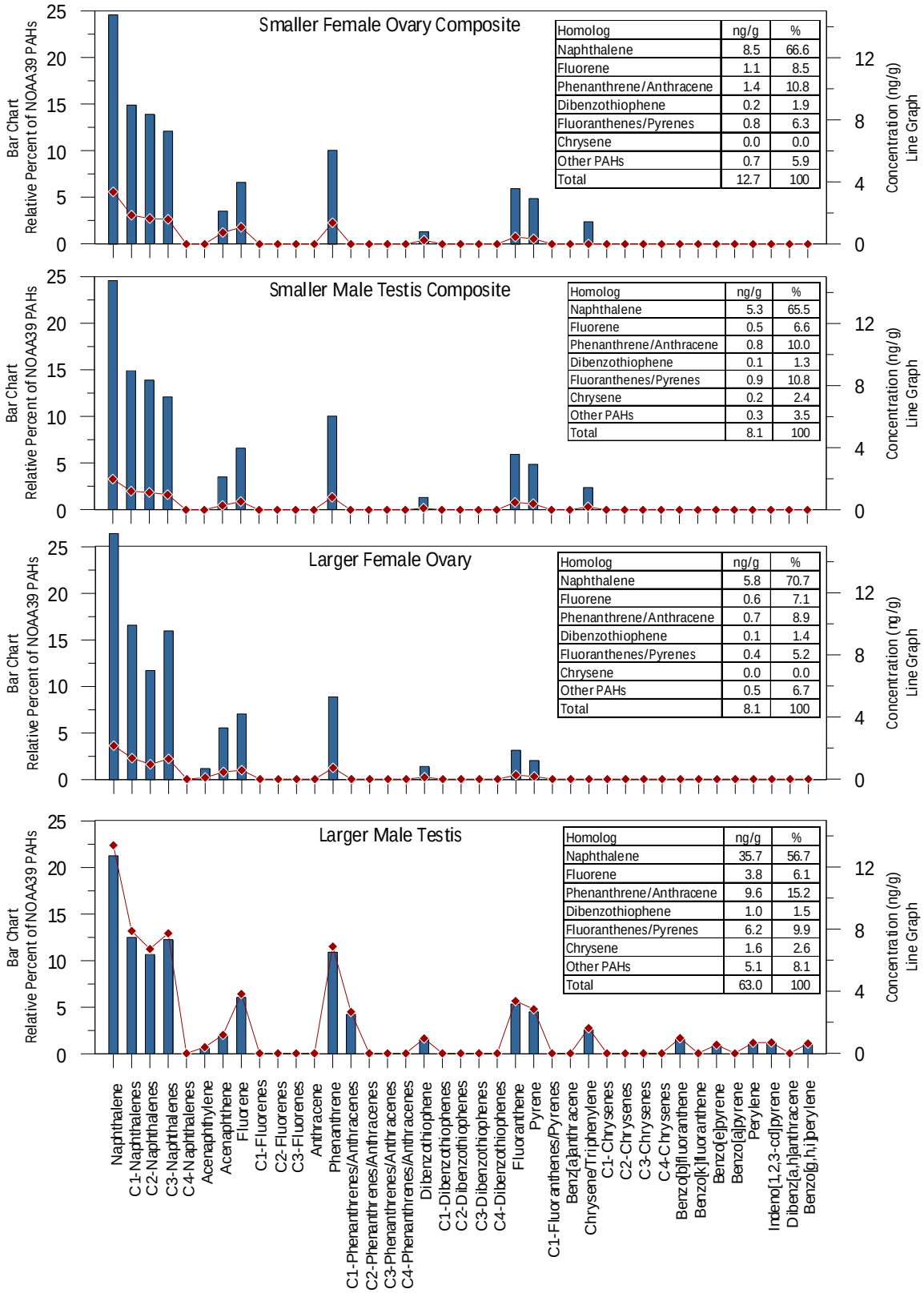


Figure 2-8. Relative and Actual PAH Concentrations in Donor Fish for 2010 Study.

2.1.7 COMPARISON OF ALL SAMPLES

Comparison of the various samples analyzed for PAH concentrations during the studies related to the oil spill was performed with a cluster analysis. The analysis was performed on the relative concentrations of the 39 PAH and alkylated homologs. A Bray-Curtis similarity index (Bray and Curtis 1957) was computed and used in the cluster analysis. Statistical testing was performed with SAS/STAT® software (SAS 2008). Clustering was performed with PROC CLUSTER using the flexible sorting option.

The dendrograms from the cluster analyses on the PAH analytes and on the samples are shown with the relative concentrations of PAHs in Figure 2-9. The resultant two-way cluster table shows four PAH clusters and three main sample clusters defined by the sample proportions.

Sample Cluster A contains all of the samples identified with the CBO signature; CBO, CBO water and eggs from 2009 study treatments, and 2007 water samples from Keil Cove and Horseshoe Cove. Cluster A is composed of PAHs in Clusters 1, 2, and 4; but the PAHs in Cluster 3 are not found in Cluster A. PAH Cluster 1 contains those PAHs which are found in most of the Cluster A samples and rarely in other samples; notably the dibenzothiophenes and chrysenes.

Sample Cluster B contains all of the donor fish samples for the 2010 study, urban water and egg samples from the 2009 study, the adult fish collected in early 2008, and one egg sample from the artificially spawned cages from 2008. These samples are predominantly composed of those PAHs in Cluster 2, particularly the naphthalenes as noted by the yellow and pink coloration of the cells. Sample Cluster C contains the remainder of the 2009 artificially spawned eggs, all of the naturally spawned eggs, and the urban UVT egg sample from the 2009 study. These samples are composed of PAHs in Clusters 2, 3, and 4; this group is the only one with significant contributions from Cluster 3.

The cluster analysis demonstrates that the samples known to be CBO group strongly together. In addition, water samples collected soon after the spill from sites noted to have moderate to heavy oiling (Keil Cove and Horseshoe Cove; NOAA/BML 2008) are also in the same cluster group and demonstrate the same signature. The other water samples from 2007 show the urban signature identified from the 2009 urban treatment as do samples of adult fish collected in both 2008 and 2010.

Notably, with one exception, all of the egg samples collected during the 2008 field study cluster together and contain PAHs which are not present in the CBO signature. The one exception (artificial spawn at Peninsula Point) clusters with the urban influenced samples due to an absence of PAHs in Cluster 3.

2.1.8 CBO CHEMICAL SIGNATURE CONCLUSIONS

The chemical analyses of various samples collected during the investigations following the Cosco Busan oil spill demonstrate that CBO has a PAH signature that can be readily distinguished from another known source (ANS) and from urban background contamination by the relative proportions of 39 petrogenic PAHs. This signature persisted in water exposed to oiled gravel and then into herring eggs, even when the initial concentrations of CBO were low. Adult fish collected in January 2008 and February 2010 showed no indication of exposure to CBO. Naturally spawned embryos collected from sites suspected to have been oiled by CBO after the spill indicate an urban background signature and not the CBO signature. The only samples in which the CBO signature was identified were the 2009 laboratory experiment CBO dosed samples and water samples collected at Keil and Horseshoe Coves after the spill but before clean-up of the sites, several months prior to the spawning season.

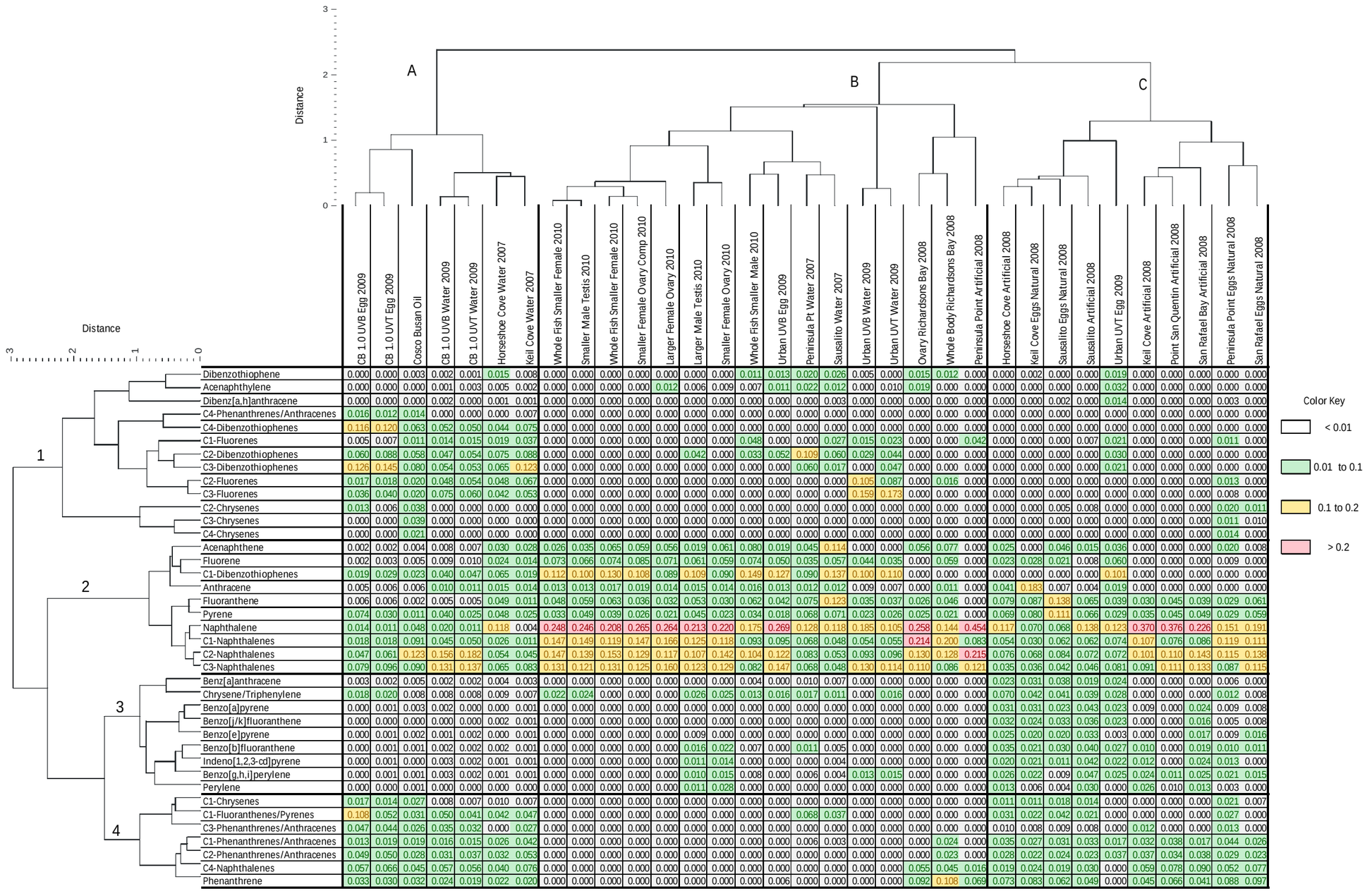


Figure 2-9. Multivariate Cluster Analysis on PAH in Study Samples.

2.2 PETROLEUM DOSE RESPONSE RELATIVE TO ESTABLISHED RESPONSE

The 2009 experiments demonstrated relationships between total PAH concentrations in water and PAH concentrations in the eggs that were significantly correlated (R^2 = 0.72 and 0.87, Experiments 3 and 4, respectively). Therefore, the compounds released from the treatment columns into the water were accumulated into the eggs based on their concentration in the water. Figure 2-10 illustrates this relationship for the 2009 study and compares these relationships to those reported from other researchers (i.e., Barron et al. 2002). The relationships (slope) of the three lines (Experiment 3, Experiment 4, and Barron et al.) shown in Figure 2-10 between total PAH concentrations in water and the concentration present in eggs were not significantly different but the intercepts of the regression equations are significantly different. This indicates that accumulation into eggs in the 2009 experiments was higher at any given water concentration than in the Barron et al. (2002) study and likely is a temperature related difference. The Barron et al. (2002) study was done at 4.9 to 6.6°C whereas the temperature in the NOAA/BML study fluctuated between 7 and 25°C in Experiment 3 and between 7 and 18°C in Experiment 4 (Incardona and Vines 2009).

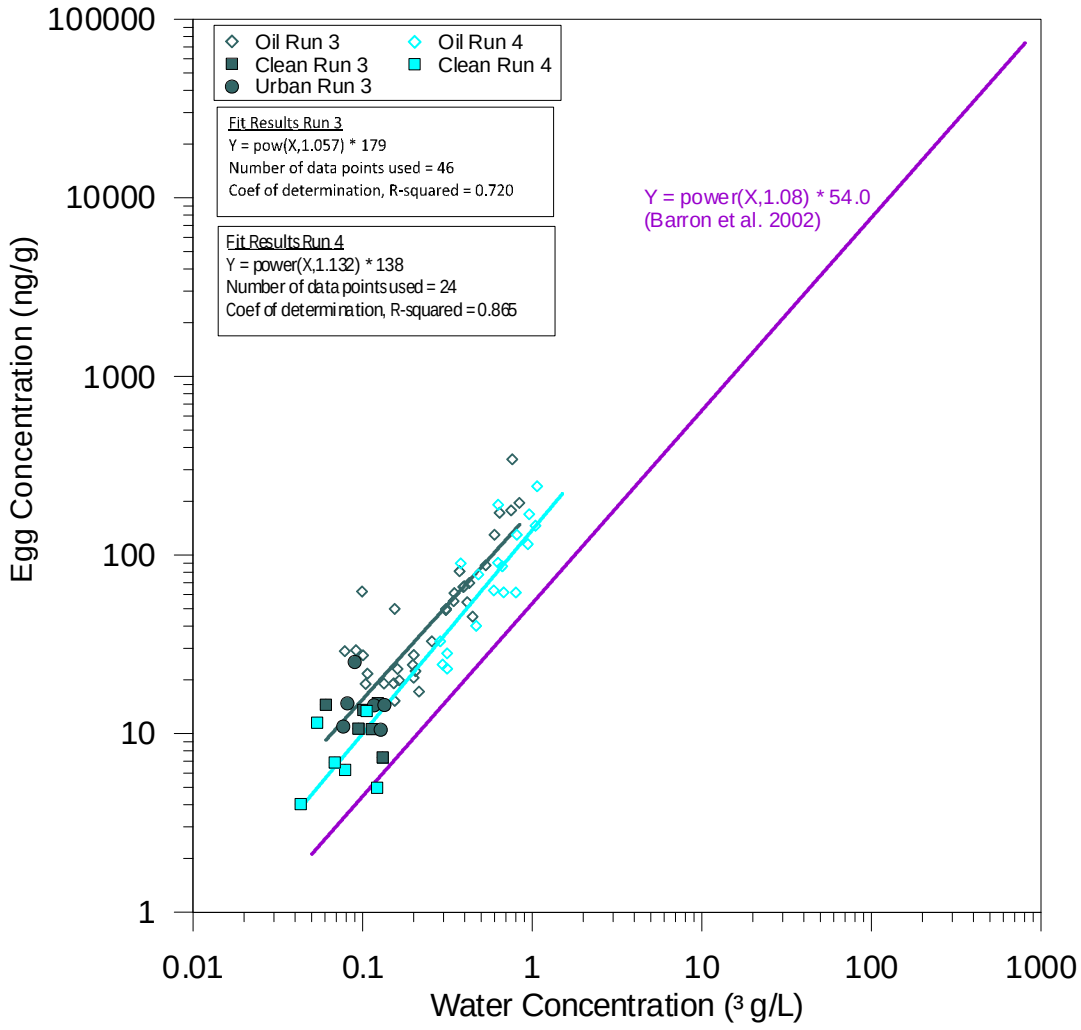
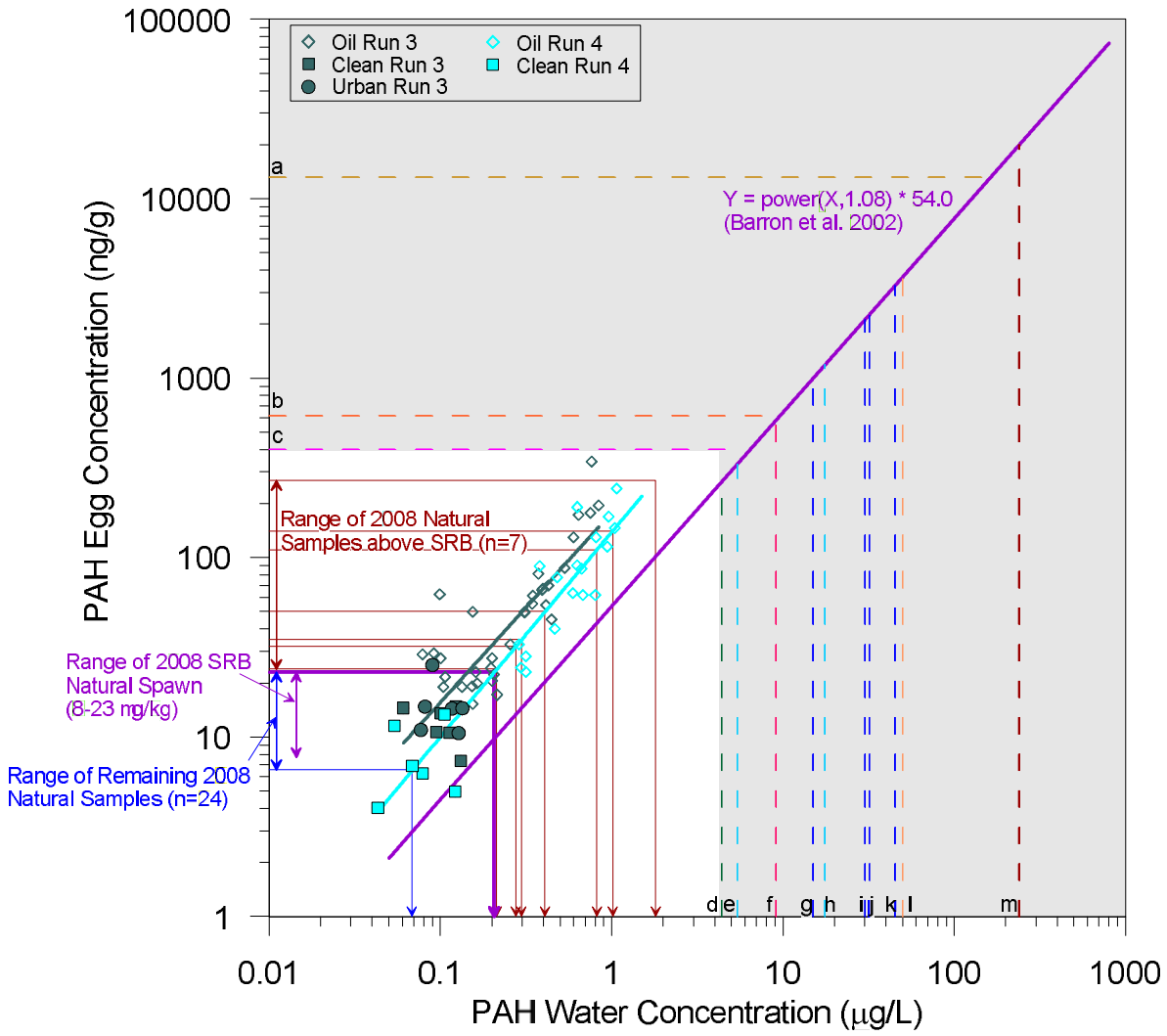


Figure 2-10. Relationships Between Total PAH in Water and Egg Tissue Compared to Barron et al. (2002).

To examine the total PAH concentrations observed in the 2008 field studies and the 2009 laboratory studies in context with the effects levels of other studies, the effects levels were overlaid on the plot as shown in Figure 2-11. This figure shows water and tissue concentrations associated with a range of developmental effects observed by other investigators relative to a variety of oil types (Barron et al.



	Author	Endpoint	Oil Source	Species
a	Brannon et al. 2006	Blue Sac Disease	ANS	pink salmon
b	Incardona et al. 2009	Decreased heart rate	ANS	herring
c	Barron et al. 2003, Carls et al. 1999	Yolksac edema NOEC	ANS	herring
d	Marty et al. 1997	Edema LOEC	ANS	pink salmon
e	Heintz et al. 2000	Marine survival LOEC	ANS	pink salmon
f	Carls et al. 1999	Yolksac edema LOEC	ANS	herring
g	Barron et al. 2003	8-day EC50 sunlight	ANS	herring
h	Heintz et al. 1999	Survival LOEC	ANS	pink salmon
i	Barron et al. 2003	4-day LC50 sunlight	ANS	herring
j	Barron et al. 2003	8-day EC50 control light	ANS	herring
k	Barron et al. 2003	4-day LC50 control light	ANS	herring
l	Vines et al. 2000	LC50 percent hatch	Creosote TPH	herring
m	Kocan et al. 1996	Physical deformities	ANS TPH	herring

Figure 2-11. Effects Levels for Total PAHs for Study Samples and Other Researchers.

2002, 2003; Carls et al. 1999, 2000; Heintz et al. 1999, 2000; Brannon et al. 2006; Vines et al. 2000; Kocan et al. 1996; Incardona et al. 2009). The maximum PAH concentration observed from the 2009 laboratory study approached the NOEC for demonstration of edema effects observed in developing herring embryos exposed to ANS but the maximum concentration was below any of the measured effects levels reported by these investigators. All of the 2008 egg concentrations (or extrapolated to water) were less than the NOEC concentrations reported by others (Barron et al. 2003 and Carls et al. 1999 for yolk-sac edema); therefore, based on the literature no effect from oil would be expected. Comparison of the highest concentration of CBO spiking (1.0 g/kg) used in the 2009 laboratory study to the literature effect values indicates that those levels approach the adverse effects concentrations for developing herring.

3 ADVERSE BIOLOGICAL EFFECTS AND RELATIONSHIPS TO CONTAMINANT AND ENVIRONMENTAL STRESSORS

3.1 BACKGROUND ON ADVERSE IMPACTS TO FISH AND ESTABLISHING SOURCE OF IMPACTS

The adverse biological responses expected to result from the exposure of developing Pacific herring eggs to CBO include the onset of multiple heart defects (e.g., edema, heart arrhythmia, changes in heart beat rates), body axis defects, opacity of yolk sac, early stage death or interference in the normal development of the eggs and hatching mortality. Since these adverse biological responses are not necessarily specific to a particular stressor it is necessary to identify commonality between patterns of response and contaminant concentrations to directly implicate one or more causes of the abnormalities recorded from the 2008 investigation conducted by NOAA and BML.

Many stressor types ranging from natural environmental variables to specific chemical contaminants or mixtures of stressors can display similar responses. The blue-sac syndrome is an anomaly produced in fish eggs and early yolk sac stages that exhibits many of the expected responses to CBO exposure. The blue-sac syndrome was originally described as a general syndrome that was related to large losses of trout during their development in hatcheries (Wolf 1954). It is characterized by mixtures of abnormalities including fluid retention (edema) with retarded development and lethargic movements and the appearance of opacity and other changes in the yolk appearance. In advanced cases there are also changes in heart rates and respiration with severe hepatic and renal blockages and severe cardiac pathology identified with histological preparations (Schereschewsky 1935; Dietrick 1938). Early work on trout raised in hatcheries revealed that a principal cause of blue-sac syndrome was elevated concentrations of waste ammonia-related products surrounding developing eggs that were hydraulically isolated from flowing water. The build-up of waste products in more stagnant water surrounding the eggs produced the syndrome in a high percentage of fish in these areas of isolation (Wolf 1956). Blue-sac syndrome has been well characterized for more than a century and was recognized as the most serious disease of young fishes prior to this discovery of the relationship of the syndrome to ammonia-related waste products and hydraulic isolation (Hofer 1896; Wolf 1956).

While these earlier research efforts concentrated on the effects of ammonia-related waste product impacts, ammonia is not the only potential cause of this syndrome. More recent work has also used this syndrome to describe early responses of many different species of fish to various stressors including further work on ammonia, petroleum hydrocarbons, organotins, copper, retene, dioxins, UV light and various plant extracts (Wolf 1956; Berry et al. 2007; Gonzales-Doncel et al. 2008; Hodson et al. 2007; Brinkman et al. 2009; Brinkworth et al. 2003; Adema-Hannes and Shenker 2008; Heintz et al. 1999; Barron et al. 2004; Strähle and Jesuthasan 1993). Other responses added to the earlier observations of effects described as blue–sac syndrome include body axis defects (scoliosis, lordosis and kyphosis), thickening of the body, delayed or arrested development of the tail area of developing embryos, abnormal fin development, craniofacial abnormalities, arrested development and hatching of incomplete but living organisms and various forms of tissue opacity (Schein et al. 2009; Boudreau et al. 2009; Ramachandran et al. 2006; Kocan et al. 1996; Couillard et al. 2005; Middaugh et al. 1998; Zhang et al. 2008; Tillitt et al. 2008). There are also indications that certain physical stressors (temperature, salinity, pH, dissolved oxygen; UV light without contaminant uptake) can also create responses consistent with blue-sac syndrome (Strähle and Jesuthasan 1993; Morley and Batty 1996; Johnston et al. 2001).

Thus, this array of abnormalities representing disruption of fish embryo development may be a result from a complex maze of causative agents. In the case of petroleum hydrocarbons, multiple sources of PAH contaminants may add to the complexity: PAHs may be derived from non-point urban

contributions via storm drains, run-off or aerial fallout, from specific spills of petroleum or alternative uses of petroleum based compounds (e.g., creosote), from the presence of black carbon (e.g., coal) or from natural products from burned wood (pyrogenic; Oros and David 2002; Neff 1979; Simoneit 1984).

The contaminant concentrations or level of stress obtained in a controlled experiment with known sources of stress produce a suite of responses that are dependent on or correlated to the water or tissue concentrations and/or non-chemical stressors in that study. Determination that a contaminant was directly responsible for observed biological effects must demonstrate that the effects are above those levels due to environmental stressors to which the larvae would be subjected to in the natural environment, regardless of a contaminant impact.

High quality video and photographic images using specialized dissecting microscopes were used in all biological response assessments (2008, 2009, and 2010 datasets). The images provided by NOAA/BML were used to document observations on the development of herring embryos from natural spawning at Peninsula Point, Keil Cove, Sausalito, and San Rafael in 2008. Additional documentation was also provided on an experimental subtidal exposure of artificially spawned herring contained in meshed cages at these same locations as well as at Point San Quentin and Horseshoe Cove. For this study, the primary data captured by NOAA was associated with potential heart abnormalities and included still and video imaging while the BML group evaluated spawning success and the presence of larval abnormalities via still images. This information served as the initial assessment of adverse effects associated with the CBO spill and was provided to NewFields for review. The NewFields review identified environmental stressors as well as laboratory artifacts that interfered with establishing a pattern of biological responses that might be singly attributed to CBO exposure. While the overall conclusions by NOAA/BML and NewFields were in agreement that adverse effects to herring development were evident in the 2008 data, there was no agreement on the probable cause(s) of the effects.

Making comparisons based on measurements of equivalently developed young is key to producing a standardized dataset. Asynchronous development of herring embryos occurs even in cohorts and the time to hatch may vary significantly in response to environmental factors (temperature, salinity, pH, etc). Interrupting that development process by dechorionation of the eggs prior to natural hatching may result in evaluation of immature specimens of widely varying development stages. Additionally, collection of naturally spawned herring eggs results in unknown time of fertilization that may have occurred over several tidal cycles which introduces additional developmental variability to assessments of naturally spawned embryos. All of these factors contribute to difficulties in comparing data from different researchers and studies.

3.2 DEVELOPMENTAL STAGING

NewFields followed the generally accepted practice of embryo and larval aging and based our comparisons on development stages rather than increments of time (e.g., days post hatch; Kimmel et al. 1995, Sherrill et al. 2009; Parichy et al. 2009). Several good studies of organogenesis and post hatch staging are available; these studies detail the span of anatomical relationships and physiological changes that are associated with various embryonic and larval growth stages (Kimmel et al. 1995; Hill and Johnston 1997; Sherrill et al. 2009). Morphological and physiological development occurs rapidly during organogenesis and post hatch stages making assessment of early life stages very difficult when different development stages are represented. Table 3-1 summarizes noteworthy developmental changes from the embryogenesis stages (stages o, p and q; Hill and Johnston 1997) to post-hatch larvae that were analyzed in the NewFields review of the Cosco Busan 2008, 2009 and 2010 herring studies.

Table 3-1. Rapid Developmental Changes During Embryo Organogenesis Stage Through Post Hatch Larval Stage

Stage	Dominant Visible Characteristics	Organs Undergoing Progressive Development
Pre-Hatch (Dechorionated) Organogenesis (o,p,q)	Rotation of head (compressed pericardium) Large, round yolk sac Spinal curvature	Head (size & shape) Heart Otic vesicles Eye – various stages of pigmentation Pectoral Fin Jaw Primordial kidney Primordial liver
Larvae at Hatch	Larvae are linear Yolk sac is elliptical Larvae swim Pectoral fins elongated Melanophores evident along ventral edge & near pectoral fin	Swim bladder Fins, pectoral fin girdle Further development of liver, pronephros & urinary tract
Hatch to 96 hph	Yolk sac diminishes Lower jaw protrudes Melanophores vivid	Gastrointestinal tract Urinary tract Liver Swim bladder

3.3 Assessment of Biological Injury Caused by Environmental Stressors or Petroleum Contaminants

Numerous scientists have focused on occurrences of early life stage abnormalities of herring and other teleosts that are associated with environmental conditions (Griffin et al. 2004, 2009; Berry et al. 2007; Brinkworth et al. 2003; Hershberger et al. 2005; Morley and Batty 1996; Tytler and Ireland 2000; Tytler et al. 1996; Johnston et al. 1998) and/or anthropogenic contaminants (Incardona et al. 2004, 2009; Kocan et al. 1996; Gonzalez-Doncel et al. 2008; Carls et al. 1999; Barron et al. 2003; Vines et al. 2000; Marty et al. 1997). From this body of literature, several recurring patterns of stress and injury have emerged: mortality, edema, spinal abnormalities, hemorrhages, reduced growth, abnormal development (eye, craniofacial, skeletal), cardiac or respiratory dysfunction, delayed or precocious hatch, and symptoms of immunotoxicity or genotoxicity. These can be abstracted into the following categories of abnormalities:

1) Lethality -Early or late stage embryo mortality
   Evidence: tissue opacity and/or necrosis
2) Developmental - Retarded growth of organs
   Evidence: small or unpigmented eyes, stunted growth of trunk
3) Morphological - Disruptions in musculoskeletal systems
   Evidence: abnormal craniofacial or spinal development (body axis defects)
4) Physiological -Reduced functioning of organ systems; can produce non-viable embryos
   Externally visible examples: alteration of heart or respiration rates; pericardial, yolk sac and or coelomic edema; lethargy

Table 3-2 summarizes the endpoints assessed for the individual Cosco Busan studies and indicates where direct comparisons can be made between the studies. The overarching question is whether exposure to CBO causes a distinct pattern of biological effects and whether known environmental stressors may cause similar patterns of anomalies. The 2010 laboratory experiments conducted by NewFields focused on anomalies associated with environmental stressors observed at two sites representative of the 2008 field study. These stressors included condition and health of gametes, temperature, salinity, ultraviolet radiation and simulation of stranding stress (combined temperature shock, ultraviolet (UV) exposure, and potential desiccation caused by direct exposure to air). All embryos were allowed to hatch naturally and then prepared for high quality digital imaging. The various stressors applied to the treatments are

summarized in Table 3-3, treatment codes shown are used in the following sections. The comparisons between 2008, 2009, and 2010 datasets highlight similarities and differences detected with the various endpoint assessments.

Table 3-2. Summary of Endpoints Measured by Researchers During Cosco Busan Studies.

Endpoint		2008 NOAA/BML Field Study		2009 NOAA/BML Laboratory Study		2010 NewFields Laboratory Study
		NOAA/BML	NewFields	NOAA/BML	NewFields	
Survival/Lethality	Embryo Mortality (Early Stage)			✓Paradise Cove natural spawn		✓
	Embryo Mortality (Late Stage)			✓	✓	✓
	Larval - % Hatch	✓		✓		✓
	Larval - % Normal Hatch	✓		✓		✓
Developmental	Growth – Standard Length				✓	✓
	Abnormal Development	✓		✓	✓	✓
Morphological	Musculoskeletal, Craniofacial Defects	✓	✓	✓	✓	✓
Physiological	Pericardial Edema	✓	✓	✓	✓	✓
	Yolk Sac Edema		✓		✓	✓
	Cardiovascular Function	✓	✓		✓	✓ Controls only
Note: NOAA/BML did not differentiate the occurrences of pericardial and yolk sac edema.						

Table 3-3. Stressors Applied in 2010 Laboratory Experiment.

Treatment Code	Fertilization Salinity	UV Exposure	Incubation Salinity	Incubation Temperature	Air Exposure
Control	16 ppt	No	16 ppt	12 °C	None
CUV	16 ppt	Yes	16 ppt	12 °C	None
A-16	16 ppt	Yes	variable 24 to ~30 ppt	11 °C with periodic increases up to 22 °C	None
A-28	28 ppt	Yes	variable 24 to ~30 ppt	11 °C with periodic increases up to 22 °C	None
A-TS	28 ppt	Yes	variable 24 to ~30 ppt	11 °C with periodic increases up to 24 °C	Exposure during extreme temperatures
B-22	22 ppt	Yes	variable 14 to 24 ppt	11 °C	None

3.3.1 LETHALITY

Determining the stage of lethality is important and can help identify the stressors that contributed to abnormal embryogenesis. Mortality of developing embryos can occur immediately after fertilization or at any stage in development prior to hatching (Figure 3-1). The stage of development where mortality occurs provides an indirect demonstration of the type of development defect that is disrupted. Interference in fertilization success may indicate eggs or sperm are in poor condition or not developed sufficiently, possibly reflecting condition of parental stock. A lack of egg hardening and swelling of activated eggs can be related to disruption of Na/K channels and the uptake of excess water; whereas, disruption of cell differentiation and interference with early epiboly stages can indicate adverse conditions within the developing egg as well as exposure to UV light.

Fertilization success is determined by the presence of a fertilization membrane surrounding the dividing cells. If the eggs or the sperm are damaged or if they have not developed properly then the success of fertilization and initial separation of the fertilization membrane will not occur. A description of viable gametes contained in guidance literature (Dinnel et al. in prep) indicates that gametes should be taken from 'ripe and runny' gametes.

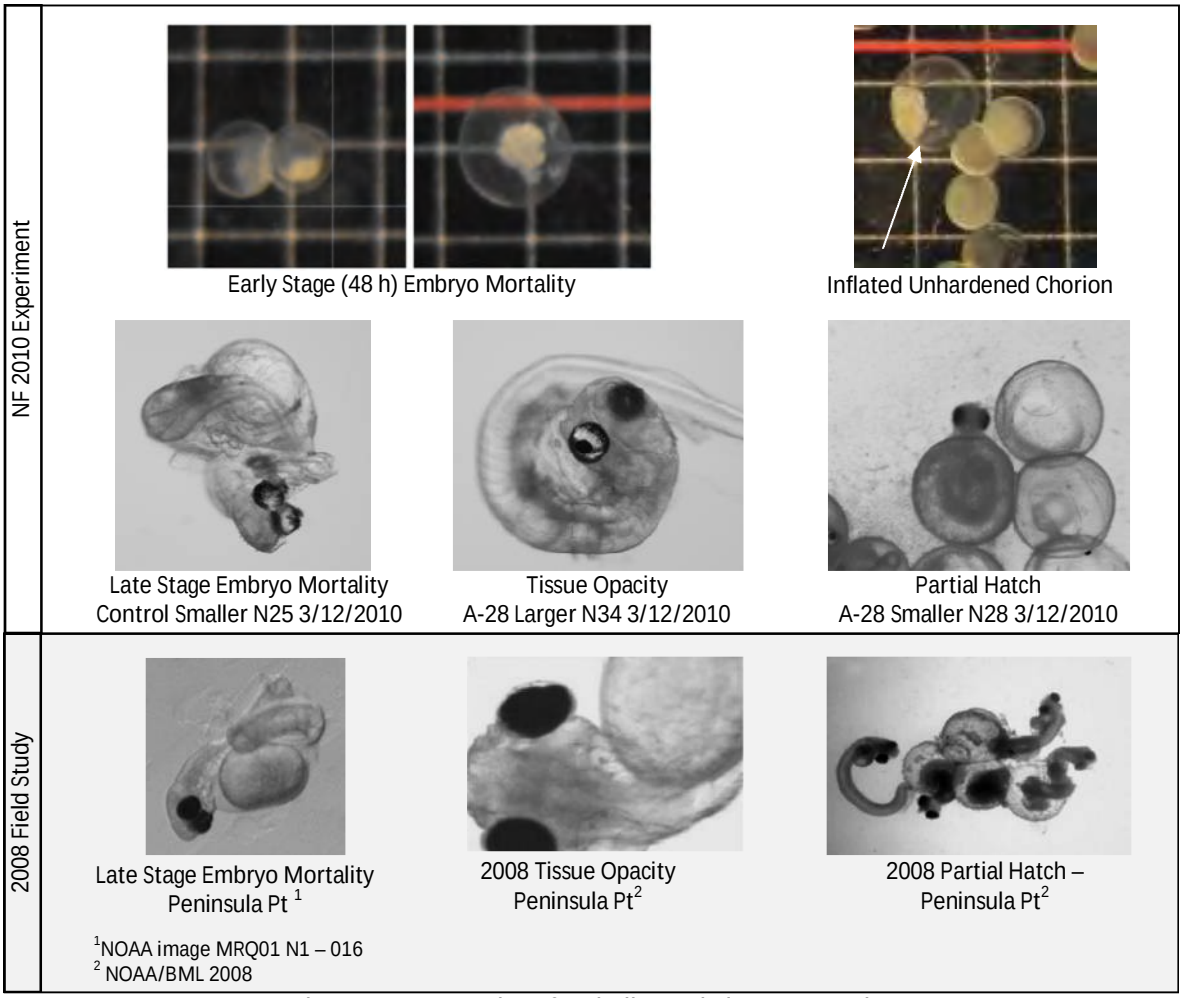


Figure 3-1. Examples of Lethality and Tissue Necrosis.

Early Embryo Mortality

The frequency of early embryonic mortality was not assessed in the 2008 field or 2009 experimental studies. Information is only available from the Paradise Cove natural spawn collection in 2009 and the NewFields 2010 laboratory study. Normal development is readily apparent after 48 h and can easily be distinguished from non-fertilized eggs and those eggs that are not progressing through the blastula stage. Results from the 2010 NewFields laboratory study were compared to data collected by BML from the 2009 Paradise Cove natural spawn (Table 3-4). The early embryo mortality observed in natural spawn collected in 2009 was similar to that observed in 2010 from the smaller donor size group, perhaps reflecting the influence of similar environmental factors; whereas, the higher percentage of early mortality in the embryos from the larger donor fish is likely attributable to the poorer quality of the eggs (i.e., not 'ripe and runny'; NewFields 2010b, Dinnel pers. comm).

Table 3-4. Early Embryo Mortality.

2009 Paradise Cove[1]	2010 Smaller Donor Size[2]	2010 Larger Donor Size[2]
28% (range 5% – 68%, N=8)	29% (range 17% - 55%, N=6)	52% (range 42% - 66%, N=6)

[1] Data from Incardona and Vines (2009)
[2] Data from NewFields (2010b)

UV exposure in the absence of other contaminants has been linked to injury during several phases of embryogenesis. Strähle and Jesuthasan (1993) describe one type of early effect due to UV exposure as resulting in a separation of the blastoderm from the yolk cell, which forms a vesicle resting on the disintegrating yolk mass. The occurrence of the disintegrated yolk mass with a residual of dense tissue mass was noted in the early development of 2010 herring embryos, similar to the description noted above (Figure 3-1).

Another type of early mortality to eggs was also observed in 2010 with less frequency. In these cases, the chorion was very inflated and had little tissue mass remaining; this effect may be due to lack of hardening of the chorion after activation (Figure 3-1, top right). This response has been associated with interference of hardening enzyme by salts for freshwater fish (for example, NaCl, KCl, $CaCl_2$, or $MgCl_2$), as well as oxygen deficiency (Zotin 1958).

Late Stage Embryo Mortality

In the 2010 laboratory study, late stage embryo mortality was evaluated at 8 dpf. The number of non-viable, eyed embryos was recorded with an Olympus microscope and digital imaging system. Results from this evaluation were compared to the 2009 column studies for the clean control samples (Table 3-5). Although there is a lot of variation among the various control samples, it appears that under the best conditions, approximately 3% of embryos will exhibit late stage mortality without exposure to a contaminant source. Higher mortalities observed may have been due to less than optimum condition of donor egg or sperm material (NewFields 2010b).

Table 3-5. Late Stage Mortality in 2009 and 2010 Studies.

Study	Sample[1]	Non-viable Eyed Embryos (%)	
		Without UV Exposure	UV Exposure
2009 NOAA/BML	CLEAN - Experiment 3	2.6	2.1
2009 NOAA/BML	CLEAN - Experiment 4	16.0	38.8
2010 NewFields	Control - Smaller	3.1	2.7
2010 NewFields	Control - Larger	11.6	4.5

[1] 2009 data provided by NOAA, 2010 data from NewFields

3.3.2 Developmental and Morphological Abnormalities

Developmental and morphological abnormalities occurred during the 2010 experimental exposures to non-chemical stressors. These abnormalities ranged from differences in body length to alterations in the embryo body curvature and interruption or lack of development of the tail area of the embryo; examples observable in developing embryos are shown in Figure 3-3. Larval growth was measured as standard length and numbers of abnormally developed larvae were recorded. NewFields also determined the number of specimens that were not linearly oriented at hatch and indicated level of severity. The main types of axis defects are scoliosis, kyphosis, and lordosis; distinct bends in head or tail region are also included in this category. These anomalies were observed in 2010 laboratory and 2008 natural spawn (Figure 3-2) as well as in the 2009 experimental imagery. Additionally, non-lineal body axis occurred with a 'failure to straighten' phenomena wherein an otherwise normal looking

embryo failed to straighten in response to touch with a probe and remained coiled (noted in 2008 and 2010 results).

Figure 3-3. Examples of Developmental Defects in Embryos

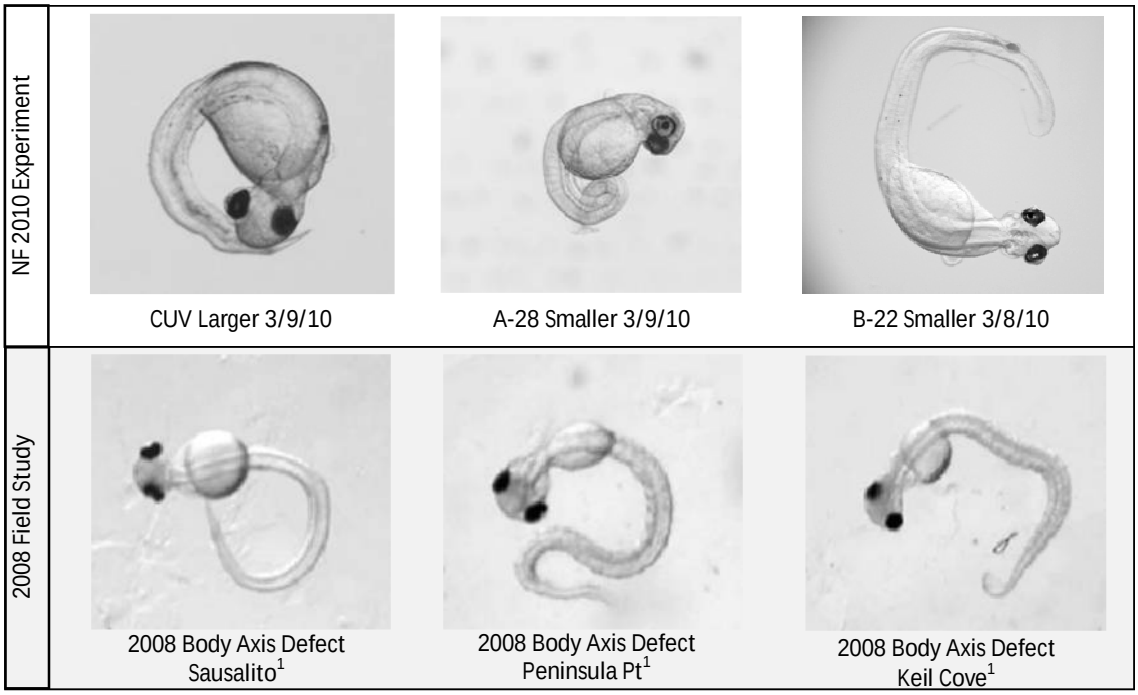


Figure 3-2. Examples of Morphological Abnormalities in Hatched Larvae.
[1]NOAA/BML 2008

UV exposure has been implicated as a causative agent in disruption of epiboly processes which may result in grossly retarded development in the trunk and tail region of the embryos (Strähle and Jesuthasan 1993). Exposure to PAHs has been implicated in causing body axis defects (Incardona et al. 2005); however, several studies reporting PAH association with body axis defects have used dechorionated embryos which have not reached full development. These types of trunk and tail development abnormalities were observed in the 2010 experiments (in the absence of any chemical stressor) as well as in the 2008 and 2009 experiments.

Temperature may affect development of eggs and larvae by altering the rates of many biochemical and tissue level processes (Morley and Batty 1996) and metabolic rates. Incubation temperature may have a direct effect on growth, time to hatch, and influence the development of fins and associated musculoskeletal system in larvae (Johnston et al. 2001). Growth, as measured by larval length at hatch, may be affected by metabolic rates during embryogenesis. Additionally, the size of eggs initially spawned has been shown to influence larval size at hatch (Blaxter and Hempel 1963). At hatch, larvae have large primordial dorsal and ventral fins which are gradually adsorbed and replaced by dorsal, anal and caudal fins (Johnston et al. 2001); the effectiveness of swimming performance may be impacted and affect survival.

Severe defects were observed in all treatments in the 2010 laboratory study, ranging from 1% to 8% of the larvae; similar to the occurrences observed in the clean, urban, and lower oil dose treatments from

the 2009 NOAA/BML laboratory study and in natural spawn at Paradise Cove in 2009 (Figure 3-4). For the most part, the 0.3 g/kg and 1.0 g/kg treatments in 2009 showed higher incidence of defects than found in the 2010 study. Body axis defects in the 2009 study were shown to be partially related to the amount of oil exposure, but largely due to factors undocumented in that study (NewFields 2010a). The results from the 2010 laboratory study indicate that changes in salinity and temperature can account for observances of up to 10% incidence of defects.

3.3.1 PHYSIOLOGICAL STRESS

Several metrics are used to measure physiological stress. Cardio-respiratory function can be assessed by determining heart rates and comparing to data representing a normal, baseline range in addition to determining the incidence of fluid accumulation, i.e. pericardial edema. Disrupted kidney and urinary tract function is often coupled with the outward manifestation of fluid build-up in the yolk sac or coelomic regions. Evaluation of cardiac function was not routinely evaluated for the Cosco Busan studies (the 2008 field study is the only one that incorporated a detailed investigation into cardiac stress). Edema assessments are the only metric that is available from all studies. Edema assessments made by NOAA and BML on imagery produced from the 2008 and 2009 studies were based on qualitative judgments and put pericardial and yolk sac edema into one category, referred to as "edema."

The distinction of pericardial and yolk sac edema is important since these endpoints may represent different etiologies. Disrupted physiological functions are responsible for edema and may be caused by impairments to any interactive organ systems. Edema can occur singly (either yolk sac or pericardial) or in combination (yolk sac and pericardial, or coelomic). Herring embryos are able to maintain osmotic balance without aid of developed kidneys and it has been reported that dechorionated embryos can maintain osmotic balance, at least in a freshwater species (Hill et al. 2004). It should be pointed out however, that the comparison of a freshwater fish (Danio rerio) to herring, a euryhaline species, may not correlate as far as osmotic regulatory functions are concerned since the osmotic gradients between blood and the surrounding water environments are vastly different (Bonga and Lock 2008).

At hatch, herring larvae have cardiac, respiratory, and renal systems that are not fully developed but are closely linked and serve the main functions of blood pressure regulation, oxygenation, and blood filtration as well as osmotic regulation. The bulbus arteriosus maintains blood pressure while skin cells similar to branchial chloride cells presumably function to supply oxygen (Sherrill et al. 2009; Tytler and Ireland 2000); gill development occurs during metamorphosis of larvae into juveniles. The pronephros functions as a primordial kidney system, but yolk-sac larvae may not develop direct urinary tract connection to the external environment for a day or so after hatching. Figure 3-5 portrays the principal physiological configuration at the yolk-sac larval stage.

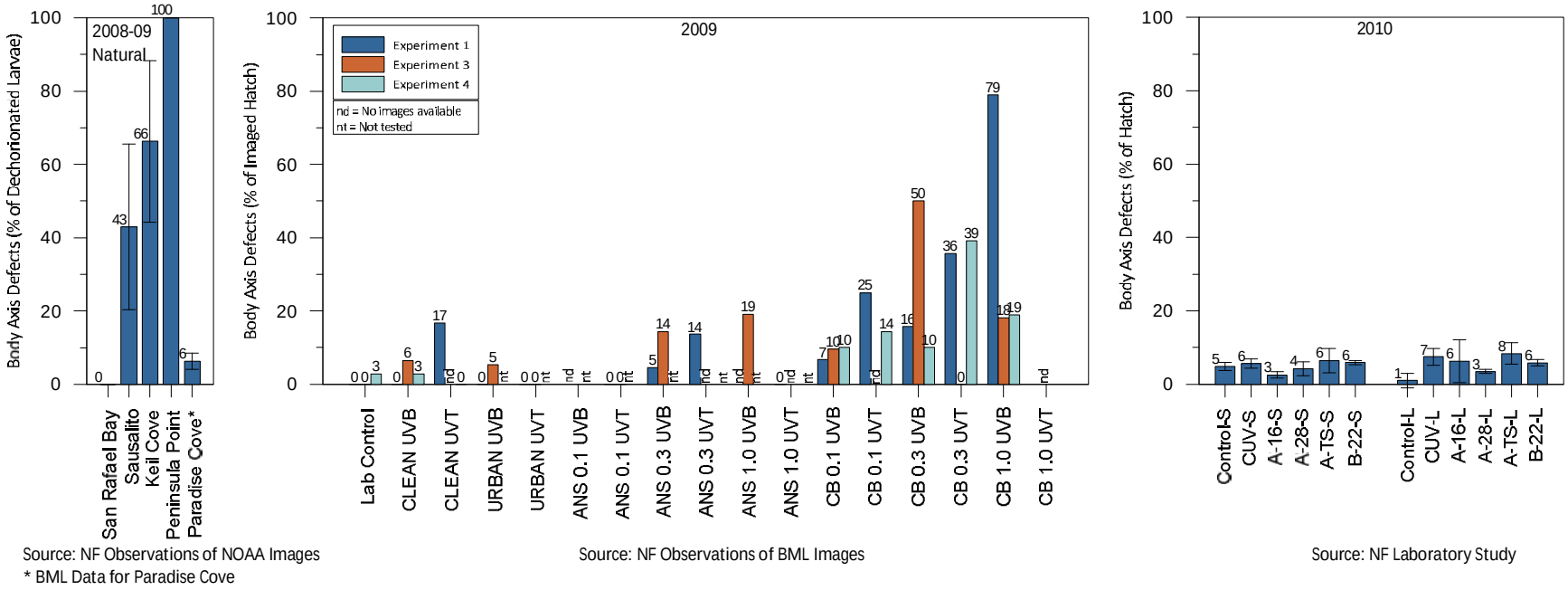


Figure 3-4. Body Axis Defects in 2008 Natural Spawn, 2009 and 2010 Laboratory Studies.

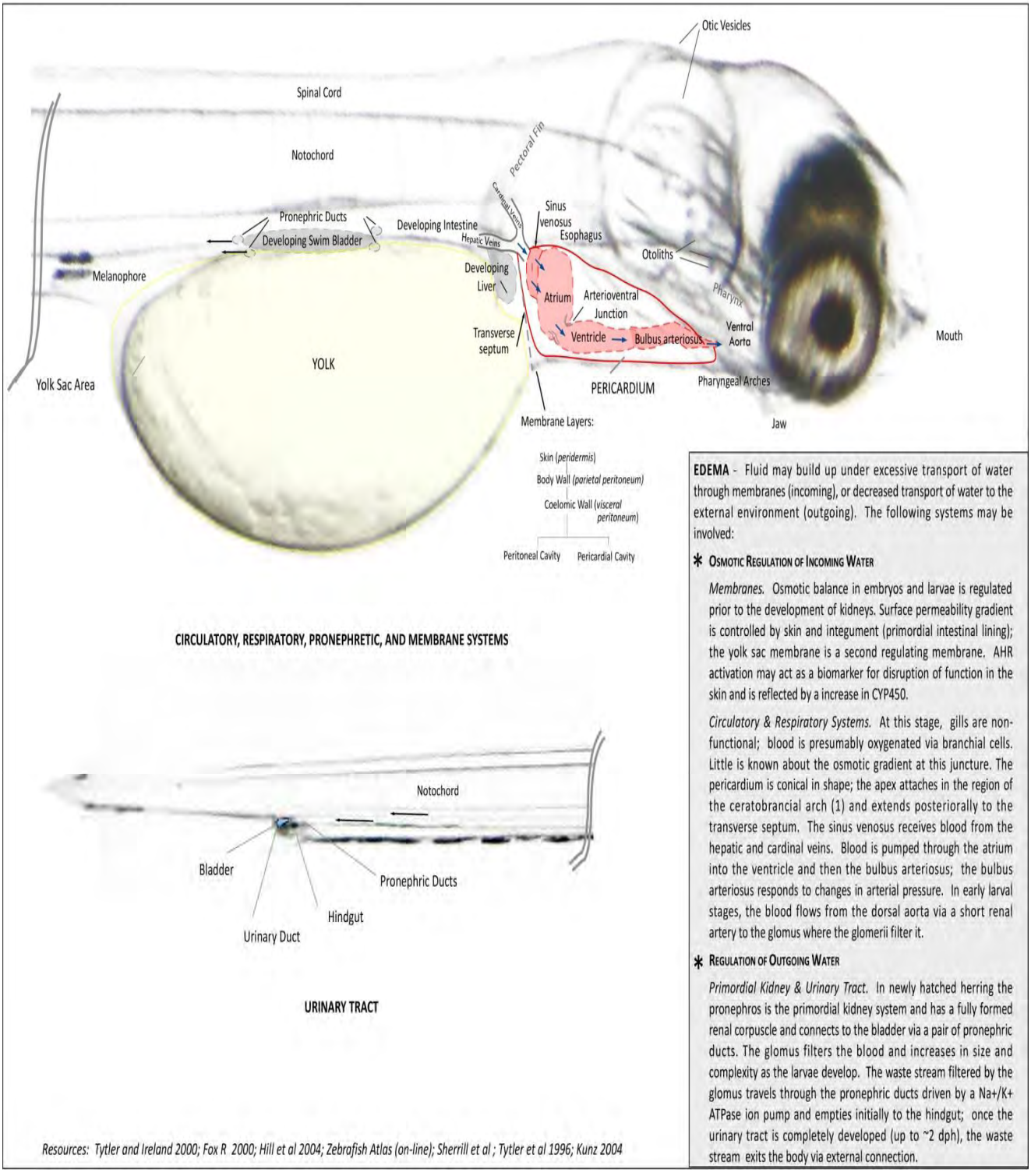


Figure 3-5. Configuration of Principal Organ Systems Contributing to Edema.

In the 2010 laboratory experiments, photomicrometry image resolution was sufficient to determine the location of the transverse septum which facilitated determination of the posterior wall of the pericardial cavity. The identified location of the transverse septum (proximal to the anterior edge of the pectoral fin near the 6th somite) was used as the definition point for separating the yolk sac area from the pericardial cavity, resulting in a more accurate evaluation of pericardial edema during various stages of development (Figure 3-6). NewFields recorded the incidence as well as the intensity of pericardial and yolk sac edema for larval specimens (examples shown in Figure 3-7) in all treatments as an indicator of physiological stress. For the most part, instances of pericardial and/or yolk sac edema were distinguished from each other. This meristic evaluation was extended to a subset of data from the 2009 experiments and 2008 field image assessment to quantitatively compare the occurrences of these types of edema.

Yolk Sac Edema

- Symptom: cellular or tissue level dysfunction of membranes resulting in accumulation of fluid contained within the yolk sac membrane; a secondary condition may be caused by consumption of yolk during post-hatch growth while yolk sac membrane is not absorbed at the same rate as the yolk is consumed.
- Causative agents: disruption of osmotic balance between external fluids and fluids contained within various locations within the body; principle sources fluid intake are skin or integument (gut). Contaminants may cause disruption of biochemical pathways, specifically, act as AhR antagonists (demonstrated by CYP1A induction). Environmental stressors that may be related to osmotic disruption includes changes in temperature, salinity, and pH. These potential stressors may operate by changing hydrostatic pressures between tissue reservoirs; or, presence of under-developed urinary tract which permits fluid to build up before being released.

Pericardial Edema

- Symptom: swelling of pericardial cavity
- Causative agents: cardiovascular dysfunction; renal failure; AhR antagonism

Dysfunction of the cardiovascular system has been suggested as a causative factor for pericardial edema as well as other maladies. Altered function of the cardiovascular system may be a result of pathologic changes in cardiac cells causing retardation of cardiac contractility (e.g., bradycardia). It has been recognized that the atrium is a major site for pathogen recognition and destruction via antigens and macrophages in reticuloendothelial cells (Sherrill et al. 2009). Incardona et al. (2004, 2009) reported that exposure to PAHs caused cardiac arrhythmia in embryonic herring (representing dechorionated embryos 7 to 11 DPF) and produced a high incidence of pericardial edema. Suboptimal water quality conditions including hypoxia can also affect cardiovascular health and function and has been associated with epinephrine release, increased respiration, diuresis, decreased growth, and compromised immunity (Sherrill et al. 2009). Observations of cardiovascular disease in fish have been associated with outward symptoms occurring in multiple body systems; Table 3-6 summarizes the range of symptoms that may be manifestations of cardiac disease (adapted from Sherrill et al. 2009). Retardation of general circulation caused by lack of swimming motion has also been implicated as causing increased fluid retention.

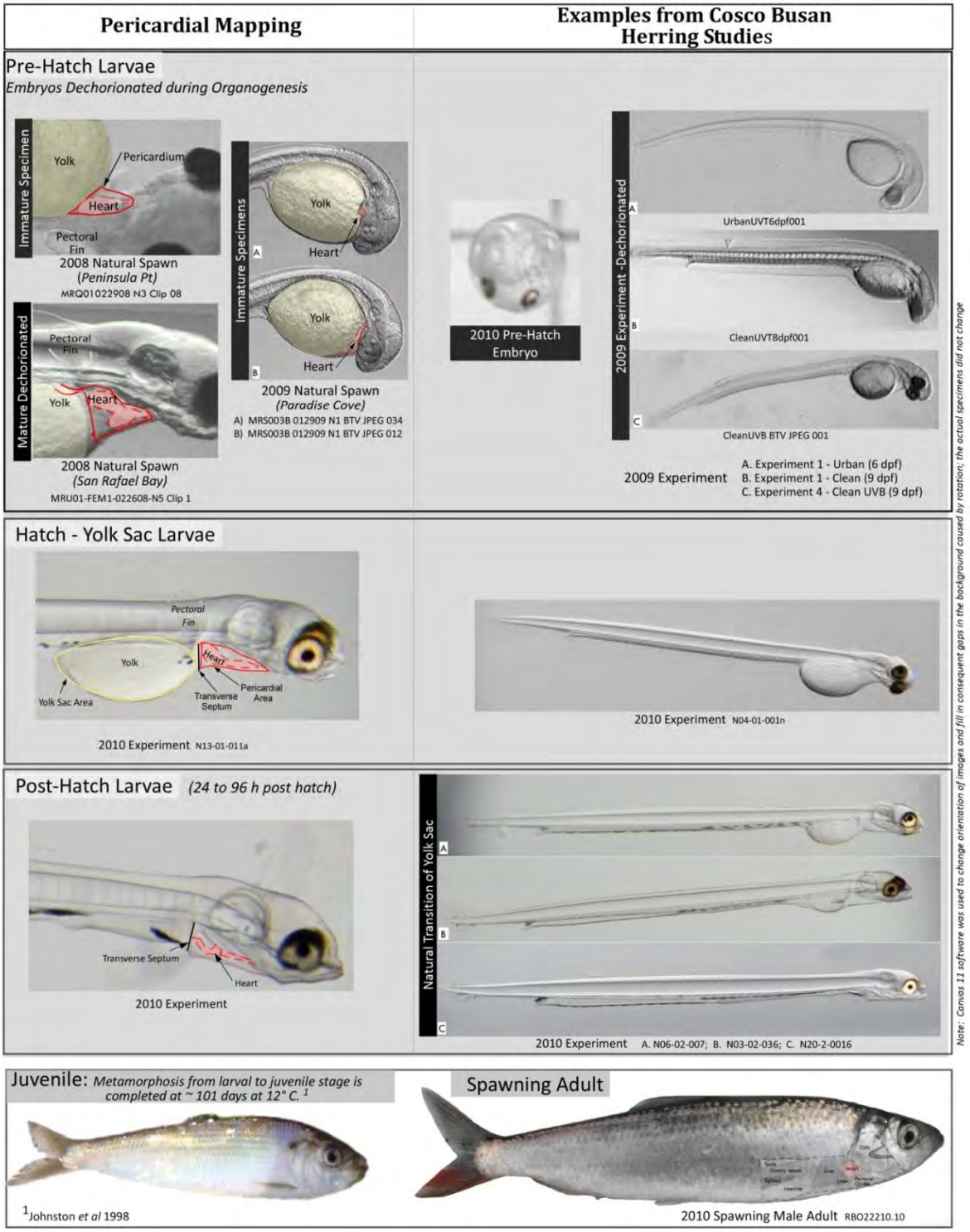


Figure 3-6. Pericardial and Yolk Sac Alterations with Growth.

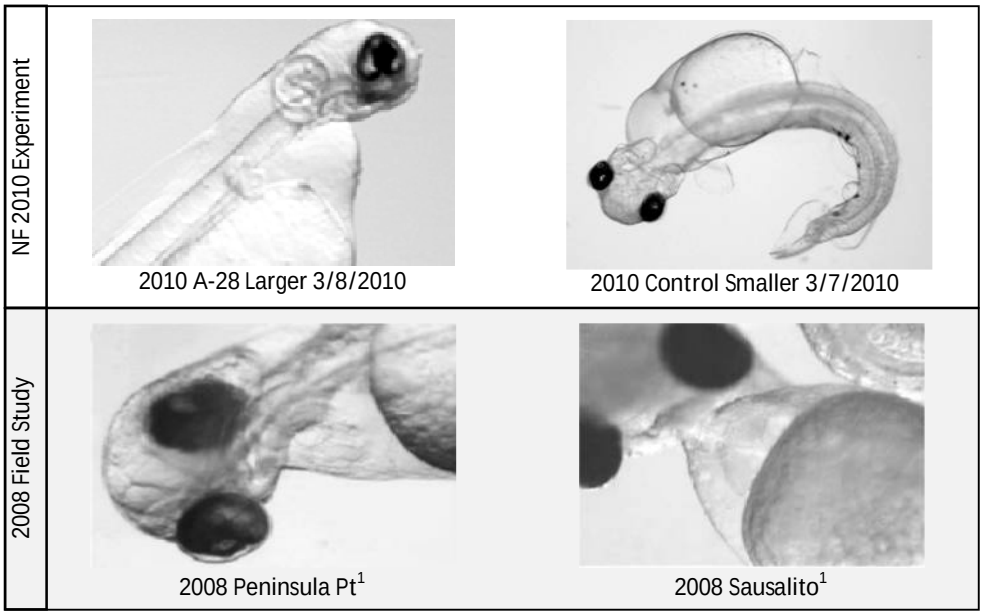


Figure 3-7. Physiological Dysfunction: Pericardial Edema.
[1]NOAA/BML 2008

Table 3-6. Signs of Cardiac Stress (based on Sherrill et al. 2009).

Symptom	Physiological System Affected	Observed in Cosco Busan Studies	
		Qualitative	Quantitative
Inactive or irregular swimming, buoyancy problems	Musculoskeletal & neurologic	Yes (NF 2010)	Yes (NOAA/BML only- 2008 subset)
Reduced weight gain, weakness		Not Assessed	Not Assessed
Hemorrhagic scales or lesions	Integumentary, scales	Not Observed	Not Assessed
Changes in heart & respiratory rates	Cardiorespiratory	Not Assessed	Yes (2008 subset -NOAA, NF; 2010 NF Controls only)
Coelomic cavity edema	Coelom	Yes	Yes
Exophthalmia, cloudiness	Eyes	Yes	Not Assessed

Co-Occurrence of Yolk Sac and Pericardial Edema

The term "ascites" has been used to describe the accumulation of fluid around viscera within the body cavity; a condition that has also been termed "subcutaneous edema" as illustrated by a parasagittal section of field collected herring larvae from an oiled site in Prince William Sound (Marty et al. 1997). Both yolk sac and pericardial edema are present. Osmoregulatory dysfunction has been implicated as the cause of coelomic edema where both the pericardial area and yolk sac demonstrate extreme swelling (exhibiting the characteristic symptoms of blue sac disease). Research has shown that AhR antagonists can cause disruptions to the osmotic regulatory capacity of the skin (Hill et al. 2004). Hill et al. (2004) formulated a model representing the interactivity of causative agents that may act on different target sites and may result in pronounced edema, and postulates on the potential for cascading fluid buildup in one sector may cause damage to other systems. This model has been modified and is presented in Figure 3-9. It should be noted that it is very difficult to evaluate pericardial edema in circumstances when coelomic edema may be overriding the pericardial area (Figure 3-8), particularly when specimen is not oriented laterally; histological examination may be required.

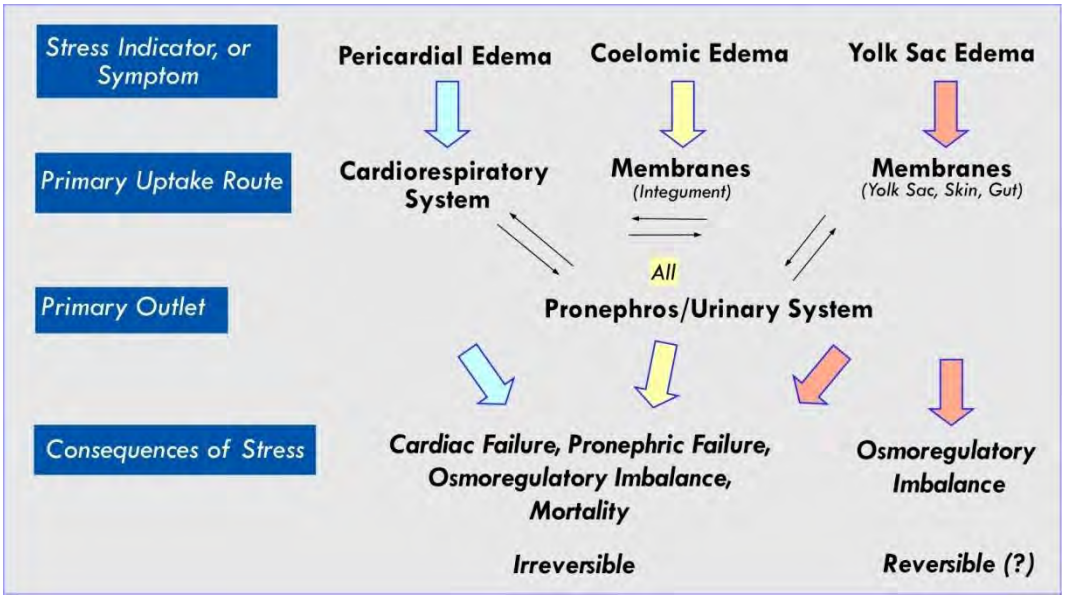


Figure 3-9. Functional Model of Edema.

Figure 3-8. Example of Coelomic Edema Overriding the Pericardium.

Comparison of Edema Occurrence with Contaminant and Environmental Stressors

Edema occurs in both the pericardium and yolk sac areas of developing larvae as discussed above. Both types of edema were observed in the 2008 NOAA/BML field study, the 2009 NOAA/BML laboratory study, and the 2010 NewFields laboratory study; although with differing frequencies. Examples of pericardial edema and yolk sac edema are shown in Figure 3-10 and Figure 3-11, respectively. The 2010 study found significantly higher occurrences of yolk sac edema in the treatment with thermal shock and air exposure (A-TS) as shown in Table 3-7Error! Reference source not found. (NewFields 2010b). The stress of air exposure and warm temperatures in addition to the stresses of changing salinity and temperature during incubation likely weakened the developing embryos. The frequency of occurrence of both types of edema in the 2010 laboratory study was comparable to that observed in the samples from naturally spawned eggs in 2008 for both pericardial edema and yolk sac edema (Figure 3-12).

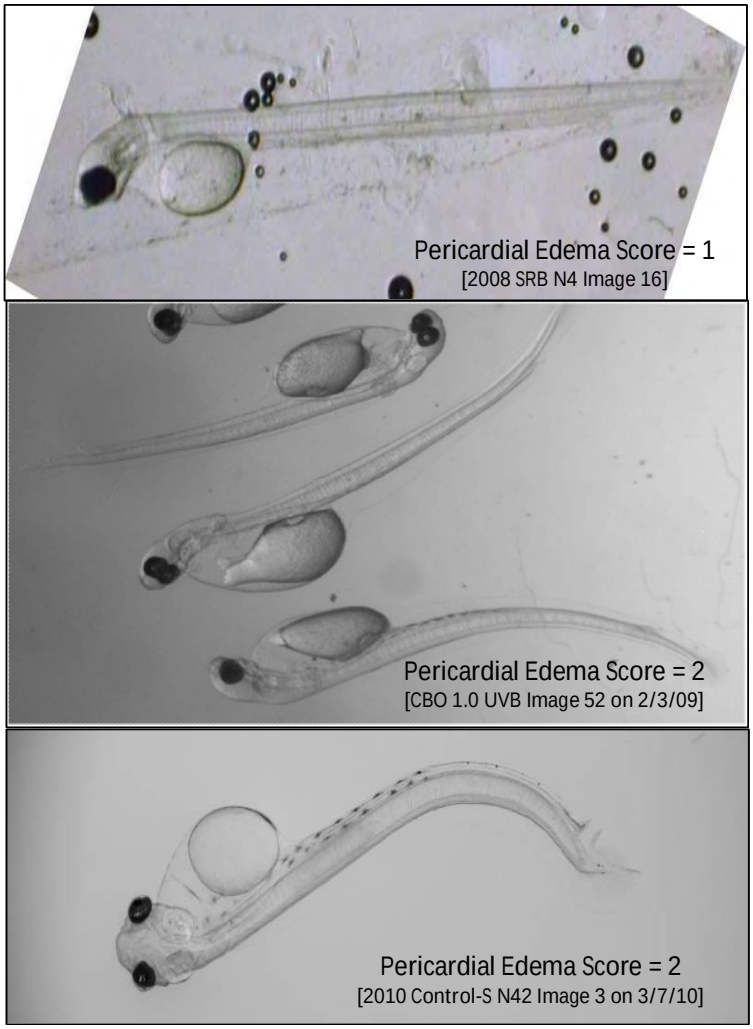


Figure 3-10. Examples of Pericardial Edema.

Pericardial edema was observed in few larvae in 2008 and 2010 while in the 2009 laboratory study pericardial edema was observed in up to 100% of the larvae exposed to fresh CBO and ANS (CBO 1.0 UVB, ANS 1.0 UVT; Experiment 1). As can be seen in Figure 3-12, the frequency of occurrence of pericardial edema varied widely, but treatments with oil exposure, especially in Experiment 1 where fresh oil was used, were well above the range reported for either the 2008 field study or the 2010 laboratory study (1 to 3% occurrence).

Table 3-7. ANOVA Results on Frequency of Occurence of Edema, 2010 Laboratory Study.

Donor Size	Measure	Prob>F	Min. Sig. Difference	Control	CUV	A-16	A-28	A-TS	B-22
			All Larvae						
Smaller	Pericardial Edema (%)	0.671	2.4	1.5	0.6	0.6	0.0	0.6	1.3
	Yolk Sac Edema (%)	0.042	10.7	4.6	4.6	8.8	14.0	16.7	3.7
Larger	Pericardial Edema (%)	0.526	2.3	0.0	0.0	1.0	0.2	0.9	1.5
	Yolk Sac Edema (%)	0.006	16.7	0.0	4.0	15.3	14.3	32.9	10.7

Shading shows treatment mean comparisons to Control based on Dunnett's one-tailed test, purple shaded cells indicate significantly larger means.

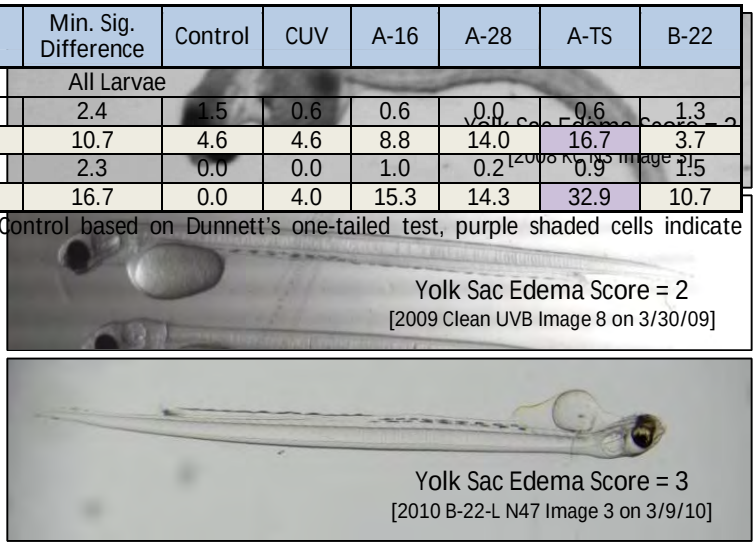
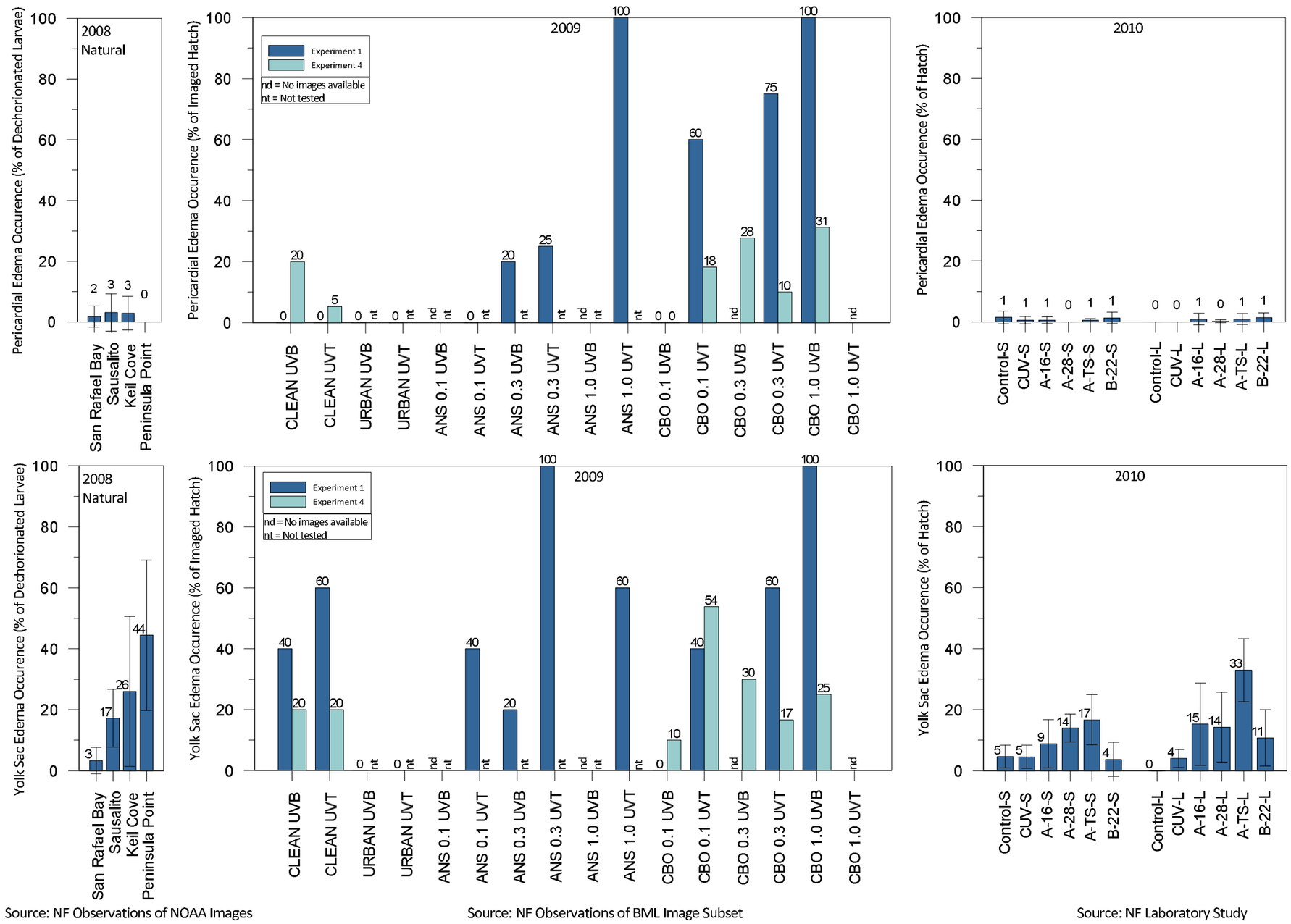


Figure 3-11. Examples of Yolk Sac Edema.

In contrast, yolk sac edema occurrence was roughly similar in all three studies. The known salinity and temperature stresses applied in the 2010 laboratory study produced yolk sac edema in up to 33 ± 10% of the hatched larvae (A-TS-Larger). This rate is similar to the 44 ± 25% observed in dechorionated larvae from Peninsula Point, the location upon which the A-TS salinity, temperature, and air exposure regime was based. Occurrence of yolk sac edema in the other treatments with a range 4 to 17% of hatched larvae was of similar magnitude to samples from Sausalito (17%) and Keil Cove (26%). Samples from the 2009 laboratory study had rates of yolk sac edema occurrence of up to 60% in clean samples and ranging from 0 to 100% range in the oiled samples (based on a random subset of the images provided to NewFields by BML).

In the 2009 laboratory study, only a few samples had larvae with yolk sac edema without pericardial edema (clean, urban, ANS 0.1 UVT, Experiment 1; CB 0.1 UVB, Experiment 4); pericardial edema occurrence was observed in the 0.3 g/kg and 1.0 g/kg oil dosed samples. This is indicative of the influence of oil in the development of pericardial edema as noted by Carls et al. (1999) and Heintz et al. (1999, 2000) whereas the 2010 laboratory study demonstrates that variations in temperature and salinity can induce yolk sac edema. The 10 °C variations noted for temperature in the 2009 study (Incardona and Vines 2009) reproduced in Figure 3-13 are similar to those achieved in the 2010 study (Figure 3-14), therefore yolk sac edema of a similar magnitude may likely be related to the temperature changes as well as other non-oil related factors discussed in NewFields (2010a; Part 2 report on 2009 data).

NewFields observations of edema based on the NOAA images from the natural hatch in 2008 differ from the edema reported by NOAA/BML. This difference appears to be due to the NewFields quantitative method of differentiating yolk sac edema from pericardial edema. Comparable measurements and scoring on images provided by BML for hatched larvae and NOAA for the dechorionated assessments using our refined procedures are the basis for the comparison in Figure 3-12.



Source: NF Observations of NOAA Images

Source: NF Observations of BML Image Subset

Source: NF Laboratory Study

Figure 3-12. Percent Occurrence of Pericardial (top) and Yolk Sac (bottom) Edema in 2008, 2009, and 2010 Studies. Percent occurrence is shown above each bar. Experiment 3 not reassessed for edema.

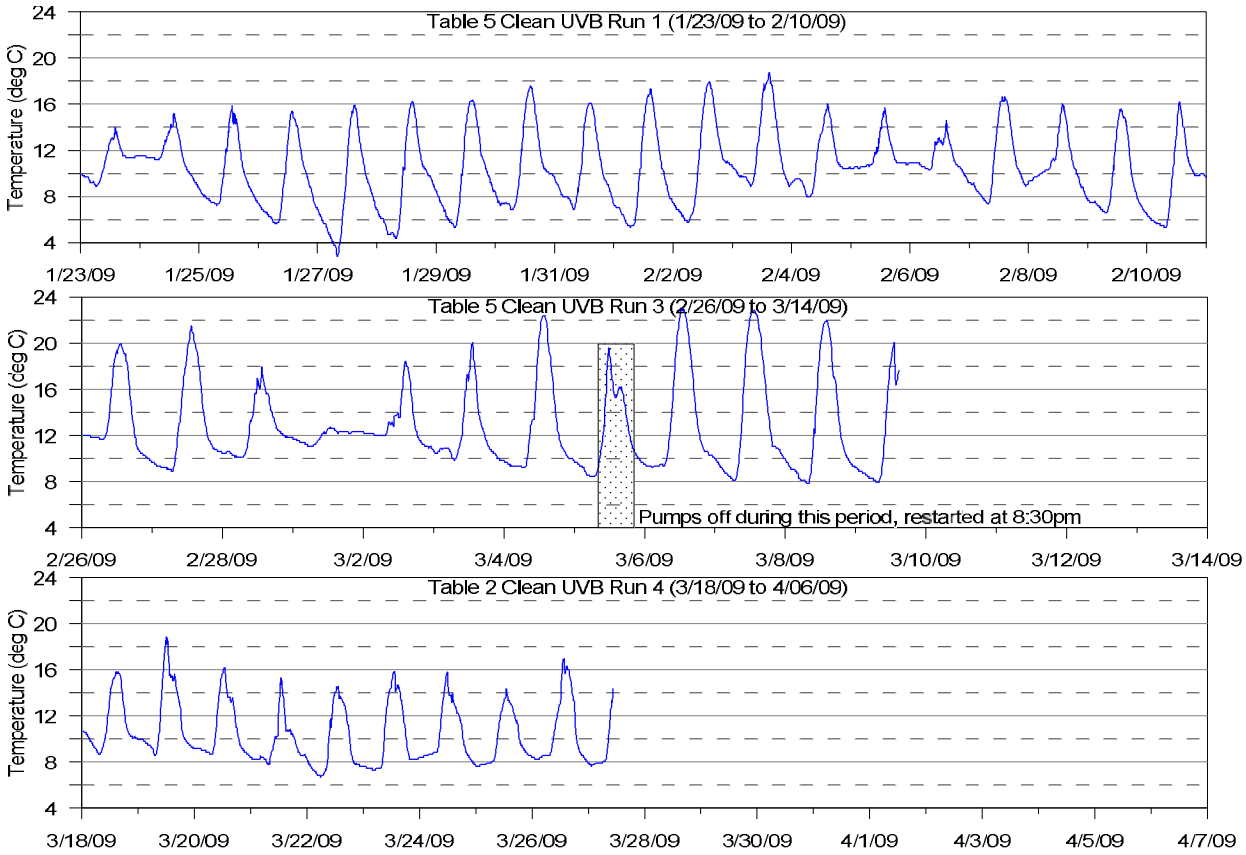


Figure 3-13. Temperature Records from 2009 NOAA/BML Laboratory Study.

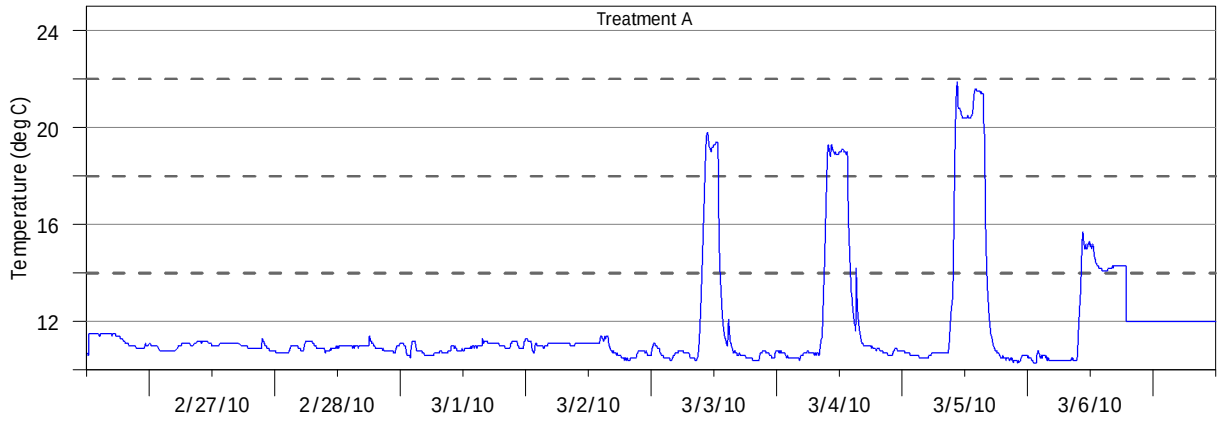


Figure 3-14. Temperature Record from 2010 NewFields Laboratory Study.

3.4 SUMMARY OF BIOLOGICAL SIGNATURE

Abnormalities observed in the 2008 natural herring spawn are consistent with the influence of different salinities at fertilization, variable salinity and temperature conditions at the study sites during development, the impact of air exposure during warm daylight hours and the health and condition of the gametes from the donor fish that were reproduced in the 2010 laboratory study. The much higher occurrence of pericardial edema in 2009 does appear to have a relationship to PAH contamination, from both CBO and ANS, and the virtual absence of pericardial edema in the 2008 larvae indicate that the adverse development observations made during 2008 were not associated with CBO exposure. Additionally, the presence of yolk sac edema and the extremely low occurrence of pericardial edema suggest that the 2008 abnormalities are due to non-PAH stressors.

4 DISCUSSION

The scientific data collected between 2007 through 2010 were used to address premises central to building a causal relationship assessment. The salient points from this review are summarized below; the following paragraphs discuss the arguments used to determine the degree of causality between CBO exposure and biological effects on early life stage development of Clupea pallasi after the Cosco Busan oil spill.

4.1 CBO CHEMICAL AND BIOLOGICAL SIGNATURES

The Pacific herring eggs spawned in 2008 and collected from the intertidal areas characterized as being exposed to CBO (in 2007) were not exposed to CBO during their development. The chemical exposure signature in the developing eggs is consistent with urban background and burned wood or creosote and not with CBO. The developing eggs were instead exposed to urban San Francisco Bay PAH contamination (Peninsula Point and San Rafael) and/or urban Bay PAH contamination augmented by sources enriched with fluoranthene and pyrene that may have come from burned wood or creosote (Keil Cove and Sausalito). Experiments conducted with known CBO exposure in 2009 produced a diagnostic chemical signature of CBO which is distinctly different from ANS and urban sources of PAH exposure under either transmitted or blocked UV light and at all concentrations of CBO. The absence of the CBO chemical signature in 2008 means that the developing eggs were not exposed to CBO and were also not exposed to any of the measured or unmeasured chemical components contained in CBO.

A distinct biological signature was developed during the 2009 and 2010 studies that distinguished the effects of petroleum contamination from other stressors. The biological signature associated with petroleum related effects is the incidence and severity of pericardial edema. While yolk sac edema was present in developing eggs of the 2008 spawn events pericardial edema was not present except for organisms with extensive body axis defects, a similar result as found in the non-chemical stressor study in 2010. The presence of yolk sac edema, extreme body axis defects and early mortality in developing eggs observed in 2010 in the absence of petroleum was also observed in natural spawn events of 2008 and 2009. The absence of the petroleum related pericardial edema response combined with the incidence and intensity of yolk sac edema, extreme body axis defects and early mortality of developing eggs are all consistent with effects in response to factors other than CBO exposure. Neither the biological nor the chemical signatures for CBO petroleum exposure were present during the 2008/2009 natural spawn development observations.

4.2 APPLYING THE CAUSAL RELATIONSHIP CRITERIA

Probability and Time Order Did the aftermath of the 2007 CBO spill result in identifiable fuel oil concentrations at herring spawning locations in central San Francisco Bay in 2008?

After the 2007 release of fuel oil from the Cosco Busan a series of sampling events documented the distribution of the spilled oil. NOAA produced summaries of cooperative observations made by the SCAT teams at various beaches and shorelines throughout the North and Central Bay Region. The areas highlighted as potential locations for CBO related adverse effects that warranted further assessment included Peninsula Point, Keil Cove, Sausalito, and Horseshoe Cove with San Rafael Bay and Point San Quentin as reference areas. Pacific herring were selected as a surrogate test organism for assessment because their intertidal spawning habits were most likely to result in exposure to surface or stranded petroleum, their responses and sensitivity to petroleum contamination have been studied extensively, they are a key ecosystem component in San Francisco Bay and would be sensitive surrogates for assessing the effects of CBO impacts on other species within the Bay. Establishing the potential risk of exposure to Pacific herring during anticipated intertidal/shallow subtidal spawning (January through

March 2008) included the collection and analyses of CBO samples for characterizing a chemical signature of the oil. Parallel efforts were also made by collecting water and sediment samples to compare PAH signatures with CBO. These collections demonstrated that the CBO signature in oil samples and some of the water samples were essentially the same while the sediment PAH chemical signature was very different having much higher concentrations of higher molecular weight PAH compounds than either the CBO or the urban bay water signatures.

The SCAT team observations and collection of samples in 2007 documented the locations that were exposed to CBO at the time of the spill. The samples of water also demonstrated that CBO PAH profile in water samples could be distinguished from urban sources of PAH contamination in San Francisco Bay.

Strength of Association Has it been shown that CBO fuel oil can cause consistent pattern of adverse biological effects?

The details of the observations that were made for the 2008 assessments conducted by NOAA/BML included testing with artificially spawned gametes that were deployed in shallow subtidal environments confined in meshed containers. These samples evaluated whether adult herring were exposed to CBO and whether that exposure was sufficient to cause adverse biological effects in the developing embryos. Very detailed observations with video and still photographic documentation of dechorionated embryos were combined with PAH chemical signature information. Minimal observations of abnormalities were observed in combination with PAH chemical signatures that were not consistent with CBO or with the PAH signature in adjacent sediment. We conclude that embryos were not exposed to CBO or bioavailable sediment PAH and that adults used as donors for the gametes were likewise not exposed. Additionally, the urban PAH chemical signature in the developing eggs was not at concentrations that would indicate adverse biological effects.

Also in 2008, naturally spawned eggs were collected in targeted intertidal areas closely associated with the SCAT designated sites identified in 2007. The PAH signatures in eggs of natural spawn in the intertidal environments also corresponded with urban and not with CBO or sediment PAH signatures. Additionally, total PAH concentrations were less than the LOEC for the lowest literature derived response and thus did not attain high enough concentrations to predict adverse effects on developing herring based on historical information. These combined data indicate that the herring adults and the developing eggs did not contain chemical signatures that indicated exposure to CBO nor were the PAH concentrations observed in the tissues of the developing eggs sufficient to cause the level of adverse effects observed.

Specificity Is CBO the only causative agent to create this pattern of effects?

The experimental demonstration of adverse effects to non-chemical stressors conducted in 2010 and the natural responses of the 2009 Pacific herring spawn in the high intertidal at Paradise Cove coupled with the lack of a CBO chemical exposure signature implies that the 2008 Pacific herring adverse effects were not a response to CBO but to a variety of non-chemical stressors enhanced by an urban signature that in two of the four cases may also contain contributions of PAH from local burning of hardwoods or the presence of creosote pilings.

Consistency of Association Have the same cause-and-effect relationships for CBO occurred under different conditions?

There was no sign of exposure to CBO in the developing eggs and the concentrations of PAH were insufficient to produce the observed adverse effects, therefore the effects in 2008 must be unrelated to CBO exposure. An alternative hypothesis (not supported by any of the data) was that exposure to CBO did occur but that the PAH compounds in CBO were not taken up by developing eggs as normally

predicted or that an unmeasured CBO contaminant affected the development of the eggs and this was the source of the adverse effects that were observed. Because the 2009 laboratory study demonstrated that CBO in water with PAH concentrations as low as 0.2 µg/L would result in a recognizable CBO signature in the egg tissue, the lack of CBO signature in the eggs collected in 2008 means that CBO was not present and therefore unmeasured CBO contaminants were also not present. Because CBO exposure is not implicated, the adverse effects observed in 2008 had to be due to some other factors not contained in CBO. Initial evaluations indicated the assessments in 2008 were confounded by a wide range of potential sources of error or stress ranging from laboratory artifacts to the impact of variable or elevated temperatures, salinity differences, condition of spawning adults, and effects of variable periods of air exposure resulting in solar (UV) impacts or dessication on herring development (NewFields 2009a). The 2009 generator column study with CBO, ANS and Urban contamination and the 2010 alternative contributing factor study that addressed UV, salinity, temperature, air exposure and donor fish/egg health were designed to investigate the impacts of these various stressors on the 2008 herring spawn.

Uptake of PAH from water into tissues of the developing eggs was equivalent to historical information indicating that the PAH compounds contained in CBO are biologically available in a manner consistent with other sources of petroleum (see Figure 2-10 and NewFields 2010a). While there were associations of effects with petroleum hydrocarbon PAH concentrations in eggs the multiple regression with this factor and the principal component analyses (PCA) performed to evaluate the 2009 experiments did not predict the scale of the observed responses in the 2009 studies (NewFields 2010a). Four loading factors using PCA were identified with oil factors explaining ~25% of the variation while light and temperature each also explained ~25% of the variance and the placement on the table and the donor fish group explained ~11% of the variance. Using these loading factors and multiple regression analysis resulted in combined explanation of variance for various biological response measures ranging from 0.14 to 0.72 R^2. There was little consistency of explanation of variation between experimental runs except a large unexplained percentage ranging from ~25 to 75% of the observations made by the various investigators. These results imply that the observations made in 2009 were highly influenced by a number of variables ranging from the oil produced from the generator column to many non-chemical based stressors and measurement artifacts.

Because the non-oil factors implicated in the generator column studies were involved with the effects observed in 2008 as well as 2009, those factors were investigated in the absence of oil in 2010. These experiments matched the fertilization salinities, rates of temperature and salinity cycling, air exposure and the condition of donor adult gametes on the 2008 herring spawning class. It was demonstrated that the development of Pacific herring of two different age classes demonstrated abnormalities that were indiscernible from those observed in 2008. These abnormalities occurred in the absence of CBO petroleum signatures in the eggs of the donor fish and the concentrations of PAH in those eggs were also equivalent to the 2008 egg burdens. The PAH signatures in the 2010 eggs were consistent with the urban generator column signatures obtained in 2009 and the eggs of herring developing at the selected locations in San Francisco Bay that were suspected to be impacted by CBOS. All of these signatures indicated that the eggs from the spawning classes of herring in 2008, 2009 and 2010 were not exposed to CBO.

A major effort of the 2010 study was to quantitatively distinguish between yolk sac and pericardial edema and to also apply those observation methods to 2008 and 2009 studies. There are different etiologies of the onset of each of these forms of edema and it was necessary to separate them. Yolk sac edema occurred when the yolk sac was being resorbed and fluid was either retained or concentrated in the emptying space. This accumulation of fluid is retained in the yolk sac area until the urinary tract is connected and the fluid can be evacuated. Pericardial edema occurs in response to exposure to petroleum compounds that interact with the cardio-respiratory system through the primordial gills.

Pericardial edema is a signature for petroleum and other forms of bioavailable contaminants that enter the blood stream through the developing gills or integument. The presence of yolk sac edema is a broad but possibly reversible response to many types of non-chemical as well as chemical stressors.

The 2010 studies also demonstrated that salinity at fertilization, varying salinity and temperatures combined with stranding stress, and the condition of the donor gametes induced yolk sac edema at similar rates to those observed in the 2008 study. Pericardial edema was only observed in 1% of the 2010 larvae; these larvae also exhibited severe body axis defects, both in the treatments as well as in the Control samples. Similarly, pericardial edema was observed in approximately 2% of the 2008 larvae compared to much higher occurrences with exposure to oil in 2009 (ranging from 10 to 100% of larvae per treatment).

Predictive Performance Does experimental evidence support the cause-and-effect relationship; it there a dose-relationship?

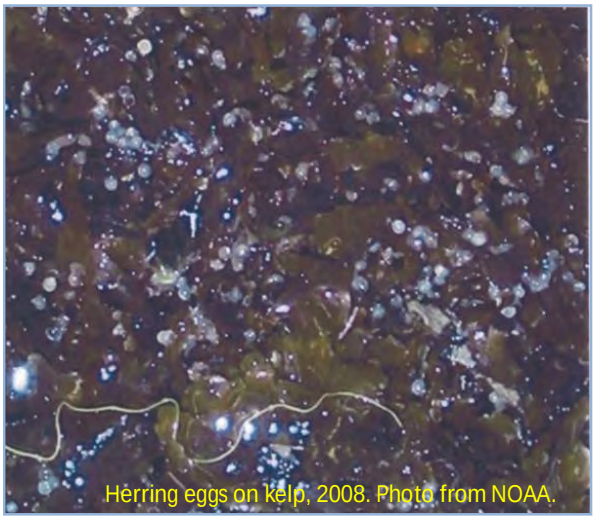
The 2009 generator column studies conducted by NOAA and BML were designed to demonstrate that 1) responses comparable to those observed in the 2008 assessments could be produced under controlled laboratory conditions using CBO and 2) that the chemical signature and PAH concentrations obtained from CBO exposure would replicate the observations in 2008. The experiments demonstrated that CBO exposure would produce an identifiable CBO chemical signature of PAH compounds that was different than the urban signature and different than the signature of the 2008 assessment. This 2009 CBO chemical signature was discernable at all exposure concentrations in the 2009 generator column study. The 2009 studies also demonstrated that the 2008 signatures were consistent with the signatures produced from the urban columns but not the CBO columns. The PAH concentrations in egg and water samples from all concentrations of CBO columns, including the highest, were less than the body of scientific literature documenting effects on various species throughout the world. There were observed effects of pericardial edema with CBO exposure but this effect was not observed in 2008 nor was it observed in the 2010 non-oil exposures, except in the cases of larvae with extreme body axis defects. These extreme body axis defects, arrested trunk development or failure of uncoiling, have been documented to occur in the presence of UV, elevated temperatures, and suspended solids (Strähle and Jesuthasan 1993; Morley and Batty 1996; Johnston et al. 2001; Griffin et al. 2009). In addition to body axis defects these factors also cause early developmental mortalities (within two days of fertilization) which were also observed during the 2010 studies. The results of the 2009 study indicated that CBO exposure is discernable even at the lowest concentrations of exposure and that the biological response of increased pericardial edema can be seen even in the compromised Experiments 3 and 4 when CBO is present.

The concentrations of PAH in 2009 generator column studies with the highest concentration of CBO is consistent with the LOEC for herring edema responses available in the literature. Lower concentrations of CBO exposure also showed pericardial edema responses that were not observed in 2008 assessments. All CBO exposure concentrations produced a diagnostic chemical signature of exposure even in those eggs showing no adverse effects. The generator column studies in 2009 demonstrated that pericardial edema could be observed in response to the two higher exposure levels of CBO in the water and in the tissues of the developing eggs and that those concentrations of total PAH were not observed in the eggs of the naturally spawned herring in 2008.

Coherence Were CBO concentrations in 2008 above known toxic thresholds?

Concentrations of PAHs in developing embryos collected in 2008 from naturally spawned eggs were consistently below those levels found to produce adverse effects (Barron et al. 2002, 2003; Brannon et al. 2006; Carls et al. 1999, 2000; Heintz et al. 1999, 2000; Incardona et al. 2009; Kocan et al. 1996; Vines

et al. 2000). In addition, as discussed above, neither the CBO chemical signature nor the biological signature of pericardial edema was present in the 2008 natural spawn.


Herring eggs on kelp, 2008. Photo from NOAA.

A summary of the individual criteria used to examine causality between the CBO spill and the adverse effects to herring in 2008 is shown in Figure 5-1. Together these criteria present a weight of evidence to determine the outcome of this investigation. When CBO is present, an identifiable chemical signature using the relative concentrations of the 39 PAH and alkylated homologs will occur even at concentrations of PAH as low as 0.2 µg/L (Experiment 3 - 0.1 CBO UV blocked). The PAH compounds in developing eggs demonstrate equal uptake from water as other sources of PAH and result in an identifiable chemical signature. The concentrations in the tissues of the eggs in 2008 (25 to 280 µg/kg) predict little to no effect from PAH exposure based on historical information that indicates the LOEC for yolk sac edema at ≥ 400 µg/kg (Barron et al. 2003 and Carls et al. 1999).

Pericardial edema was observed in developing eggs exposed to CBO during the 2009 experimental study at total PAH concentrations in excess of 0.6 µg/L in the water, resulting in tissue concentrations of >79 µg/kg, both of which are lower than the literature would suggest. These responses in organisms exposed to a lower level of PAH contamination suggest that either there are unmeasured contaminants in the CBO that increase the response of exposed organisms or that there are other contributing stressors in the experimental array enhancing the adverse effects. Despite this potential the chemical signatures of these PAH compounds in the 2008 eggs indicate that they were not exposed to CBO before or during development but were exposed to urban sources of PAH contamination. Additionally, the types of abnormalities observed during 2008 are consistent with the influence of different salinities at fertilization, variable salinity and temperature conditions at the study sites during development, the impact of air exposure during warm daylight hours and the health and condition of the gametes from the donor fish. The presence of pericardial edema in 2009 does appear to have a relationship to PAH contamination and its very low incidence (0 to 3%) during the 2008 assessments indicate that the adverse development observations made during 2008 were not associated with PAH exposure. Additionally, the presence of yolk sac edema and the low incidence of pericardial edema suggest that those 2008 abnormalities are due to non-PAH stressors. In addition to those factors that were investigated during 2010, non-studied factors such as the presence of suspended solids soon after fertilization when the eggs are still sticky will result in high percentages of body axis defects (Griffin et al. 2009).

The observations and conclusions presented in the previous section imply that pericardial and yolk sac edema can be used to distinguish between petroleum contamination and other forms of stress. This conclusion is different than some past investigations where both pericardial and yolk sac edema have been described to represent exposure to petroleum and where additional abnormalities are also produced during those exposures (bent spines, kyphosis, lordosis, scoliosis; arrested development, early embryo mortality, etc.). A potential reason for this discrepancy is perhaps due to the relatively low concentrations of PAH exposure in the experimental generator columns, the addition of other stressors during the experimental demonstrations, and the quantitative method that we used to distinguish between pericardial and yolk sac edema.

We hypothesize that adverse biological effects increase as the number and strength of stressors increases. In other words, negative biological effects are sequentially manifested in adjacent organs as the extent of biological damage increases. Based on the developmental studies that were conducted between 2008 and 2010 it appears that pericardial edema begins to occur at relatively low concentrations of PAH as determined in the compromised tests conducted in 2009 (as low as 0.2 µg/L in water and egg tissue levels of 20-40 µg/kg). Pericardial edema is a sentinel of PAH related effects and begins at lower concentrations than previously documented (>3.5 µg/L and 400 µg/kg in egg tissue). The additional stressors involved in the 2009 experiments likely enhanced this apparent response to lower concentrations of PAH. The 2010 multiple stressors study showed that yolk sac edema can occur on its own or in association with pericardial edema. Yolk sac edema is manifested with fluid build-up primarily related to a combination of yolk sac adsorption, diffusion across osmotic gradients, and the reduced ability of the fish to excrete significant fluid levels until the nephritic system develops. Enlargement of the pericardial area or yolk sac may induce vertebral defects that cause body axis contortions which can result in poor swimming movements. Therefore pericardial edema may be the initial response to PAH exposure that triggers a cascade of effects resulting in abnormal development of larvae.

The 2008 samples show little or no pericardial edema indicating lack of exposure to effects based concentrations of PAH and the chemical signature in the tissues of these developing eggs show no CBO exposure. There were significant effects observed during the 2008 assessment but these effects were related to other factors consistent with the intertidal conditions during the spawn events (salinity, temperature, stranding and UV) and with laboratory artifacts and conditions that impacted the assessment and required additional work to remove those impacts. The 2010 experiments accounted for many of those impacts but there are still unexplained factors that contributed to the 2008 studies; however these factors are not related to CBO.

The final conclusion from all of the studies conducted from 2007 through 2010 is that CBO was not the primary cause of disrupted development of Pacific herring embryos in 2008. This conclusion is based on the absence of a chemical PAH signature that was demonstrated to occur when CBO was present at less than effects based concentrations. It is also based on the lack of the biological signature of response to an exposure to petroleum, pericardial edema. Further, the types of effects that were observed in 2008 are consistent with those observed in the non-petroleum stressor experiment conducted in 2010. Although the intensity of some of the 2008 effects were greater than observed in 2010, some of it can be explained by the lower degree of air exposure employed during the experiment than occurred in the intertidal environment in San Francisco Bay and the unmeasured impact of suspended solids that were not evaluated during this study but which have been documented as impacts during fertilization (Griffin et al. 2009).

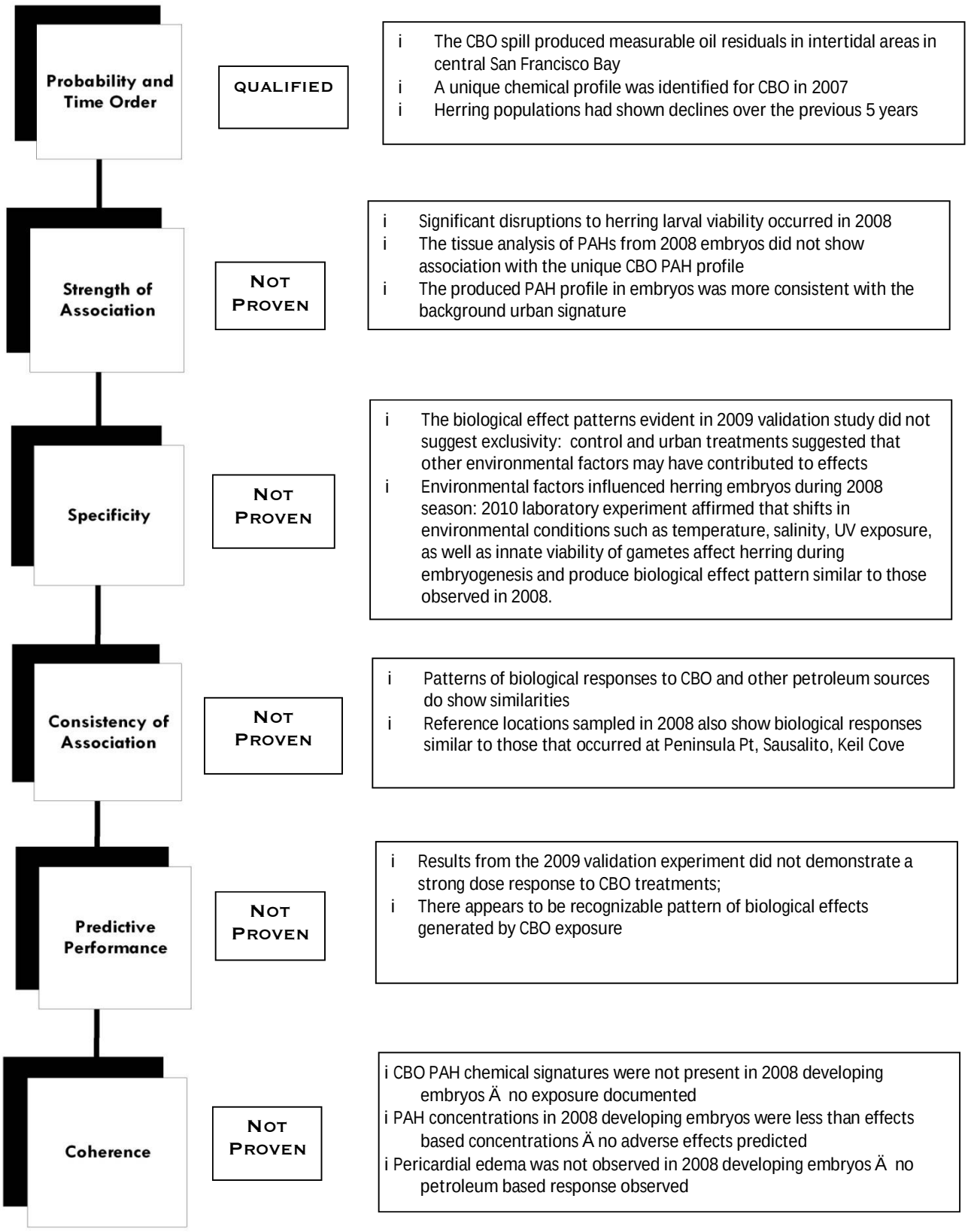


Figure 5-1. Weight of Evidence after Critical Examination of Causal Framework.

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