

***Biological Review of 2009 Experimental Data on  
Cosco Busan Oil Effects on Herring***

***Part 1: Test and Data Validation***

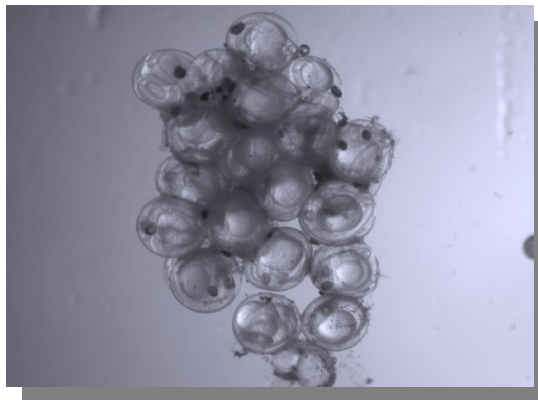
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**EXECUTIVE SUMMARY**

**Cluster of Herring**  
**Embryos**  
Three principal quality assurance review inquiries are addressed in this report relative to the experimental program performed by NOAA/BML in 2009. Interpretation of the data output from these studies is dependent on assessing data qualifications before relationships between adverse biological responses can be assigned to any particular cause, including petroleum. The following QA/QC inquiries are addressed separately; concluding statements are made based on the outcome of the following investigations:

1. Are the tests of acceptable quality to validate the performance of the experimental runs?
  - a) Were test organisms healthy? Did test conditions provide for adequate health during experiments?
  - b) Were the experiments isolated from extraneous sources which may contribute to the toxicity relationships observed?
2. Are the test conditions maintained within guidelines established for early life stage tests?
3. Are the experiments repeatable?

**Question 1.** Test data must be validated by demonstrating that the test organisms were of appropriate health and sensitivity and that the laboratory conducting the test can perform the test under ideal conditions. Test validation for early life history studies with developing eggs and larvae require that a minimum of 65% of the developing organisms hatch successfully as normal larvae. The laboratory control treatment is a surrogate to determine if the test organisms are healthy and if laboratory testing conditions support adequate water quality to maintain health for the duration of the test period. The laboratory control was only performed for experimental runs 3 and 4. The laboratory control for experiment 3 attained adequate normal hatching (77.7%) whereas experiment 4 did not achieve minimum standards. This determination validates experiment 3 and indicates that the test population for experiment 3 was healthy and that the laboratory conducted the tests appropriately for only this experiment.

Because of the complexity of the experimental design, a second 'method' control was used for these studies. These controls ("clean" controls with or without transmission of ultraviolet light) established whether the generator column procedures maintained outdoors were under sufficient control to allow the experiments to be conducted without introducing experimental artifacts. These controls were used for all four experiments but

only the first experiment had adequate normal hatching (91 and 88% for clean UVB and UVT respectively).

These test results indicate that data from Experiment 1 are the most useful data for evaluation. Although a laboratory control was not performed for this test, the clean control indicated that the outdoor column generation procedure did not produce artifacts that impacted the test results. Experiments 2-4 had inadequate control or experimental control survival to normal hatching indicating that the data produced during these experiments is flawed and that experimental artifacts were associated with the outdoor testing procedures reduced normal development. It is unfortunate that experiment 1 was treated as a 'dry run' of the procedure and did not have chemistry samples collected. The remaining tests (experiments 2-4) do not pass quality control evaluation and their test results would not be accepted by ASTM, USEPA, WDOE, or Standard Methods.

**Question 2.** Standard test procedures have been developed for early life history fish testing for multiple species of fish, including Pacific herring. These protocols indicate standard water quality conditions that must be maintained in order to minimize the potential of laboratory artifacts reducing the value of the information that is collected. The factors that may have contributed to the failure of the generator column testing (oil exposures) were several. It is incumbent upon a laboratory to demonstrate or discuss these conditions to indicate why they would not cause adverse effects that invalidate the tests. Those pertinent discussions and documentation have not been released by the laboratories performing the experiments.

One condition that negated acceptable control for water quality assurance is the lack of temperature control with ranges of  $>15^{\circ}\text{C}$ . This trend of temperature exceedence was observed daily during the conduct of the tests and greatly exceeded the acceptable range of  $\pm 3^{\circ}\text{C}$  over 24h periods as recognized by ASTM, Standard Methods, WDOE and USEPA. Rapid change in temperature is a stress that is not allowed for biological testing performed for regulatory purposes. Additionally, there was a significant amount of time the temperature also exceeded maximum levels acceptable for herring tests ( $18^{\circ}\text{C}$ ). Temperatures above this range have been found to adversely affect Pacific herring obtained from San Francisco Bay. Both the maximum and the rate of change in temperature demonstrate that the outdoor generator column work in 2009 was flawed and resulted in conditions that could contribute to the extremely low survival and normal hatching rates observed for experiments 2-4.

A second set of water quality parameters were out of QC bounds: dissolved oxygen saturation and the concordant response of pH. Dissolved oxygen saturation greatly exceeded 100% (maximum of 431%) for experiments 3 and 4; dissolved oxygen was not assessed for experiments 1 and 2. ASTM recommends that dissolved oxygen saturation be maintained between 60 and 100% to minimize gas-bubble disease. This saturation was greatly exceeded probably due to the release of oxygen by algae during the day (oxygen use by algae in the dark was not assessed) and also resulted in pH drift.

The third water quality issue was salinity. The target salinity for optimum growth and hatching success of herring is 16‰. However, it has been noted that salinities during a spawn event should be mirrored in laboratory tests. The 2009 column studies were conducted at 22‰. The combination of temperatures in excess of  $18^{\circ}\text{C}$  and salinities in excess of 16‰ have been demonstrated to produce adverse effects.

The last water quality related issue was the algal fouling on the outer surfaces of the developing eggs. Photographs of the developing eggs showed extensive coverage of algae on the outer surface of eggs that should be essentially transparent. The algal blooms were extensive and occurred on the slides containing the embryos in sufficient densities to not only influence the water quality measures but also most likely affected the osmotic regulation during embryonic growth by covering the chorion. The algal densities intensified over time (experiment 4 = experiment 3 > experiment 2 > experiment 1). This fouling of the eggs demonstrates that the outdoor generator columns and the presence of the fouling on controls as well as treatment containers create another problem with interpreting the results of all experiments.

All of these factors indicate that the testing procedure employed during 2009 was unable to maintain acceptable water quality limits. Lack of control of these factors contributes significantly to the poor results in the outdoor laboratory controls. The indoor controls indicate that acceptable herring response could be obtained under controlled conditions for one of the test (experiment 3) but the outdoor generator columns were not under control except perhaps in experiment 1 that did not have water quality measured or chemistry samples taken to demonstrate exposure of the eggs to oil from the columns.

**Question 3.** Treatments with Alaska North Slope Crude petroleum (ANS) were used as a 'positive' control. This source of petroleum has well-characterized response patterns using the assessment endpoints that were evaluated. These assessment endpoints included body axis defects, pericardial and yolk sac edema, head to trunk angle, and pigmentation differences. Successful demonstration of the expected responses based on chemical concentrations in the tissues of test organisms exposed to ANS would suggest that the data produced even in the presence of the artifacts indicated above might provide useful conclusions. The ANS syndrome could not be replicated in these experiments. Treatments with an urban control (without addition of oil) showed a gradient of effects, some remarkably similar to the oiled treatments and the clean control.

**Conclusion:** Based on the poor survival to normal hatching in controls (lab and clean control generator) these data would not be acceptable for use in regulatory programs conducted by USEPA and state agencies. The range in water quality characteristics are extreme and laboratories conducting tests with this lack of control are obligated to explain why the data is not compromised in technical reports provided for these regulatory programs. This species (Pacific herring) is not a test species that has been used in many regulatory programs but it is used in the state of Washington as a standardized test organism for regulatory programs, it has been used extensively in Alaska for experimental work on the effects of petroleum, and it has been used as an experimental fish for a wide range of investigations. The NOAA/BML studies indicate that the organism can be used under laboratory conditions and adequate survival can be obtained (experiment 3). It also was found to be able to develop and successfully hatch into normal larvae using the experimental generator columns (experiment 1). Unfortunately, chemistry samples were not taken for experiment 1 so a direct measurement of response to tissue concentrations of petroleum cannot be made and the generator column work for experiment 3 provided very poor development and survival to normal hatch for the experimental controls (<37%). Part 2 of the report on the 2009 generator column work will examine the measurement endpoints that were collected and develop a best fit multiple regression statistical

assessment of the probable causes of the low survival and adverse responses that were observed in 2009.

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## INTRODUCTION

### BACKGROUND SURROUNDING 2008 AND 2009 STUDIES

The studies conducted by the Bodega Marine Lab (BML) and National Oceanic and Atmospheric Administration (NOAA) in 2008 and 2009 were designed to demonstrate the range of effects potentially caused by the Cosco Busan oil spill in San Francisco Bay in November 2007. The spill released an estimated 54,000 gallons of oil into San Francisco Bay. A fuel oil slick expanded over the next several days and, based on trained SCAT team observations, was found to visibly contaminate shorelines of the Central Bay with discontinuous patches of oil along bays and headlands (NOAA and BML 2008; Lankford et al. 2008). Quantitative, defensible chemical and biological associations are needed to support the hypothesis that oiling on intertidal and shallow subtidal sediments from the spill in November of 2007 had a direct and measureable adverse effect on the development of Pacific herring embryos into larval fish during the 2008 spawning season. Pacific herring (*Clupea pallasii*) were chosen as the test species for several reasons. First, they spawn on kelp and algae in shallow subtidal areas where surface oil is most likely to accumulate. Second, herring have been used as a surrogate testing organism by numerous researchers to demonstrate species sensitivity to low levels of petroleum compounds (NOAA 2007). Third, it was suggested by NOAA and BML that these organisms would serve as surrogate or proxy species for quantifying the amount of damage to nearshore spawning fish species in San Francisco Bay (NOAA and BML 2008). NOAA, BML and other Trustees conducted the following activities in order to compile relevant data needed to form the basis of a resource damage assessment.

NewFields conducted an independent data validation assessment of the images and data provided by NOAA and BML. The assessment in this document was conducted prior to receipt of the November 2009 report submitted by Incardona et al. (2009) although information on the size of the donor fish from the NOAA/BML report was added during revision.

### MILESTONES - 2007

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- Collected samples from Cosco Busan and the water column within days of the spill (2007)
- Established a chemical ‘fingerprint’ of the Cosco Busan oil and detected the chemical fingerprint in water samples collected from different locations within San Francisco Bay. (Douglas 2009a)
- Characterized (semi-qualitative) the degree of oiling along shorelines and headlands by members of NOAA’s specially trained SCAT Teams (Shoreline Cleanup and Assessment Technique) in 2007

The initial chemical results of the water column samples, collected within days of the spill, documented the presence of identifiable Cosco Busan (CB) oil within parts of the Bay. This oil signature could be “chemically fingerprinted” and identified at very low concentrations in the water column after initial weathering had occurred. The SCAT teams *visually* identified relative levels of shoreline oiling within the Central Bay area (NOAA and BML 2008). It is NewFields’ understanding that chemical analysis was not

performed in conjunction with the visual inspection of selected locations. These areas were selected for further study to examine potential effects of stranded oil that might be present during the spawning events of Pacific herring and the development of their embryos to newly hatched larvae during the spawning season in 2008.

#### MILESTONES - 2008

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- Harvested gametes from adult herring in San Francisco Bay were analyzed for presence of PAHS. The levels of PAHs in ovaries were lower than those reported for Puget Sound herring (NOAA and BML 2008). Gametes from adults were also artificially fertilized and placed at shallow subtidal sites chosen by the SCAT teams to represent potential subtidal areas that may have had various levels of oiling in 2008. Harvested naturally spawned eggs from intertidal/shallow subtidal sites suspected to have various levels of oiling were also examined. Additionally, the naturally spawned eggs were chemically analyzed to demonstrate exposure to the contaminant of concern “weathered” Cosco Busan oil.
- Transported the naturally spawned eggs and the artificially spawned and subtidally exposed eggs to a laboratory to determine developmental viability of the eggs, embryos, and larvae. NOAA examined a subset of early embryonic stages by dechoriation of developing embryos, whereas BML allowed a subset of eggs to proceed to hatching under laboratory conditions.

The 2008 experiments and field/lab observations were designed to demonstrate the onset of abnormal development and growth similar to a widely recognized syndrome of effects documented to occur in Pacific herring as well as many other species of fish after exposure to petroleum (Pearson et al. 1985, 1995; Brown et al., 1996; Hose et al., 1996; McGurk et al., 1996; Norcross et al., 1996; Marty et al., 1997; Middaugh et al., 1998; Carls et al., 1999, 2005; Heintz et al., 1999, 2000; Incardona et al., 2004, 2005, 2006, 2009). These studies used established early life history methodologies developed for rearing and exposing developing eggs to chemicals of potential concern until hatching. These procedures have been developed over the past 20 years and have been standardized during the past 10 years [ASTM 1998 for a suite of fish species, Dinnel et al. (2005, 2008) for Pacific herring].

Multiple effects were evaluated for field and laboratory efforts using many high quality video and still pictures of the developing and dechorionated embryos evaluated by NOAA and BML for the 2008 efforts (NOAA and BML, 2008). The results reported by NOAA and BML in 2008 found “in general, markedly abnormal biological responses were observed widely in samples of embryos naturally deposited in the intertidal zone at oiled sites. Less severe effects were observed in caged embryos incubated farther from shore in the shallow subtidal zoned of oiled sites.” (NOAA and BML 2008).

NewFields Biological Assessment of the 2008 data found abnormal development occurred in some samples collected from the intertidal areas but found no correlation or concordance of effects associated with the SCAT team ranking of oil or by the concentrations of petroleum compounds measured in the tissue of the developing eggs collected from locations within the intertidal or subtidal areas (NewFields 2009). In fact, the PAH distribution in the developing eggs from these locations matched more closely an urban pyrogenic PAH fingerprint that is distinct from weathered CB oil (b 2009).



The inconclusive and disparate results observed in 2008 led to a series of controlled laboratory experiments conducted in 2009 designed to demonstrate a biological signature response that could be used to demonstrate exposure to CB oil.

#### MILESTONES - 2009

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- Harvested adult herring for chemical analysis of gametes (2009).
- Conducted four experiments using an outdoor array of columns containing oiled gravel using the procedures developed by NOAA at Auk Bay, Alaska and others (Marty et al., 1997; Short et al., 1997; Carls, et al., 1999; Incardona et al., 2007, 2009). The treatments included various combinations of laboratory controls, clean controls, urban controls, ANS crude oil concentrations, and CB oil concentrations under UV blocked (UVB) and transmitted light (UVT) exposures.

#### ***EVALUATION OF 2009 LABORATORY EXPERIMENTS***

The 2009 experiments also assessed early developmental endpoints, although these endpoints did not necessarily reflect the same parameters evaluated in the 2008 experiments. CB oil was added to gravel at specific concentrations (0.1, 0.3 and 1.0 g oil/kg dry sediment) and placed in generator columns using methods developed by NOAA and others. The columns deliver a continuous flow of oil-contaminated seawater to produce exposures in chambers containing herring eggs incubating on glass slides. Weathered ANS crude oil was included in three of the four experiments to provide a reference toxicant to compare results to those previously published documenting the PAH syndrome of effects on developing fish. This is a positive control where an expected response is necessary to demonstrate repeatability of the dosing procedure and consistency with past experiments. Figure 1 is schematic diagram of the experimental array of generator columns and exposure chambers.

NOAA examined a subset of embryos ranging from 6 days post fertilization (dpf) to 9 dpf and subsequently dechorionated the embryos for examination. BML allowed a subset of the embryos to continue to develop to hatch using incubators (hatch typically ranged from 9 to 13 dpf). Larvae specimens were then scored for hatching success and morphological deformities.

# Plan view of oiled gravel column exposure system

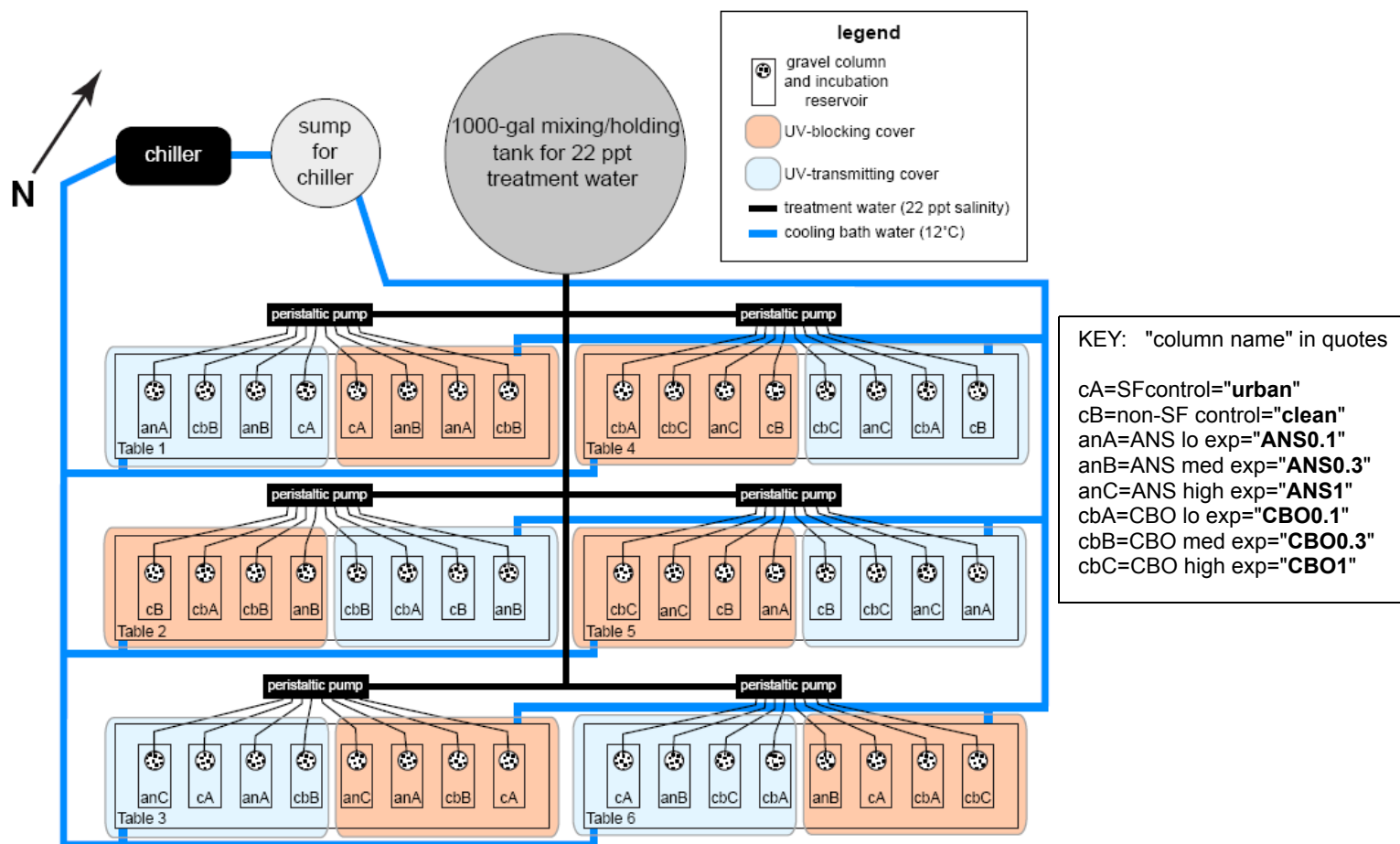


Figure 1 Schematic of Column Array

Three additional types of controls were also used during the course of the experimental runs to validate different aspects of the testing process.

- An indoor laboratory control was included as part of the test design for all of the experiments. A laboratory control is used to demonstrate the overall health of the test organisms under controlled indoor laboratory conditions which is a required assessment procedure to document the validity of tests (ASTM 1998). Embryo and larval images of the laboratory controls were provided to NewFields for Runs 1 and 2. However tabular data relative to hatching success and larval development were not provided for these runs. No conclusive statements regarding the health or viability of the test organisms can be made for Run 1 or 2. Imagery and data were provided for Runs 3 and 4, and an assessment of the health and viability of the test organisms were performed for these two experimental runs.
- A 'clean method' control was included in the experimental runs in order to assess whether the outdoor exposure system and presence or absence of transmitted UV light had effects that were separate from the effects of oil and presence or absence of UV light.
- An urban control was included in experiments 1, 2, and 3 to determine if San Francisco Bay background levels produced a notable effect on Pacific herring development in order to compare to any effect of the CB oil acting alone under experimentally controlled concentrations. The gravel for this urban control was collected from an urban beach in northern San Francisco Bay believed to be outside of the influence of CB oil.

The objective of this first report is to evaluate control test results provided by NOAA and BML for the 2009 column experiments 1-4 during which herring embryos were also exposed to CB oil and, in some experiments, ANS oil at environmentally relevant concentrations (0, 0.1, 0.3, 1.0 g/kg). Additionally, NewFields conducted an *independent* review of image records provided for each experiment to determine if any biological response patterns were associated with concentrations of CBO and ANS oil and/or in the presence or absence of ultraviolet light. NewFields' biological review of the 2009 data of NOAA and BML leads to four areas which need to be fully addressed. This report focuses on test validation, experimental design, and Quality Assurance/Quality Control (QA/QC). Other areas including comparability of data collected with previous studies and linkage to the 2007 spill event are in preparation and will be reported in an ensuing document.

ASTM, USEPA, and/or WDOE have QA/QC testing criteria that must be met for a test to be considered acceptable for interpretation. These agencies also provide testing guidance to aid in establishing criteria that may vary depending on the elements of the experimental plan. The following attributes were addressed in the evaluation of quality requirements derived from standard testing protocols:

- 1) *Are the tests of acceptable quality to validate the experimental runs?* Validation of the test is based on adequate response of test control (laboratory) while validation of the outdoor exposure method is accomplished with the clean control. The urban control will separate the

potential contributing factors of background urban signatures from the test results with petroleum hydrocarbon exposures.

- 2) *Are test conditions maintained within guidelines established for early life stage tests?* Usability of the data is determined by establishing and maintaining water quality conditions within narrow ranges that have been demonstrated to produce healthy population of test organisms in the control exposures.
- 3) *Are the experiments repeatable?* Are reference toxicant test results comparable to other published studies? The testing procedures used and the data generated should be repeatable and verifiable by other researchers. Standard tests, such as reference toxicant exposures are used to ensure the test organism is responding to a known contaminant in a similar manner when compared to other data. In this case, ANS treatments were considered a reference toxicant test. A second report will address the comparability of 2009 column data results with previously published responses of herring to ANS exposures.

The first attribute, acceptable control survival, indicates the test population is healthy, the laboratory demonstrates sufficient experience by performing the test adequately, and the testing procedures and water quality conditions are managed to permit further evaluation. The laboratory control, clean method control, and urban control data were used in combination to address this first attribute. The second attribute, maintenance of water quality within established limits prevents synergistic relationships between contaminants and undue stress placed upon test organism by varying exposure conditions. Fluctuating water quality conditions can result in poor survival of the test organisms. The last attribute, demonstrating comparable responses of test organisms to known reference toxicants indicates the health of the test population and its sensitivity to a known toxicant are similar to previous testing. A similar response of the test organism to a known toxicant signifies the current tests are an accurate representation of organism response to the contaminant.

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## **VALIDATION OF 2009 COLUMN EXPERIMENTS**

### **CONTROL TREATMENTS**

- 1) **LABORATORY CONTROL.** ASTM (E 1241 section 13.1.3) indicates that a dilution water or solvent control is required as part of the experiment, otherwise the test may be considered unacceptable. Experiments 1, 2, 3 and 4 included a laboratory control tested indoors (in an incubator) with normal lighting and temperature control. Success of this control demonstrates lab personnel are using appropriate testing protocols, under a controlled setting with healthy test organisms to achieve acceptable QA/QC. This assessment can be made for Experiments 3 and 4 but not for Experiments 1 and 2; laboratory control *data* for experiment 1 and 2 were not provided.
- 2) **Clean UVB and Clean UVT.** All four experiments included a method control (Clean Control) prepared by the addition of clean gravel to the generator columns. The clean controls were tested outdoors under ambient natural light using a UV screen to either block or transmit natural light. The clean control established whether the

combination of generator columns with ambient light either UV blocked or transmitted provided an adequate environment to conduct the test without introducing other contributing factors.

- 3) **URBAN UVB/UVT CONTROL.** Experiments 1, 2, and 3 included an urban control prepared by including intertidal gravel samples collected from San Francisco Bay to represent a non-point source urban signature from a location that had not received any of the spilled Cosco Busan oil. The results of this baseline sample demonstrate if an urban signature from a variety of different sources shows an adverse effect separate and distinct from any effects associated with CB oil acting alone.
- 4) **ANS UVB/UVT Crude Oil.** The ANS treatment was used to demonstrate the ability of these 2009 studies to replicate the same pattern of response observed for other studies.

#### **CRITERIA FOR ACCEPTABLE CONTROL RESPONSES**

Acceptable response of the test organism in the laboratory control samples indicate the test population was healthy and that the laboratory demonstrated sufficient experience by performing the test adequately and the testing methods are sufficient to permit further evaluation. ASTM guidance establishes species-specific survival to normal hatching for multiple species tested under the early life stage testing protocols (ASTM E 1241 appendices). Minimal control survival to normally developed larvae for fish species included in the guidance range from 65 to 80% of successfully fertilized embryos (ASTM E 1241 appendices). Pacific herring are not part of this specific guidance, but procedures and control survival information have been produced for Washington Department of Ecology as part of the wastewater assessment program. These methods, modeled after ASTM (Dinnel et al., 2005; 2008) have been accepted as standard procedures by the State of Washington and have also been used for testing conducted in Alaska and California. Control survival must exceed 70% for a test to be validated when Pacific herring are testing using this guidance.

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#### **TEST ACCEPTABILITY**

#### **NOAA/BML ASSESSMENT**

Four experiments were performed during the 2009 experiments. Experimental Run 1 (23 January through 10 February 2009) was considered a dry run or practice to show the test design was acceptable for testing. Minimal water quality data were collected and no chemistry data were provided for this dry run. A subset of photographs of organisms exposed to the various treatments and a partial summary of data on the percent normal hatch for all treatments were provided. NOAA and BML concluded the test design was acceptable for conducting a series of column exposures. Experimental Run 2 (13-28 February) was terminated early when laboratory control treatments were determined to be unacceptable. Notes collected from the NOAA data sheets report "run aborted due to high rates of abnormal early embryos in the lab incubator controls." This run was therefore excluded from further consideration. Experimental Run 3 (26 February through 10 March) included a partial set of images, summaries of test results and chemistry data for water and eggs. Because of excessive algal growth throughout the test system on 11 March through 13 March, the test system was dismantled and the containers and generator

columns were cleaned with a concentrated bleach solution followed by rinsing in sodium thiosulfate (documented in water quality notes on March 12<sup>th</sup> and 13<sup>th</sup>). Experimental Run 4 (conducted 18 March through 6 April) had a reduced testing design omitting the ANS treatments and the urban control. Water quality data were provided with the summary data along with photographs of dechorionated embryo's and newly hatched fish. Analytical data for water samples and egg tissues were also provided.

#### ***NewFields Biological Assessment***

Laboratory Controls. Guidance values of >70% survival to normal hatch in laboratory controls have been established by Dinnel et al. (2008). NewFields was provided laboratory control images only for Experiments 1 and 2 with no summaries of laboratory control survival for either experiment. The lack of data relative to hatching success precludes the use of these first two experiments. Survival to hatching of normal larvae for the BML data was 77.4% in Experiment 3 but only 57.3% in Experiment 4. The NOAA data reported viable eyed development of >80% for dechorionated embryos for Experiments 3 and 4.

Run	NOAA Viable Eyed (%)	BML Normal Hatch (%)
Experiment 3	96.6	77.4
Experiment 4	80.0	57.3

These observations indicate the test organism population used for testing was acceptable for Experiment 3 but not for Experiment 4. The higher percentage of viable eyed embryos reported by NOAA was based on dechorionated embryos 9 and 10 dpf (days post fertilization) whereas the BML observations of percent normal hatch were made on embryos that hatched between 10-18 dpf. It is reasonable to assume a persistent adverse impact observed in an early developing embryo would be retained in later stages. The longer period of development and exposure for the BML samples resulted in a further decline in the health of the organism at hatch in the BML data compared to the NOAA viable eyed larvae data. Based on laboratory controls conducted indoors, Experiment 3 is validated while Experiment 4 is not validated; Experiments 1 and 2 cannot be assessed (Figures 2 - 4).

Clean Controls. Experimental Runs 1 – 4 had a 'method' control identified as clean control for the outdoor generator column studies. This clean control is not a substitute for the laboratory control exposure but is used to demonstrate any experimental related effects of the outdoor test system. Adequate survival of the clean 'method' controls would indicate the outdoor exposure system provided the necessary test conditions and did not to contribute to any adverse biological effects. Conversely, if the clean control results show adverse effects, then additional contributing factors related to the testing procedures need be evaluated. The 'method' clean control had acceptable survival to normal hatching for the first experiment only (Figure 2 and 4). Neither 'method' clean control achieves adequate survival under UVB or UVT for the remaining experiments (Experiments 2, 3 and 4). The reduced survival of the clean controls suggests the outdoor exposure system with the generator columns added stress to the organisms in the last

three of the four experiments. This effect was more pronounced in the clean controls with UV transmission compared to clean controls with blocked UV. This observation could be interpreted as an impact from transmission of UV light in the absence of a contaminant. In each of the four experiments, the UVT Clean control had an adverse impact on the developing embryos that exceeded the impact on organisms exposed to the UVB Clean control. This pattern was consistent among the experiments and occurred in the absence of contaminants. The reduction in survival of the clean control (UVB and UVT) below 80% indicates the conditions in the generator column studies conducted outdoors may have impacted survival and ultimately normal development (Figures –2 - 4).

Urban Controls. Experimental Runs 1, 2, and 3 included an urban control. This urban control was included to assess whether the urban beach gravel from San Francisco Bay, with its multiple input of pollutant sources, had any adverse impact on the development of herring independent from effects observed with Cosco Busan oil. The percentage of normally developing larvae for Runs 1, 2, and 3 was 79%, 25%, and 40% respectively. These results would suggest that the mixed use urban nature of the San Francisco Bay estuary may impact the health of herring embryos.

A summary of the response of each control for each of the experimental runs is shown in Figures 2 through 4 and Table 1. There is a general pattern of decreasing survival to normal hatch from the first through the fourth experiment, indicating increasing problems with the experimental conditions. Table 1 summarizes the experimental control responses and actual water quality conditions for Runs 1 – 4; ASTM guidance for early life stage toxicity testing of fish and specific guidance for herring published by WDOE are included for reference.

Table 1 Comparison of Standards for Early Life-Stage Toxicity Testing for by ASTM and WDOE Requirements and Experimental Ranges Attained during Testing

Requirements and Experimental Ranges Attained during Testing						
Criteria			Experiment 1	Experiment 2	Experiment 3	Experiment 4
Measurement	ASTM 1241	WDOE <sup>1</sup>				
Laboratory Control <i>Mean Survival to normal hatching</i>	>65 to >80%	>70%*	No Data	No Data	77	57
Clean Control <i>Mean Survival to normal hatching [UVB/ UVT]</i>			91/88	55/46	37/8.9	30/11
Temperature(°C) <i>Time Weighted</i>	±3°C	12°C	3.1 to 19.8	8.3 to 23.1	6.8 to 24.5	6.7 to 18.9
Dissolved Oxygen (%) <i>Time Weighted</i>	60-100% Saturation		Not assessed	Not assessed	112-431	98-207
Test Chamber pH	Measured		Not assessed	Not assessed	8.0 to 9.9	7.2 to 8.5
Incoming pH	Measured		Not assessed	Not assessed	7.68-7.93	7.44-7.87
Salinity		16‰	Not assessed	Not assessed	21-23	21-23
NOAA/BML Experimental Qualifiers			Dry Run – no chemistry for water or eggs	Terminated - failure of egg development	Algal fouling	Reduced experimental design

<sup>1</sup> Test validation for embryo development is validated and accepted by WDOE for routine application to effluent testing (Dinzel et al. 2008).

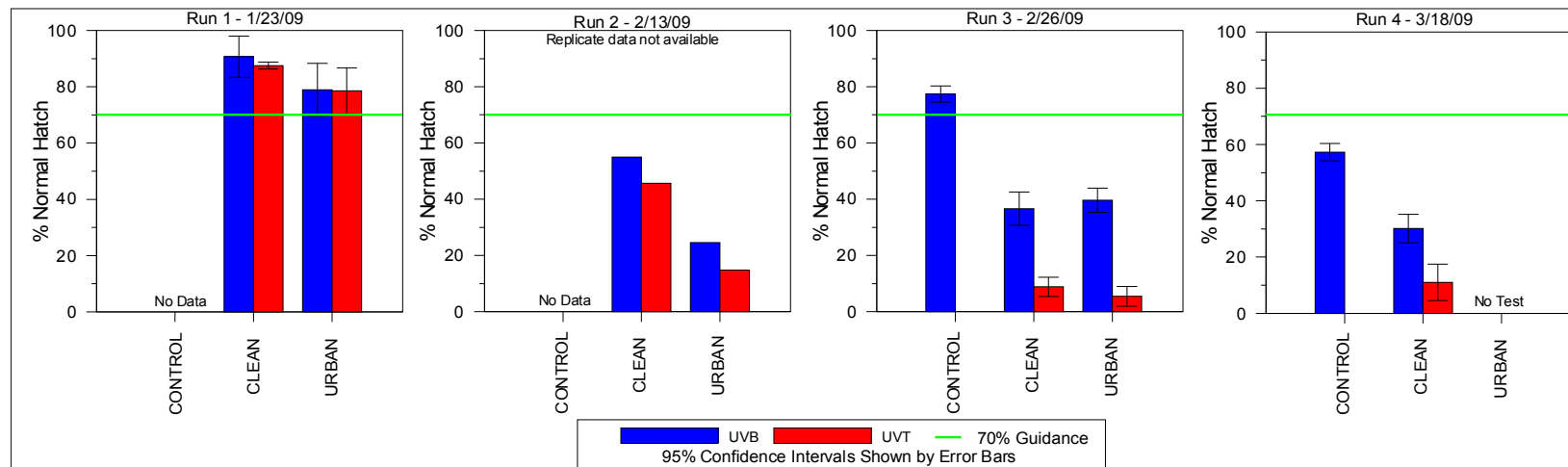


Figure 1. BML Assessment of Percent Normal Hatch in the Control Samples

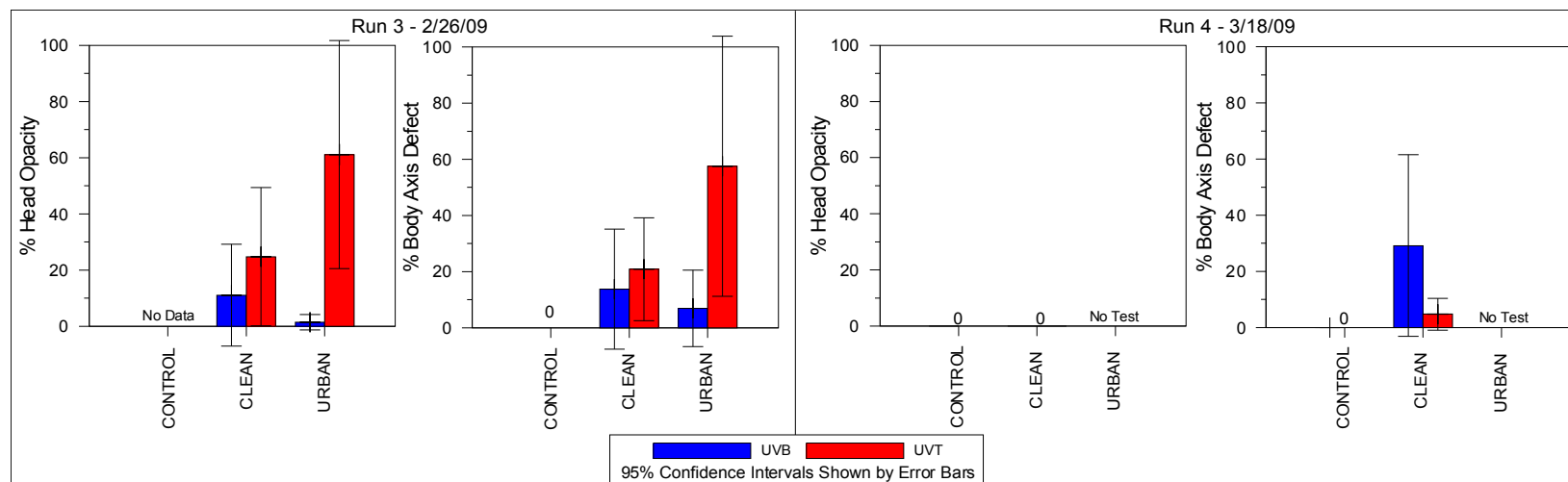


Figure 2. NOAA Assessment of Abnormalities in the Control Samples



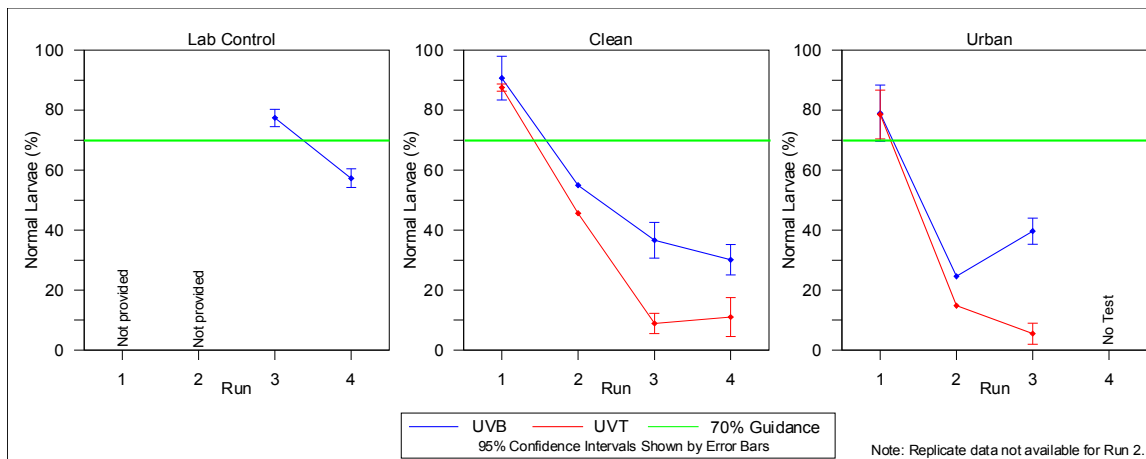


Figure 4. Percentage of Normal Hatch for Each of the Controls during Experiments 1 - 4.

## WATER QUALITY VARIATIONS

Demonstration of acceptable water quality conditions for testing provides confidence that additional stress on test organisms caused by fluctuating water quality conditions does not compromise test results through additive or synergistic responses to combinations of factors. NewFields review of the water quality data indicates several water quality parameters were not maintained within recommended limits of either ASTM 1241 or Dinnel et al. (2005, 2008) during testing. These included test temperature, salinity, and the combination of dissolved oxygen saturation and pH. Temperature, salinity and dissolved oxygen saturation have specific guideline limitations that have been established; pH does not, but general guidance under other protocols for marine testing is to monitor and comment about pH values which exceed a range of 0.2 to 0.3 units.

### TEMPERATURE

The targeted test temperature was 12°C, the optimum temperature for populations of Pacific herring from the San Francisco Bay region (Dinnel et al. 2008, Vines et al. 2000, Alderdice and Velsen 1971). The average temperature measured using a continuously monitored temperature data logger ranged from 10.5 to 12.9°C for Experiments 1-4. However, the target temperature range was not controlled in the test chambers and consequently temperatures increased or decreased based on daily weather conditions. The overall range of temperature experienced by the developing embryos during each of these tests averaged ~15°C (range 12.2 to 17.7°C; see Figure 5). The rate of temperature change often exceeded 10-15°C over short periods of time (~6h). This trend was observed on a daily basis throughout the testing programs. This temperature range exceeds the ASTM guidance of  $\pm 3^\circ\text{C}$  change in temperature over any 12-h period and this is also the preferred maximum range of change for periods of 72h (ASTM E1241 10.4.3). The influence of temperature on stocks of Pacific herring obtained from the San Francisco area during the 2006/2007 spawning period, prior to the Cosco Busan spill event, indicates that temperatures  $> 18^\circ\text{C}$  should be avoided. In all cases, the temperature ranged to above  $18^\circ\text{C}$  for all outdoor experiments which, under controlled experimental conditions, resulted

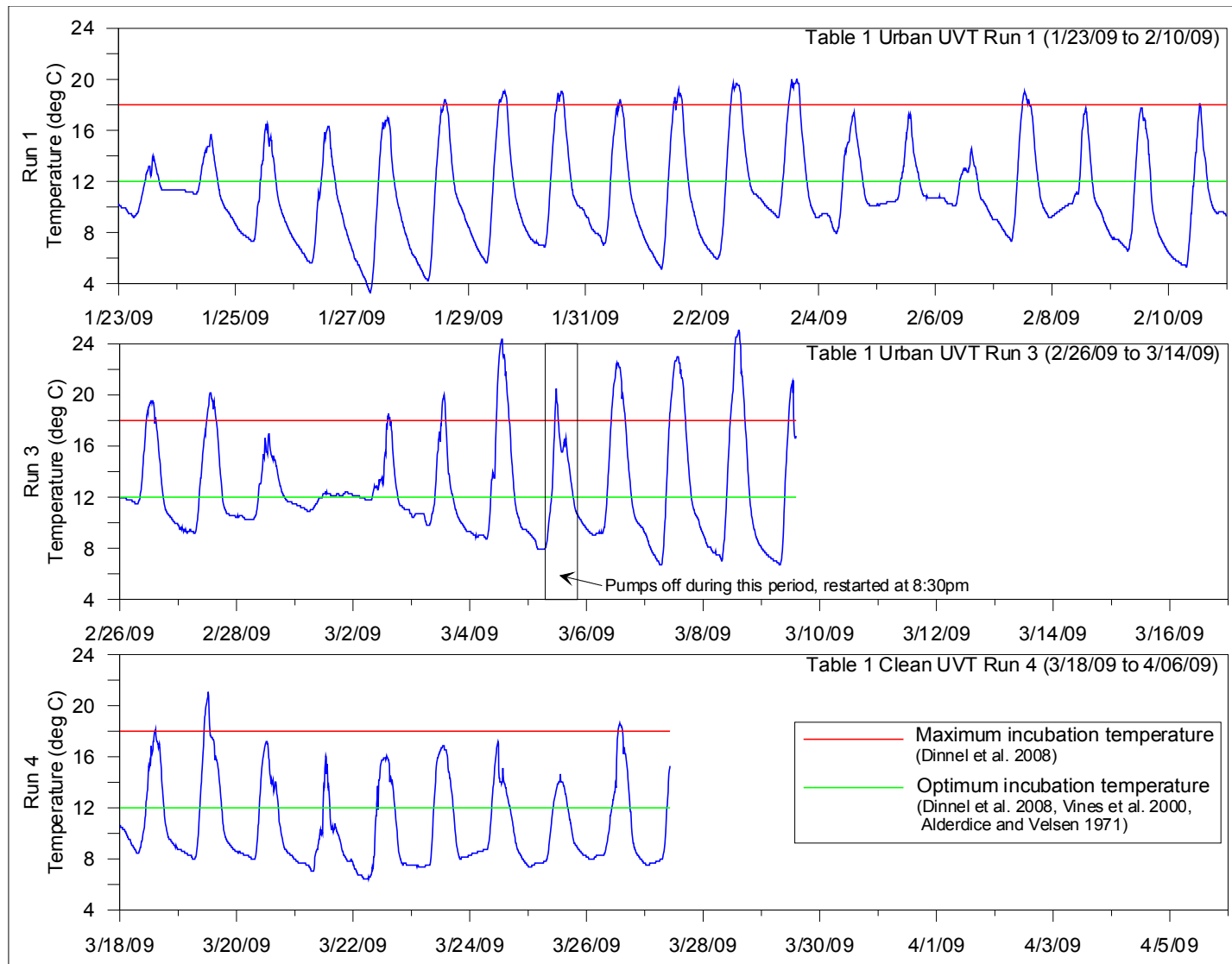


Figure 5. Fluctuating Temperatures over Time for Experimental Runs 1, 3, and 4

in reduced hatching success of normal larvae (Dinnel et al. 2008). Table 2 summarizes data contained in the methods development reported by Dinnel et al. (2008). This table shows the percentage of live hatch for two different collection periods in 2007 as denoted by two data points reported in each column labeled "live hatch." Noteworthy from this data set is only 8% of combined live normal hatch is reported at 20°C.

Table 2 Reduced Hatching Rate Occurs with Increased Temperature (*adapted from Dinnel et al. 2008*)

**San Francisco Bay 2007<sup>1</sup>**

<b>Temperature °C</b>	<b>Collection 1</b>			<b>Collection 2</b>		
	<b>Live Hatch (%)</b>	<b>Normal Live Hatch (%)</b>	<b>Hatch (dpf)</b>	<b>Live Hatch (%)</b>	<b>Normal Live Hatch (%)</b>	<b>Hatch (dpf)</b>
20	29	7.8	6	12	2	6
18	82	69	6	32	27	7
15	84	71	10	63	57	9
12	84	80	11	22	22	13
10	67	53	14	51	50	14

<sup>1</sup> Tests were run at 16 ‰.

## SALINITY

Salinity for Pacific herring has been found to effect the normal development of eggs to viable eyed larvae at hatching. Optimal salinity for hatching in the San Francisco Bay region is generally 16-20‰ with decreased hatching observed above 24‰ (Cherr and Pillai 1994; Griffin F.J. et al. 2004). The combined influence of elevated salinity and temperature indicate embryo development is affected when salinity is 30‰ and temperature > 14°C (Dinnel et al. 2008). Mortality under these conditions is 50.8% with 19.8% of hatched larvae categorized as abnormal. The maximum temperatures observed with each of experiments ranged from 18.9 to 24.5°C. Observations of the combined influence of salinity and temperature show effects of these water quality attributes are typically synergistic in nature. The combination of increased temperature and salinity are expected to produce a greater effect than those observed for temperature only (Alderdice and Velsen 1971; Vines et al. 2000). The higher temperatures, combined with the 22-23‰ salinity observations made during the water quality assessments, suggest the potential for adverse effects associated with the combination of these two water quality parameters with temperature variation being the major component.

## DISSOLVED OXYGEN AND PH

ASTM (E 1241 10.4.3) indicates that super saturation by dissolved gases should be avoided to prevent gas-bubble disease. ASTM recommends dissolved oxygen saturation should be between 60 and 100% saturation. Each of the experiments with measured water quality characteristics that were assessed (Experiments 3 and 4) had super saturation of dissolved oxygen continuously above this range. In Experiment 3, there was one sample that had a dissolved oxygen saturation measure of 431%.

Trends in saturation of oxygen do not show patterns related to the level or presence of petroleum in the experimental array, as indicated by Figure 6, presenting data summarized from Experiments 3 and 4. The combination of increasing percent DO

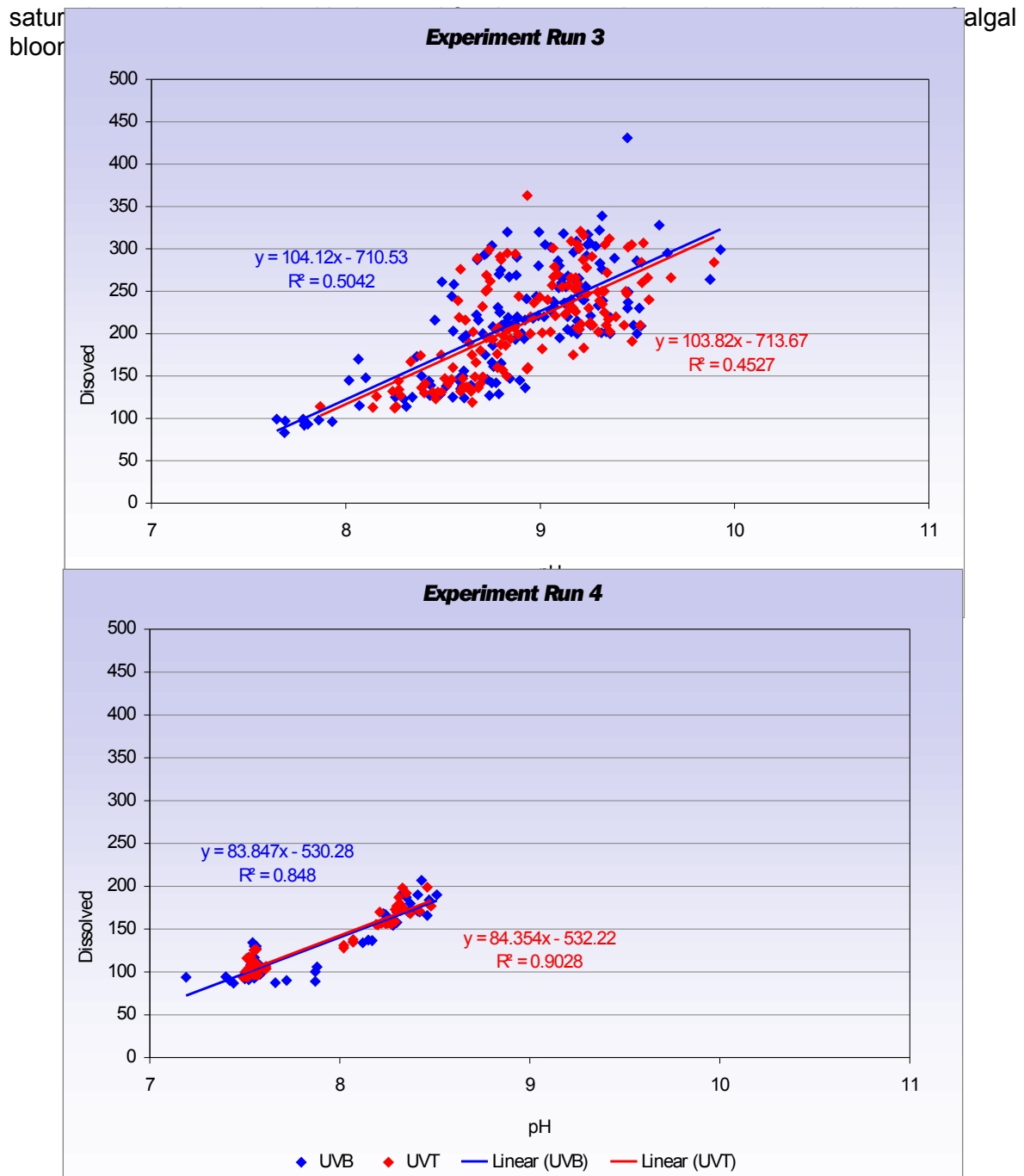


Figure 7 Pattern of Dissolved Oxygen vs. pH for Experimental Experiments 3 and 4.

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**WATER QUALITY ISSUES****TEMPERATURE**

Temperature fluctuated in the outdoor environment based on the influence of the solar energy on the water in the shallow test containers of the experimental array. Temperature was not controlled within a limit of  $\pm 3^{\circ}\text{C}$  during the experiment, but fluctuated  $\pm 10^{\circ}\text{C}$  over periods of four to six hours. This extreme temperature range would affect test results and would not be acceptable under ASTM protocols. Additionally, the maximum temperatures attained on a daily basis exceed the thermal tolerance range for Pacific herring embryos from San Francisco Bay, California provided by Dinnel et al. (2008). Herring obtained from San Francisco Bay during the 2007 spawning (first quarter of 2007) indicate test temperatures in greater than  $18^{\circ}\text{C}$  only 8% normal live hatch are reported (Dinnel et al. 2008). For all four column experiments, the test temperatures were above the thermal tolerance limit throughout much of the test and the rate of temperature change exceeded acceptable limits based on ASTM protocols.

**SALINITY**

The targeted salinity was approximately 22‰, chosen by BML/NOAA to be consistent with salinity values in the spawning area of San Francisco Bay. However this salinity was above the optimum of 16‰ reported by others for maximum egg and larval survival (Alderdice and Velsen 1971, Cherr and Pillai 1994). Researchers have noted the combined influence of temperatures in excess of  $18^{\circ}\text{C}$  and salinities greater than 16‰ produce increased adverse effects (Alderdice and Velsen 1971; Vines et al. 2000); therefore these are likely contributing factors regardless of the amount of oil present in the test chambers.

**DO AND pH**

Percent saturation of dissolved oxygen was measured daily in the early afternoon and exceeded guidance values for each of the outdoor experimental runs. A similar pattern was observed for each experiment; the daily measurements showed increasing dissolved oxygen saturation and pH over time. This effect is almost certainly related to algal blooms that occurred during the experiments and contribute oxygen to the test waters via photosynthesis during the day. It also indicates that algal blooms continued to increase through time, resulting in higher oxygen saturation near the end of the experiments.

**ALGAL BLOOMS INFLUENCE ON WATER QUALITY**

Algal blooms have dramatic effects on water chemistry, most notably pH and dissolved oxygen (DO). When algae remove carbon dioxide during photosynthesis they raise the pH by increasing the level of hydroxide ions. The opposite reaction occurs during respiration when carbon dioxide is produced lowering hydroxide and lowering the pH. Therefore, high pH ( $> 8.0$ ) can be an indicator of photosynthesis by large quantities of algae.

Algal blooms produce large amounts of oxygen during photosynthesis that may lead to supersaturated levels of DO in the water column. Conversely, during respiration, algal blooms remove the DO from the water column which may lead to little or no oxygen in the water column. These conditions can also be created when a large quantity of algae die and decompose. Super saturation of DO ( $\geq 110\%$  saturation) can be an indicator of

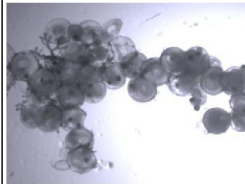
photosynthesis by algae, particularly during mid-to-late afternoon. The combination of elevated pH and super saturation of oxygen and the observations of algal blooms in the test containers support these statements. A subset of photos showing algal growth on slides containing the herring embryos from each control is shown in Figures 8a and 8b.

The concentration and percent saturation of oxygen and pH at night were apparently not measured. Had these parameters been measured or, if the data are available from night time assessment, the concentrations of dissolved oxygen should be much lower than during the day. Phytoplankton and algal blooms during daytime produce dissolved oxygen through the use of carbon dioxide that helps cause pH to increase. During the night oxygen is consumed and we must assume that the dissolved oxygen saturation would decrease when respiration by the plants occurs rather than photosynthesis. These observations support the anecdotal observations of algal stimulation made by NOAA/BML researchers but also support the observations that we made on the amount of algal fouling of eggs and glass slides on the photographs that were assessed.

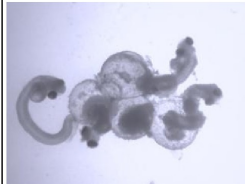
Super saturation of DO can have a direct effect on developing embryos by gas bubble disease. A larger impact may be the fouling of eggs by algae during their development. UV blocked and transmitted light show no difference in the relationship of DO saturation and pH indicating that the amount of algal stimulation is similar among the test treatments regardless of UV exposure (Figure 7). There is a reduction in the rate of increasing DO saturation per day in the last experiment but the saturation and pH levels still indicate that algal stimulation occurred during the testing, even though shade cloth was added at day 6 to reduce "...the exploding algae growth..." (BML Water daily water quality notes, pg 55, dated 3/24/09). This lag may have been a direct result of cleaning the generator columns to reduce algal growth. The influence of transmitted or blocked UV light on the production of dissolved oxygen saturation and increasing pH due to algal stimulation is not substantially different.

**Qualitative Algae Assessment  
2009**

**Natural Solar Radiation – BML 2008**  
(Embryos collected from SF Bay Sites)



1] MRR20 N8 3/02/08



2] MRQ01 N2 3/7/08



13] MRU01 N4 2/26/08

Run 1 1-23-09 (limited photos available)	NOAA	BML
Urban UVB		 1] BML 2/9/09 00003
ANS 1.0 UVT	 2] NOAA 1/30/09 ovo1	
CBO 1.0 UVB	 3] NOAA 1/30/09	
Run 3 2-26-09	NOAA	BML
Lab Control	 4] NOAA 3/8/09 BTV jpeg 001	 5] BML 3/4/09 (6 dpi) Column 00003
Clean UVB	 6] NOAA 12 3/7/09 BTV jpeg a	 7] BML 3/11/09 Column 00012
Clean UVT	 8] NOAA 3/7/09 23 BTV jpeg b	 9] BML 3/12/09 Column 00010
Urban UVB	 10] NOAA 3/7/09 05 BTV jpeg a	 11] BML 3/12/09 Column 00005
Urban UVT	 12] NOAA 3/7/09 34 BTV jpeg a	No Image

**Figure 3a Qualitative Assessment of Algae Highlighting Treatments in Experiment 1 and 3 (Eggs Collected from SF Bay Sites in 2008 added as Reference for Natural Solar Radiation)**

2009



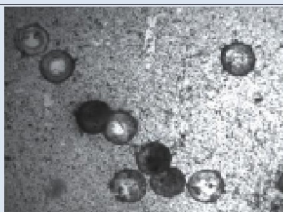


Run 4 3-18-09	NOAA	BML
Lab Control	 <p>14] NOAA - 3/27/09 Slide 25-001</p>	<p>No Image</p>
Clean UVB	 <p>6] NOAA - 3/27/09 Slide 5-002</p>	 <p>15] BML - 4/6/09 Slide 02</p>
Clean UVT	 <p>16] NOAA - 3/27/09 Slide 24-003</p>	 <p>17] BML - 4/06/09 Slide 03</p>

Figure 8b Qualitative Assessment of Algae Highlighting Treatments in Experiment 4



## **CONCLUSIONS: WATER QUALITY CONTROL**

Water quality characteristics for these experiments are not within acceptable testing limits.

- Time-weighted average temperatures are exceeded by ~10°C on a daily basis. The combination of the increased temperatures and the above optimal salinities may have negatively impacted survival to normal hatch and normal development of herring.
- Time-weighted dissolved oxygen concentrations are outside of the acceptable range of 60-100% saturation with many values in excess of 200%. There are no measurements of dissolved oxygen at night in the water quality records provided when algal oxygen consumption would be highest. The earliest morning measurements collected around 0730 indicated a much reduced saturation of 92 to 96%.
- pH is required to be measured but an acceptable range is not indicated; however, pH fluctuations through a range of 7-10 is clearly unacceptable ( ASTM E 1241).
- Excessive algal growth can create suboptimal testing conditions, interfere with the respiration of developing eggs, and interfere with contaminant interaction with test species.
- If a laboratory cannot maintain acceptable water quality limits then the measured responses of the test organisms are influenced by the test methods. *All four tests should be discarded based on unacceptable testing conditions (USEPA and ASTM).*

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**QUALITY ASSURANCE/QUALITY CONTROL REVIEW**

Quality Assurance/Quality Control requirements provide confidence in the data results through a system of quality control checks on data collection methods, laboratory analysis, data reporting, and appropriate corrective actions to achieve compliance with established performance and data quality criteria. This section presents a review of the QA/QC procedures NewFields used to ensure that project data are defensible and usable for their intended purpose.

**QA/QC OBJECTIVES FOR MEASUREMENT DATA**

The overall QA objective is to review chemical and biological laboratory analyses and reporting to ensure that data is of a quality consistent with its intended use. This section evaluates status of the 2009 column experimental data in light of standard goals for data precision, accuracy, and completeness, as well as representativeness and comparability of 2009 laboratory analyses.

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**PRECISION AND ACCURACY**

The NOAA evaluation of the 2009 experiments did not include measurement of embryonic heart rate, arrhythmia, distance of the heart atrio-ventricular junction (AV) to the ventral edge of pericardial space, or computer-based planimetry determinations of the area of heart, the area of pericardial space, or the percentage of pericardial space occupied by the heart. NOAA measurement endpoints for 2009 differed from those used in 2008 and included viable eyed embryos, necrotic eyed embryos, CNS opacity, body axis defects, and edema. BML measurement endpoints were consistent in 2008 and 2009, and were based on hatching success and morphological features such as scoliosis, edema, opaque head, bent head, opaque yolk sac, and jaw deformities. NewFields evaluation of the 2009 images will be reported in a subsequent data report.

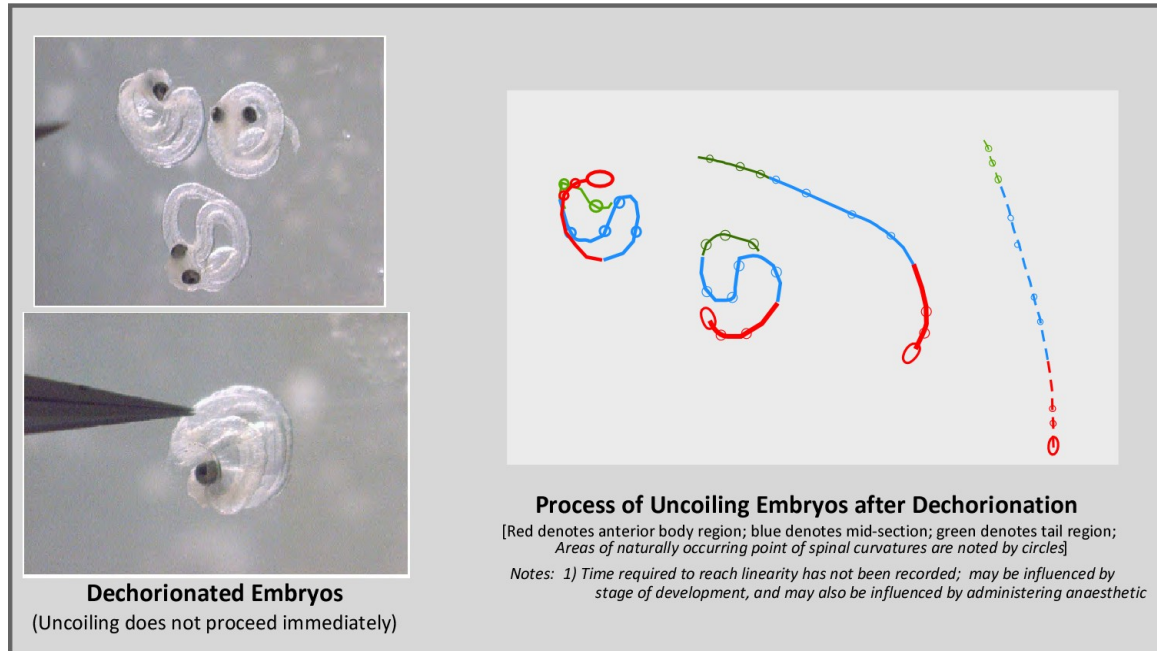
**ENDPOINT MEASUREMENT**

There is ambiguity surrounding how the 2009 endpoints are measured; detailed methods of measurements are needed to compare results.

1. Body Axis Defects. Are BAD influenced by the process of dechoriation? For example, do dechorionated specimens have underdeveloped musculature that prevents or delays the linear body line. It appears that some determinations of body axis defects may be a result of underdeveloped musculature, or effects of anesthetizing agents, fixative agents, or rapidly changing temperature conditions (Figures 9a and 9b).
2. Pericardial and Yolk Sac Edema. What is the interaction of these two metrics? Two processes may be involved in either membrane swelling: osmotic fluid balance and kidney or circulatory system irregularities (Hill et al. 2004). A pervading question is: Is swelling influenced by fluctuating salinities?

## BODY AXIS DEFECT - CONSIDERATIONS

### I. Muscle Development Disrupted by Dechoriation



### II. Swimming Musculature

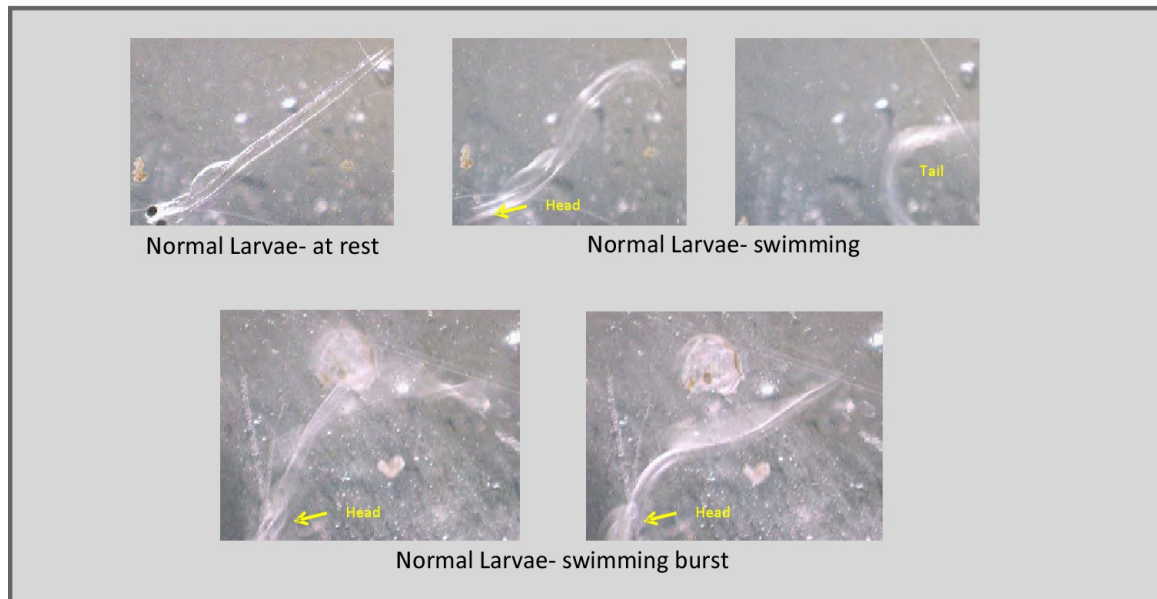


Figure 9a. Factors Potentially Affecting Body Axis Determinations: State of Musculature  
(Development, Exposure to Anesthetics or Fixative Agents)

### III. Thermal Stress

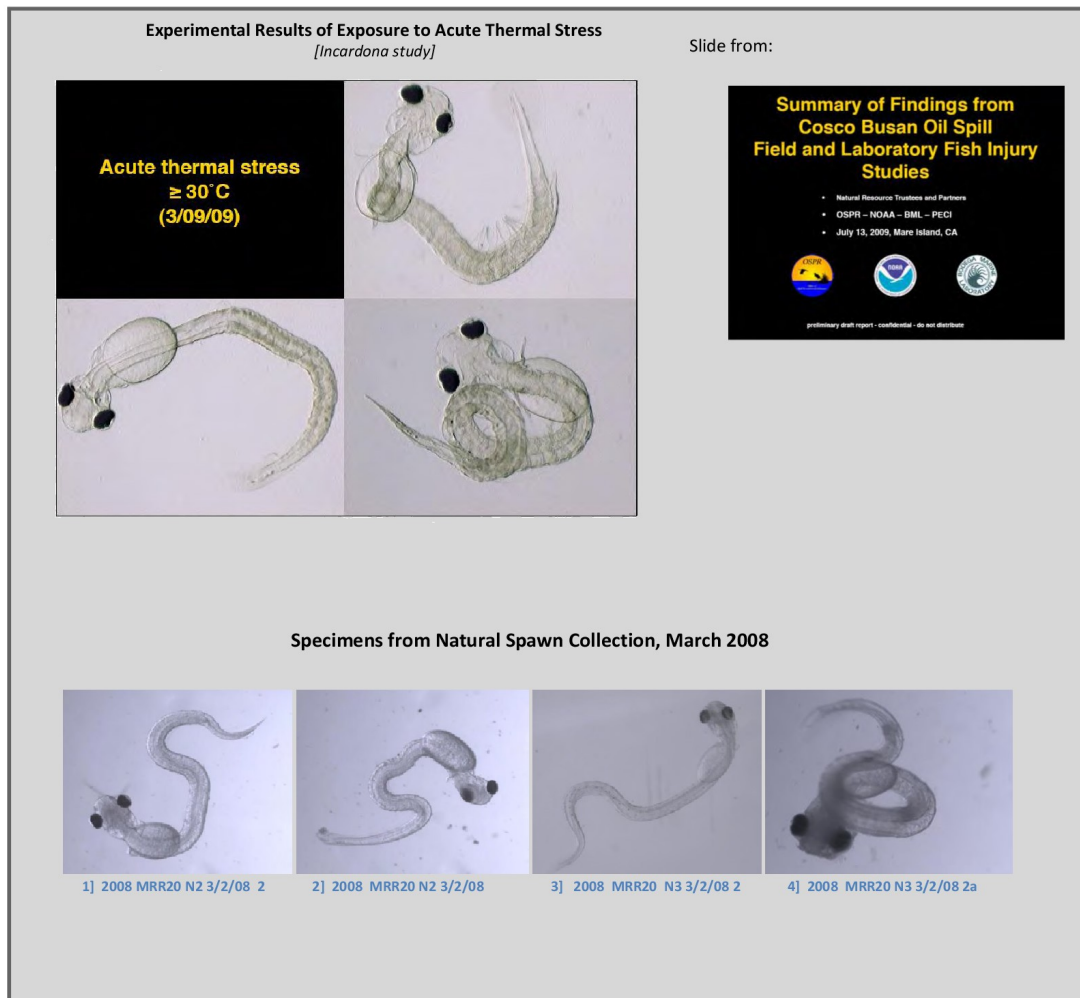


Figure 9b. Factors Potentially Affecting Body Axis Determination: Thermal Fluctuations

Figure 9b shows body axis defects related to rapid thermal stress (Incardona 2009 Powerpoint Presentation) and also specimens collected from Keil Cove in 2008.

#### **AGE DEPENDENT ISSUES**

1. Issues related to a head to trunk angle (HTA) and shape of the pericardial region. The HTA normally changes with the development of the embryo. When the HTA is less than  $\sim 110^\circ$ , the pericardial area is compressed.
2. Issues related to possible differences in absorption of the yolk sac after hatch depending on the age of the larval specimen. Can the absorption of the yolk sac in older specimens be construed as yolk sac edema? Figure 10 illustrates two contrasting assessments of yolk sac edema from published papers.

3. Guidelines to establish normal baseline at various stages of development are lacking.
4. The Data Report prepared by NOAA and BML for 2009 described the size of the donor fish used for Experimental Runs 1, 2, 3, and 4. The size of the fish varied between runs. It appears from the weight data provided that 2 year females were used for Runs 2, 3, and 4 while older fish probably 4 or 5 years of age were used for Run 1. Hershberger et al. (2005) concluded abnormalities observed in herring collected from Cherry Point in Washington State may be attributed to age structure of the spawning class. For example, “eggs from age-2 females at Cherry Point may not be completely mature and their fertilization may result in the development of larvae with gross signs of developmental abnormalities.” (Hershberger et al. 2005).

Table 3 provides the average weight, number of females used, and collection location for each run.

Table 3 Donor Fish Used for Experimental Runs

Experimental Run	Average Fish Weight (g)	# of Fish Used to Pool Gametes	Collection Location
1	104	5	Richardson Bay
2	60	26	Point Chauncey
3	61	33	Point Chauncey
4	63	13	Richardson Bay

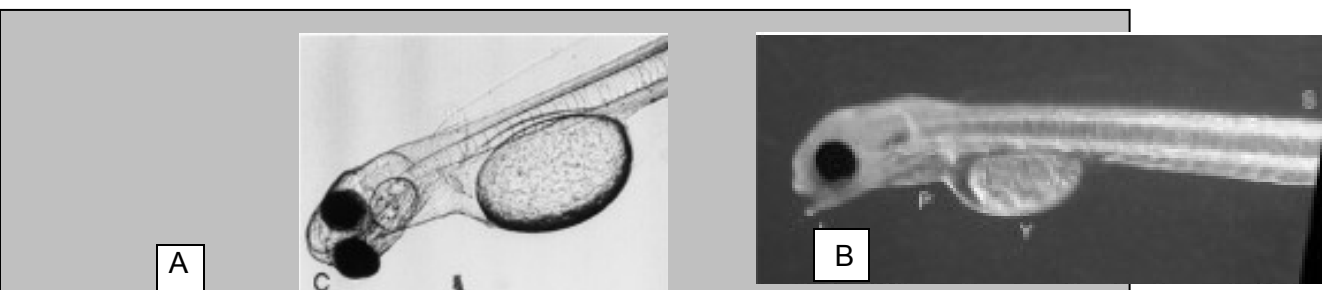


Figure 10 Differing Evaluations of Yolk Sac Edema

[Image A (Middaugh et al 1998) was termed “normal”, whereas image B (Brown and Carls 1998) was listed as having “normal jaw, normal pericardium, yolk edema”.

## **INTRODUCTION OF EXPERIMENTAL ARTIFACTS**

1. Microscope Illumination. Sources of artificial illumination can influence the assessment of opacity:

Lighting patterns from BML hatched herring images have 3 attributes:

- Lighting brightest from upper right quadrant and darkest to left
- Alternating bands of dark and bright lighting through pictures
- Juveniles placed with head to right or left in field of lighting

Lighting patterns from NOAA images have two issues:

- Lighting brightest from lower quadrant and darker at top
- Pigmentation of embryos is bronze colored (light quality, filter or staining?)

2. Photographic Records. Photo images were not taken under consistent magnification. For example, different scales were used between NOAA and BML. Images cannot be scaled, because micrometer images were not provided for each different focal setting. Inconsistent scales preclude comparison of measured data between laboratories. Further, images showing length of specimens generally do not track with subsequent series of close-up images that emphasize specific areas like the pericardial region. Without a standardized labeling system and micrometer records an independent reviewer cannot connect full body morphological assessment with the direct evidence of edema defects
3. Chemical Additives. It appears likely that on occasion fixative agents such as agarose and/or anesthetics such as MS 222 or Methyl cellulose may have been used during the collection of imagery for the dechlorinated embryo/larvae specimens. These additives can influence osmolality of the specimen, interfere with natural musculature, and also introduce toxic chemicals which may confound toxicity assessments. Precise methodology has not been provided which leads to questions regarding the isolation of contaminant effects. Lack of detailed methodology leaves questions regarding animal condition and handling (i.e., use of anesthetic).

**EVALUATION OF NON-VALIDATED TEST DATA**

Even though the design elements did not supply the necessary outcomes to validate the testing programs, NewFields evaluated the variety of potential end-points to determine whether some aspect of the study could be used to establish a linkage between the 2009 experimental generator column work and the observations made during 2008. The first QA/QC linkage between these data sets would be to assure that the information being used to interpret data by NOAA and BML was the same or at least similar to the information provided to NewFields as images and that they were representative of the total evaluations being made. A comparison was made between the completeness of information prepared by NOAA and BML and the representativeness of the images provided to NewFields.

Table 4 summarizes the imagery received by NewFields to date for control and background samples. The images from Experiments 1 and 3 severely under represent the data generated by both BML and NOAA evaluations. The reduction in data available for independent review hampers accurate review of experimental performance and endpoint assessments.

Table 4 Incompleteness of Data Provided to NewFields.

Experiment	BML Images	Lab control	Clean UVB	Clean UVT	Urban UVB	Urban UVT
1	BML Reported # Live Hatch	<i>Not Provided</i>	173	167	230	157
	# Larvae in Images Delivered to NF	9	5	6	5	5
	Representativeness –% of Live Hatch	0.0	2.9	3.6	2.2	3.2
3	BML Reported # Live Hatch	184	157	56	142	31
	# Larvae in Images Delivered to NF	15	32	0	38	5
	Representativeness –% of Live Hatch	8.2	20.4	0.0	26.8	16.1
4	BML Reported # Live Hatch	145	154	52	<i>Not Run</i>	<i>Not Run</i>
	# Larvae in Images Delivered to NF	144	144	52		
	Representativeness –% of Live Hatch	99.3	93.5	100.0		
Experiment	NOAA Images	Lab Control	Clean UVB	Clean UVT	Urban UVB	Urban UVT
1	NOAA - # Dechorionated	<i>Not Provided</i>				
	Dechorionated larvae in Images Delivered to NF	<i>Not Provided</i>	1	2	<i>Not Provided</i>	<i>Not Provided</i>
	Representativeness – % of Dechorionated	NC	NC	NC	NC	NC
3	NOAA - # Dechorionated	<i>Not Provided</i>	94	74	68	79
	Dechorionated larvae in Images Delivered to NF	<i>Not Provided</i>	61	64	68	40
	Representativeness – % of Dechorionated	NC	47.1	100.0	100.0	50.6
4	NOAA - # Dechorionated	72	67	61	<i>Not Run</i>	<i>Not Run</i>
	Dechorionated larvae in Images Delivered to NF	72	67	61		
	Representativeness – % of Dechorionated	100.0	100.0	100.0		

Image data for two of the Experiments (1 and 3) greatly under-represents the data used to provide summaries of responses documented by NOAA or BML. Images of hatched larvae were only taken on certain days while observations were made on normal hatching for each hatching day. In the case of the controls the percentage of photographic images provided ranged from 0-20.4% of the total number of hatched larvae. If these were random selections of images then the evaluations by all three groups (NOAA, BML and NewFields) could be compared if normal hatching was similar during all hatch days. However, the images do not appear to be random selections among the various treatments.

A further evaluation of data sets for Experiments 1, 3 and 4 provided by BML emphasizes the non-random nature of the images sent to NewFields. For Experiment 1 (Table 5a), 23 of the 48 separate treatment columns had no photographic records. Further, 17 of the remaining 25 with visual records, underrepresented by an average of 40%, the percent of normal live hatch relative to the summary data provided on the entire data set. This means that the samples from 23+17 out of a total of 48 treatment columns had a selection bias for what was photographed for Experiment 1.

Experiment 3 (Table 5b) showed a similar pattern 23 out of 49 treatment columns having no images of hatched larvae provided. Again, the majority of images provided for the comparison showed a selection bias with an underrepresentation of normal development in the provided images of 13 out of 21 treatments with lower normal estimates compared to estimates based on all hatched larvae. Again, Experiment 3 showed a bias of 23+13 sets of images underestimating normal hatch relative to the total number of hatching observations.

Experiment 4 (Table 5c) rectified this difference by photographing all hatched larvae. These data can be compared between the assessments made by the researchers and an independent evaluation of the images provided.



Table 5a. Comparability of % Normal Larvae at Hatch Calculated with BML Data to the Subset of Images Delivered to NewFields for Experiment 1.

Treatment	Table	Data for Images Provided to NewFields			Full BML Dataset			Run 1 Comparison		
		Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	%Normal of Live Hatch with Images	%Normal of Live Hatch	Relative Percent Difference
Lab Control		Data not provided								
Clean UVB	T2	33	31	2	67	63	4	93.9	94.0	0.1
	T4	No images			62	61	1	No images	98.4	NC
	T5	No images			44	42	2	No images	95.5	NC
Clean UVB Total		33	31	2	173	166	7	93.9	96.0	2.1
Clean UVT	T2	12	11	1	63	58	5	91.7	92.1	0.4
	T4	No images			39	38	1	No images	97.4	NC
	T5	No images			65	61	4	No images	93.8	NC
Clean UVT Total		12	11	1	167	157	10	91.7	94.0	2.5
Urban UVB	T3	6	4	2	54	49	5	66.7	90.7	30.6
	T6	6	5	1	127	113	14	83.3	89.0	6.5
	T1	35	32	3	49	44	5	91.4	89.8	1.8
Urban UVB Total		47	41	6	230	206	24	87.2	89.6	2.6
Urban UVT	T3	2	0	2	62	59	3	0.0	95.2	200.0
	T6	14	11	3	40	31	9	78.6	77.5	1.4
	T1	No images			55	51	4	No images	92.7	NC
Urban UVT Total		16	11	5	157	141	16	68.8	89.8	26.6
ANS 0.1 UVB	T3	14	13	1	32	29	3	92.9	90.6	2.4
	T5	No images			53	48	5	No images	90.6	NC
	T1	15	11	4	51	45	6	73.3	88.2	18.4
ANS 0.1 UVB Total		29	24	5	136	122	14	82.8	89.7	8.1
ANS 0.1 UVT	T3	30	28	2	44	41	3	93.3	93.2	0.2
	T5	11	8	3	50	46	4	72.7	92.0	23.4
	T1	No images			35	30	5	No images	85.7	NC
ANS 0.1 UVT Total		41	36	5	129	117	12	87.8	90.7	3.2
ANS 0.3 UVB	T2	18	6	12	65	49	16	33.3	75.4	77.4
	T6	6	2	4	128	91	37	33.3	71.1	72.3
	T1	No images			53	32	21	No images	60.4	NC
ANS 0.3 UVB Total		24	8	16	246	172	74	33.3	69.9	70.9
ANS 0.3 UVT	T1	16	3	13	31	22	9	18.8	71.0	116.4
	T2	No images			61	40	21	No images	65.6	NC
	T6	17	12	5	52	42	10	70.6	80.8	13.5
ANS 0.3 UVT Total		33	15	18	144	104	40	45.5	72.2	45.5
ANS 1.0 UVB	T3	No images			39	21	18	No images	53.8	NC
	T4	No images			46	5	41	No images	10.9	NC
	T5	No images			39	10	29	No images	25.6	NC
ANS 1.0 UVB Total					124	36	88		29.0	NC
ANS 1.0 UVT	T3	6	0	6	39	5	34	0.0	12.8	200.0
	T4	No images			53	12	41	No images	22.6	NC
	T5	No images			33	4	29	No images	12.1	NC
ANS 1.0 UVT Total		6	0	6	125	21	104	0.0	16.8	200.0
CB 0.1 UVB	T2	16	9	7	59	49	10	56.3	83.1	38.5
	T4	No images			40	36	3	No images	90.0	NC
	T6	2	0	2	75	66	9	0.0	88.0	200.0
CB 0.1 UVB Total		18	9	9	174	151	22	50.0	86.8	53.8
CB 0.1 UVT	T2	12	0	12	63	43	20	0.0	68.3	200.0
	T4	No images			56	37	19	No images	66.1	NC
	T6	1	0	1	75	50	25	0.0	66.7	200.0
CB 0.1 UVT Total		13	0	13	194	130	64	0.0	67.0	200.0
CB 0.3 UVB	T2	22	1	21	63	28	35	4.5	44.4	162.9
	T3	48	1	47	100	28	72	2.1	28.0	172.3
	T1	No images			26	1	25	No images	3.8	NC

Treatment	Table	Data for Images Provided to NewFields			Full BML Dataset			Run 1 Comparison		
		Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	%Normal of Live Hatch with Images	%Normal of Live Hatch	Relative Percent Difference
CB 0.3 UVB Total		70	2	68	189	57	132	2.9	30.2	165.4
CB 0.3 UVT	T2	24	3	21	24	3	21	12.5	12.5	0.0
	T3	30	6	24	30	6	24	20.0	20.0	0.0
	T1	No images			11	2	9	No images	18.2	NC
CB 0.3 UVT Total		54	9	45	65	11	54	16.7	16.9	1.5
CB 1.0 UVB	T4	No images			37	1	36	No images	2.7	NC
	T5	No images			28	0	28	No images	0.0	NC
	T6	20	0	20	72	5	67	0.0	6.9	200.0
CB 1.0 UVB Total		20	0	20	137	6	131	0.0	4.4	200.0
CB 1.0 UVT	T4	No images			1	0	1	No images	0.0	NC
	T5	No images			0	0	0	No images	NC	NC
	T6	No images			0	0	0	No images	NC	NC
CB 1.0 UVT Total					1	0	1		0.0	NC

\* When Live Hatched Larvae = 0, images were made of eggs only.  
NC = Not Calculated  
Cells shaded in yellow show relative percent difference of > 20%.

Table 5b. Comparability of % Normal Larvae at Hatch Calculated with BML Data to the Subset of Images Delivered to NewFields for Experiment 3.

Treatment	Column	Data with Images Provided to NewFields			Full BML Dataset			Run 3 Comparison		
		Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	%Normal of Live Hatch with Images	%Normal of Live Hatch	Relative Percent Difference
Lab control	49	57	53	4	90	79	9	93.0	87.8	5.8
	50	1	1	0	94	86	8	100.0	91.5	8.9
Lab control Total		58	54	4	184	165	17	93.1	89.7	3.8
	12	20	10	10	52	35	17	50.0	67.3	29.5
	17	14	9	5	48	39	9	64.3	81.3	23.3
Clean UVB	27	49	33	14	57	35	20	67.3	61.4	9.2
Clean UVB Total		83	52	29	157	109	46	62.7	69.4	10.3
Clean UVT	16	0	0	0	13	9	4	NC	69.2	NC
	23	0	0	0	19	8	11	NC	42.1	NC
	29	0	0	0	24	11	13	NC	45.8	NC
Clean UVT Total		0	0	0	56	28	28		50.0	NC
	5	12	9	3	32	21	11	75.0	65.6	13.3
	40	0	0	0	41	33	8	NC	80.5	NC
Urban UVB	46	53	37	16	69	47	22	69.8	68.1	2.5
Urban UVB Total		65	46	19	142	101	41	70.8	71.1	0.5
	34	3	1	2	18	9	9	33.3	50.0	40.0
Urban UVT	41	4	1	3	13	4	9	25.0	30.8	20.7
Urban UVT Total		7	2	5	31	13	18	28.6	41.9	37.9
	7	2	0	2	8	4	4	0.0	50.0	200.0
	28	0	0	0	19	9	10	NC	47.4	NC
ANS 0.1 UVB	38	50	28	22	64	33	31	56.0	51.6	8.3
ANS 0.1 UVB Total		52	28	24	91	46	45	53.8	50.5	6.3
	1	0	0	0	11	7	4	NC	63.6	NC
	32	1	0	1	12	6	6	0.0	50.0	200.0
ANS 0.1 UVT	35	0	0	0	0	0	0	NC	NC	NC
ANS 0.1 UVT Total		1	0	1	23	13	10	0.0	56.5	200.0
	6	1	1	0	10	6	4	100.0	60.0	50.0
	20	0	0	0	58	32	26	NC	55.2	NC
ANS 0.3 UVB	45	12	4	8	57	12	45	33.3	21.1	45.2
ANS 0.3 UVB Total		13	5	8	125	50	75	38.5	40.0	3.9
ANS 0.3 UVT	3	0	0	0	21	8	13	NC	38.1	NC

Treatment	Column	Data with Images Provided to NewFields			Full BML Dataset			Run 3 Comparison		
		Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	%Normal of Live Hatch with Images	%Normal of Live Hatch	Relative Percent Difference
	24	0	0	0	10	2	8	NC	20.0	NC
	42	No images			27	3	24	No images	11.1	NC
ANS 0.3 UVT Total		0	0	0	58	13	45		22.4	NC
ANS 1.0 UVB	11	27	3	24	44	6	38	11.1	13.6	20.4
	26	0	0	0	19	2	17	NC	10.5	NC
	37	7	0	7	41	10	31	0.0	24.4	200.0
ANS 1.0 UVB Total		34	3	31	104	18	86	8.8	17.3	64.9
ANS 1.0 UVT	14	0	0	0	5	0	5	NC	0.0	NC
	31	0	0	0	6	1	5	NC	16.7	NC
	33	0	0	0	2	0	2	NC	0.0	NC
ANS 1.0 UVT Total		0	0	0	13	1	12		7.7	NC
CB 0.1 UVB	9	16	1	15	28	5	23	6.3	17.9	96.3
	18	24	12	12	28	15	13	50.0	53.6	6.9
	47	22	7	15	82	51	31	31.8	62.2	64.6
CB 0.1 UVB Total		62	20	42	138	71	67	32.3	51.4	45.9
CB 0.1 UVT	15	0	0	0	5	0	5	NC	0.0	NC
	22	0	0	0	10	1	9	NC	10.0	NC
	44	0	0	0	2	1	1	NC	50.0	NC
CB 0.1 UVT Total		0	0	0	17	2	15		11.8	NC
CB 0.3 UVB	8	16	4	12	28	9	19	25.0	32.1	25.0
	19	0	0	0	56	14	42	NC	25.0	NC
	39	37	17	20	79	31	48	45.9	39.2	15.7
CB 0.3 UVB Total		53	21	32	163	54	109	39.6	33.1	17.9
CB 0.3 UVT	2	0	0	0	3	0	3	NC	0.0	NC
	21	1	0	1	5	0	5	0.0	0.0	NC
	36	0	0	0	4	0	4	NC	0.0	NC
CB 0.3 UVT Total		1	0	1	12	0	12	0.0	0.0	NC
CB 1.0 UVB	10	8	0	8	17	1	16	0.0	5.9	200.0
	25	12	0	12	15	0	15	0.0	0.0	NC
	48	28	5	23	47	8	39	17.9	17.0	4.8
CB 1.0 UVB Total		48	5	43	79	9	70	10.4	11.4	8.9
CB 1.0 UVT	13	0	0	0	0	0	0	NC	NC	NC
	30	0	0	0	0	0	0	NC	NC	NC
	43	0	0	0	0	0	0	NC	NC	NC
CB 1.0 UVT Total		0	0	0	0	0	0	NC	NC	NC
* When Live Hatched Larvae = 0, images were made of eggs only.										
NC = Not Calculated										
Cells shaded in yellow show relative percent difference of > 20%.										

Table 5c. Comparability of % Normal Larvae at Hatch Calculated with BML Data to the Subset of Images Delivered to NewFields for Experiment 4.

Treatment	Column	Data with Images Provided to NewFields			Full BML Dataset			Run 3 Comparison		
		Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	Live Hatched Larvae*	Normal Hatched	Abnormal Hatched	%Normal of Live Hatch with Images	%Normal of Live Hatch	Relative Percent Difference
Lab Control	C1	58	53	5	58	53	5	91.4	91.4	0
	C2	87	78	9	87	78	9	89.7	89.7	0
Lab Control Total		145	131	14	145	131	14	90.3	90.3	0
Clean UVB	5	53	33	20	53	33	20	62.3	62.3	0
	9	47	25	22	47	25	22	53.2	53.2	0
	18	54	32	22	54	32	22	59.3	59.3	0
Clean UVB Total		154	90	64	154	90	64	58.4	58.4	0
Clean UVT	3	13	6	7	13	6	7	46.2	46.2	0
	15	13	8	5	13	8	5	61.5	61.5	0
	24	26	15	11	26	15	11	57.7	57.7	0
Clean UVT Total		52	29	23	52	29	23	55.8	55.8	0
CB 0.1 UVB	8	23	9	14	23	9	14	39.1	39.1	0
	12	11	6	5	11	6	5	54.5	54.5	0
	19	36	13	22	36	13	22	36.1	36.1	0
CB 0.1 UVB Total		70	28	41	70	28	41	40.0	40.0	0
CB 0.1 UVT	2	3	0	3	3	0	3	0.0	0.0	0
	14	5	0	5	5	0	5	0.0	0.0	0
	21	14	3	11	14	3	11	21.4	21.4	0
CB 0.1 UVT Total		22	3	19	22	3	19	13.6	13.6	0
CB 0.3 UVB	7	14	3	11	14	3	11	21.4	21.4	0
	11	51	28	23	51	28	23	54.9	54.9	0
	20	26	7	19	26	7	19	26.9	26.9	0
CB 0.3 UVB Total		91	38	53	91	38	53	41.8	41.8	0
CB 0.3 UVT	1	0	0	0	0	0	0	0.0	0.0	0
	13	12	1	11	12	1	11	8.3	8.3	0
	22	10	1	9	10	1	9	10.0	10.0	0
CB 0.3 UVT Total		22	2	20	22	2	20	9.1	9.1	0
CB 1.0 UVB	6	45	19	26	45	19	26	42.2	42.2	0
	10	5	1	4	5	1	4	20.0	20.0	0
	17	7	1	6	7	1	6	14.3	14.3	0
CB 1.0 UVB Total		57	21	36	57	21	36	36.8	36.8	0
CB 1.0 UVT	4	0	0	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0	0	0
	23	0	0	0	0	0	0	0	0	0
CB 1.0 UVT Total		0	0	0	0	0	0	0	0	0

\* When Live Hatched Larvae = 0, images were made of eggs only.  
NC = Not Calculated

## Overview of Experiments 1 - 4

### EXPERIMENT 1

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Experiment 1 contained experimental controls (Clean UVB and UVT), reference toxicant exposures (ANS generator columns with UVB and UVT), a comparable suite of exposures with Cosco Busan oil (CB UVB and UVT) and a field control (Urban control with UVB and UVT). Data for the laboratory control were not provided to NewFields, and no samples were collected for analytical chemistry analyses for this experiment. Although data were collected on the developing embryos and hatching success, the lack of chemistry data for water and tissue samples disconnects this first run of the 2009 study from observations made during 2008 and earlier studies with ANS. Survival to normal hatching was acceptable in the Clean control treatments for UVB and UVT which can be used to validate the test.

### EXPERIMENT 2

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The test design for Experiment 2 was similar to Experiment 1. However, this experiment was terminated due to problems with the donor fish spawning success; gastrulation development did not progress well. NOAA and BML deemed this experiment as unacceptable for data evaluation. Clean UVB and UVT normal hatching were not at an acceptable level to validate this experiment.

### EXPERIMENT 3

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The test design for Experiment 3 was similar to Experiments 1 and 2. Samples for chemical analysis were collected from the water and from tissue samples. However, this test was considered by NOAA and BML to be unacceptable for evaluation due to excessive algal growth within the generator columns. Laboratory control survival to normal hatching was acceptable but the Clean UVB and UVT generator control had poor survival to normal hatching. The larval images submitted by BML of hatched live embryos represented only a small subset of the hatched specimens. Several treatments are underrepresented and in some cases, there were no images provided for some treatments. For example there were no images relative to Clean UVT, ANS 0.1 UVT, ANS 0.3 UVT, ANS 1.0 UVT, CB 0.1 UVT, or CB 1.0 UVT. Neither was a complete set of images provided for the dechorionated larvae. The lack of images leads to only a sparse and unequal data set and precludes the use of this data to make any conclusions relative to effects of oil (whether ANS or CBO) on herring larvae.

### EXPERIMENT 4

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Experiment 4 was a smaller experimental design and contained only Cosco Busan oil treatments with UVB and UVT, the Clean UVB and UVT and the laboratory control. Water and tissue samples were collected for chemical analysis in this experiment. Survival to normal hatch in the laboratory control was unacceptable (described earlier in this document), the survival to normal hatch for the Clean UVB and UVT was also unacceptable and the surrogate reference toxicant exposure to ANS was not included in this array preventing the use of this data for any substantive interpretation. Additionally, the use of bleach to remove algae from the columns and test containers (recorded in the laboratory notebooks) can produce chloroform that can be a strong toxicant. The presence or absence of this chemical during the fourth experiment should be evaluated.

## CONCLUSIONS

The experimental design for the proposed demonstration of adverse effects of Cosco Busan oil was well planned. However, actual experimental procedures and conditions introduced unacceptable variation within each experiment which precludes usability of the 2009 dataset. Table 5 summarizes the finding from this QA/QC review. Attributes that are shaded in green meet the necessary outcomes to qualify the data or conclusions while those in yellow do not. The unshaded cells represent information that is missing from the design for each of the experiments. Experiment 1 potentially provided the most useable data set; it did not have chemistry determinations to connect this experiment array with the observations made in 2008. Therefore, based on this quality assurance quality control evaluation none of the experimental runs can be validated or compared to the 2008 data.

Table 6 Summary Evaluation of Experiments 1 through 4.

Criterion	Run 1	Run 2	Run 3	Run 4
Laboratory Control Hatching Success	<i>Images only; no data provided on hatching success</i>		<b>Pass</b>	<b>Fail</b>
Method Clean Control Hatching Success	<b>Pass</b>	<b>Fail</b>	<b>Fail</b>	<b>Fail</b>
Urban Control Hatching Success	<b>Pass</b>	<b>Fail</b>	<b>Fail</b>	<i>Absent</i>
Water Quality: Temperature Range	<b>Fail</b>	<b>Fail</b>	<b>Fail</b>	<b>Fail</b>
Temperature Rate	<b>Fail</b>	<b>Fail</b>	<b>Fail</b>	<b>Fail</b>
Salinity	<i>Not assessed</i>		<b>Fail</b>	<b>Fail</b>
Dissolved Oxygen	<i>Not assessed</i>		<b>Fail</b>	<b>Fail</b>
pH	<i>Not Assessed</i>		<i>Strong trend of increasing pH reflective of algal blooms</i>	
Reference Toxicant (ANS) Response	<i>Included</i>	<i>Included</i>	<i>Included</i>	<i>Absent</i>
PAH Chemistry in Water	<i>Absent</i>		<i>Available</i>	
PAH Chemistry in Eggs	<i>Absent</i>		<i>Available</i>	
NOAA/BML Assessment	<i>Dry run - only not used for assessment</i>	<i>Terminated - Poor survival in laboratory controls</i>	<i>Algae growth interfered with test</i>	<i>Accepted?</i>

(Green shaded areas indicate acceptable while yellow shaded represent unacceptable results.)

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