Impacts of Suspended Sediments on Fertilization, Embryonic Development, and Early Larval Life Stages of the Pacific Herring, *Clupea pallasi*

A Report to the U.S. Army Corps of Engineers and the Long-Term Management Strategy Environmental Windows Science Work Group

July, 2008

Frederick J. Griffin, Edmund H. Smith, Carol A. Vines, and Gary N. Cherr

Bodega Marine Laboratory

University of California Davis, Davis CA, 95616

Executive Summary

This report describes the results of a laboratory study designed to determine the effects of suspended sediment particles on fertilization, embryonic development, and larval survival in the Pacific herring, *Clupea pallasi*. The research was commissioned through the U.S. Army Corps of Engineers by the San Francisco Bay LTMS Environmental Windows Science Work Group. The impetus for the research was to provide scientific data regarding impacts of suspended sediments on the above Pacific herring early life stages and thus provide a scientific platform on which to base regulation of dredging in San Francisco Bay during the herring reproductive season. Controlled laboratory studies were proposed so that causative linkage between suspended sediments and any deleterious effects could be made.

Sediments from two sources were utilized in the study, commercially available Fuller's earth and San Francisco Bay sediments obtained through the U.S. Army Corps of Engineers from the dredging of the Port of Redwood City; the U.S. Army Corps of Engineers provided chemical and bioassay analysis of the SF Bay sediments. Sediments from both sources were extensively washed and filtered to particle sizes of 40-50 μm or less. In initial experiments Fuller's earth was used to establish protocols and concentration tolerances of herring early life stages to a known standard sediment. Once these were established we utilized the SF Bay sediments in subsequent studies. We examined the following variables in our studies: 1) Duration of sediment exposure (from 15 min to 2 hours, to days); 2) Timing of exposure (e.g. at fertilization, 2 hours post-fertilization, at 24 hrs post- fertilization); and 3) concentration of suspended sediments (up to 500 mg/L).

Suspended sediment particles adhered to eggs and remained attached to eggs and embryos only when particles were presented to during the first two hours after eggs were dispensed into water. During this initial two hours, the adhesion development period, particles became permanently attached to egg/embryo chorions. When embryos were exposed to sediments after this two-hour adhesion development period particles easily dislodged from the embryos. In the presence of 250 mg/L or more suspended sediment eggs aggregated into clusters or multiple layers. This was quantified by video capture and image analysis of average diameter of egg and egg cluster "footprints" in culture dishes. The significance of this adhesion development period is that it is the only time during which suspended sediment particles (at concentrations up to 500 mg/liter) negatively affect Pacific herring early life stages.

The effects of suspended sediments on Pacific herring fertilization and embryonic development to larval hatch were not clear. Although there was a statistical interaction between sediment treatment and fertilization rate as well as between sediment treatment and total larval hatch (in some experiments), high variability produced low r^2 values and low confidence in the linkage. There were, however, sublethal effects on embryonic development that culminated in reduced larval survival. Effects on eggs/embryos that were treated with 250 or 500 mg/L suspended San Francisco Bay sediment for the first two hours after eggs were dispended into water included: 1) Hatched larvae that were 5-10% more likely to be abnormal; abnormalities included larvae that would not survive because they exhibited bent spines (scoliotic) or did not exit from the tightly coiled posture of pre-hatch larvae. 2) Larvae that hatched precociously, on average one day earlier than larvae from no-sediment control

embryos. 3) Larvae that hatched at a smaller size (12% less total length), possessed larger yolk-sacs (approximately 17% greater in area), and grew at a slower rate than larvae from no-sediment control embryos. Lastly, egg/embryo treatment with suspended sediments (250 or 500 mg/L) during the initial two hours in water had negative effects on larval survival; early post-hatch mortality in larvae from treated embryos was four times greater than that of non-sediment controls (~ 8% compared to 2%) for days 2, 3, & 4 post-hatch and over a 15 day period there were almost 41% fewer mortalities in no-sediment controls than in larvae from sediment treated embryos.

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Introduction

Freshwater input is a common denominator linking Pacific herring, *Clupea pallasi*, nursery areas throughout the species range because reduced salinity (below that of full seawater) is key to fertilization and the survival of herring embryo stages (Yanagimachi 1957a, b; Alderdice and Hourston 1985; Hay 1985; Griffin et al. 1998). Pacific herring leave marine waters during Winter or Spring (latitude dependent) and enter into protected bays and estuaries that are utilized for spawning and as nursery habitat (Spratt 1981; Alderdice and Velsen 1971; Hay 1985; Barnhart 1988; see also Watters et al. 2004). In northern latitudes, like Hokkaido, Siberia or Alaska, the reproductive season occurs in late Spring, a time of lowered salinity due to input from snow and ice melt (Dushkina 1973; Iizumi et al. 1995; Williams and Quinn II 2000). In more southern latitudes the reproductive season is linked to lowered salinity produced by freshwater input due to seasonal precipitation, for example San Francisco Bay, California (Cloern and Nichols 1985; Ingram et al. 1996). Freshwater input, in addition to lowering salinity, delivers suspended sediments and dissolved nutrients that replenish substrata and increase biological productivity, also important to herring reproduction (Ruhl et al. 2001; Ruhl and Shoellhamer 2004). Growth of algae and aquatic plant species (e.g. *Zostera*, *Phyllospadix*, *Gracilaria*) that provide substrata to which herring attach adhesive eggs, as well as plankton species that serve as the base of the food web on which herring larvae feed, can benefit from sediment and nutrient replenishment. Sediment input, however, can be problematic; high sediment loads can reduce light penetration and thus primary productivity (both in the water column and on the benthos) and/or can cover and smother benthic organisms and substrata due to particle settling (Bruton 1985; Cloern 1987; Newcombe and McDonald 1991).

San Francisco Bay is a heavily urbanized estuary, dramatically altered and impacted by human disturbance throughout its watershed (e.g. by placer mining, agriculture, urbanization) as well as within the bay itself (Krone, 1979). A major result of human disturbance over time has been an increased sediment budget. The Bay is the southern-most spawning and nursery area for Pacific herring along western North America and a long-term decline in spawning biomass within San Francisco Bay (Miller and Lea 1972; Watters et al. 2004) has led to increased regulatory concern over activities that might impact reproduction. Currently suspended sediment loads, due to both sediment input with freshwater influx and re-suspension of previously delivered sediments, are greatest during the Pacific herring reproductive season. They can reach 500-600 mg/L, with the potential of localized higher concentrations due to anthropogenic activities like dredging of harbors and channels, and near shore development projects (Krone, 1979; Ingram and DePaolo 1993; Ingram et al. 1996; Watters et al. 2004; McKee et al. 2006). Several previously published laboratory studies using *Clupea* species (both Atlantic and Pacific species) have generally concluded that levels of 1,000 mg/L or greater were required to produce significant mortality in embryos or larvae (Boerhlert et al. 1983; Morgan and Levings 1989; Kiorboe et al.1981). These studies, however, were conducted with eggs that were fertilized in sediment-free water and then, following fertilization and egg envelope hardening, were transferred to sediment containing water. The studies did not investigate whether fertilization was impacted. They also did not test the impacts of sediment presence during an early critical time period in herring reproduction when eggs and embryos are adhesive or “sticky” and still forming the adhesive coat that will anchor them to substrata until larval hatching (Laale 1980; Gillis et al.

1990). Here we show that full development of the adhesive layer of herring embryos requires approximately two hours, that exposure to sediments during this 2 hr window results in attachment of sediment particles that persists through embryonic development to larval hatching, and that attachment of sediment particles impacts embryo development and larval survival at sediment concentrations as low as 250 mg/L. This study includes qualitative data, as well as quantitative data. Both are important; while the quantitative data provides statistically testable results of sediment effects on embryonic and larval success, the observational data provides background for future investigations that could lead to mechanistic explanations for the quantitative results that are presented.

Materials and Methods

Solutions

Half-strength seawater, 16 practical salinity units (psu), was made by diluting 0.45 μm filtered Bodega Marine Laboratory seawater with distilled water Salinity was monitored with a salinometer (refractometer). Half-strength calcium and magnesium-free seawater (½ CaMgF) was made by diluting full-strength CaMgF seawater (made according to Cavanough 1975) with equal parts distilled water. ½ CaMgF polyvinyl alcohol (PVA) was made by adding 0.25% PVA to ½ CaMgF. Herring eggs are not adhesive while suspended in ½ CaMgF-PVA, yet will be come adhesive if transferred to normal 16 psu seawater (Griffin et al. 1998). Seawaters were stored at 12^{o}C. All chemicals were obtained from Sigma Chemical (Sigma-Aldrich).

Sediment Handling and Storage

Sediments from two sources were utilized in the study: 1) Dredged San Francisco Bay sediment from the Port of Redwood City (SF Bay sediment) supplied by the U.S. Army Corps of Engineers, San Francisco, CA; and 2) Fuller's earth, a commercially available control sediment (F-200, Sigma-Aldrich). Composition of SF Bay sediment was 18% clay (< 4 μm), 75% silt (4-63 μm), and 7% sand & gravel (>63 μm) as determined in a 2005 analysis (USACE 2005). Chemical analyses to determine presence of contaminants were available from five sediment samples; the analyses indicated that ten metals, four butyltins, four classes of pesticides, seven PCBs, and sixteen PAHs were associated with sediment particles, but only three were above San Francisco Bay Ambient Sediment Concentration (ASC) values (USACE 2005) for any individual analysis (chromium, mercury, and silver) and all three were less than 125% of ASC values. Chromium was the only toxicant for which the average of the five chemical analyses exceeded background levels; chromium average was 130 ppm compared to the San Francisco Bay ASC values of 112 ppm. Biological toxicity testing utilized an amphipod bioassay; survival of *Rhepoxynius abronius* and *Ampelisca abdita* ranged from 84-100% and averaged 94.71 % + 4.92 standard deviation (USACE 2005). SF Bay sediment was aliquoted into glass, lidded jars (250 ml) and stored at -20^{o}C. Prior to processing, Fuller's earth was stored dry packaged by the manufacturer.

Processing of suspended sediments for experiments involved suspension, washing, and adjusting concentration to 2 g dry weight sediment /L. To obtain a final concentration of 2 g/L we began with 4 g/L of Fuller's earth and 6 g/L of SF Bay sediment; the difference in initial weights was because Fuller's earth was obtained as dry sediment, while SF Bay sediment was dredged and contained water. Weighed sediments were suspended in 1 liter of 16 psu seawater at 4^{o}C, stirred vigorously with a magnetic stir bar for 60 minutes, and the larger particles allowed to settle for 30 min. The resultant supernatant was decanted and allowed to settle overnight at 4^{o}C. Settled particles were then washed by resuspension in 1 liter of clean 16 psu seawater followed by stirring for 60 minutes and overnight settling of particles; this was done twice. After the second wash resupended sediment particles were gravity filtered twice through Whatman #2 filters. Sediment concentration of the filtered suspended sediments was determined by obtaining sediment dry weight in a known volume, as follows. A 100 ml aliquot of filtered suspended sediment was removed and suction filtered onto a 0.45 μm analytical filter. The filter was dried overnight in a 60^{o}C drying oven and the weight of the dried sediments determined. A stock suspension was then obtained by

adjusting sediment concentration to 2 g/L. Two hundred ml aliquots were then frozen at -20^{0}C until use. Early on we also developed a standard suspended sediment concentration curve by recording absorbance at 546 nm using a Biomate spectrophotometer (Thermo Spectronic) and known concentrations of suspended Fuller's earth. This standard curve was used to validate dry weight concentration determinations.

The accepted method of storage in previous studies has been sub-zero storage, either frozen intact at -20^{0}C or -70^{0}C, or freeze-dried (Norton et al. 1999). However, a more recent study questioned the efficacy of using previously frozen contaminated sediments without a post-freeze wash due to the accumulation of NH_4^+ during freeze storage (Geffard et al. 2004). To address this concern, freeze-stored sediments were thawed and washed once before use in experiments.

In preliminary experiments we utilized Fuller's earth to determine appropriate final sediment concentrations, resolve effective times of sediment treatment, and to refine lab techniques with sediments. Initial concentrations used ranged from 65-1,000 mg/L Fuller's earth. 1000 mg/L was chosen because in the literature it is the consensus lowest effective concentration and because it is near the upper limit of measured suspended sediments in San Francisco Bay (Shoellhamer et al. 2007). We saw no decline in fertilization up to 1,000 mg/L Fuller's earth. Although results were not statistically significant ($p = 0.217$), we did observe a decline in total larval hatch beginning at 125 mg/L Fuller's earth, from 68 % in non-Fuller's earth control cultures to 42 % in 125 mg/L Fuller's earth treated cultures. There was also an increase in egg aggregation or clustering in the sediment treated cultures that produced multiple layers of eggs. Lastly, there appeared to be a link between hatch decline and egg aggregation since the hatch rose from 67 % to 76 % when only non-aggregated eggs were scored. Based on these experiments we predicted that effects should be obtained at concentrations of sediment beginning at 125 or 250 mg/L and focused on sediment concentrations of 65-500 mg/L in experiments conducted with suspended SF Bay sediments

Sediment Charge Experiments

Sediment particles can possess positive or negative charges which raised the possibility that charge based attraction might be involved in egg aggregation (Hunter and Liss, 1979; 1982). As a first step we asked what the charge was on SF Bay sediment particles by determining particle-particle attraction under different net solute charge conditions Suspended sediment particles were added to 10 x 5 cm circular glass dishes containing 100 ml of 16 psu seawater that was pH 4.0 (with HCl), pH 9.0 (with NaOH), or was pH 8.0 and contained 0.25% lysine, 0.25% polylysine, 0.25% glycine, or 0.25% bovine serum albumin. Ten ml of 2g/L suspended sediment stock solution was added to each solution and the dishes were swirled for 5 seconds. Images of sediments in each solution were captured (see *Microscopy and Image Analysis* below).

Animal Collection & Acquisition of Gametes

Gravid Pacific herring were collected by hook and line or otter trawl from San Francisco Bay and transported on ice to the Bodega Marine Lab (BML). Ovaries and testes were dissected and kept moist in petri dishes at 4^{o}C until needed for experiments for up to four days (Yanagimachi et al. 1992). Some ovaries and testes were obtained from fish collected in Puget Sound, Washington. These were dissected from freshly caught fish and sent on ice overnight to the Bodega Marine Laboratory. Sperm from 2-3 testes were diluted

into 16 psu seawater and adjusted to a stock suspension (10^8 sperm/ml). Eggs were either removed from dissected ovaries with a metal spatula and directly distributed into experimental dishes or into ½ CaMgF PVA and then distributed to experiments via pipette (Griffin et al. 1998). Eggs from 2-4 females were pooled.

Fertilization and Embryo Sediment Treatments

Fertilizations were conducted in either 10 x 5 cm circular glass dishes, in 10 cm diameter 2 liter glass settling columns with 8 cm diameter pieces of 1 mm Nitex mesh placed at the bottom of the settling column, or in 1 L rectangular glass dishes. The bottoms served as substrata for egg adhesion in the glass dishes, while the Nitex became the attachment substrata in the settling columns. Eggs were introduced into dishes or settling columns containing 16 psu seawater after addition of 10^5 sperm/ml and 0 – 500 mg/L SF Bay sediment. In all but the aggregation experiments (see *Sediment-Induced Egg Aggregation Experiments* below) eggs were distributed or broadcast into water evenly to prevent multiple layer clusters of eggs; eggs that aggregated into multiple layer clusters were removed to eliminate aggregation effects on experimental results. To determine sediment effects on fertilization, eggs were co-incubated with sperm and sediments for 15 min (normal time for maximum fertilization; see Yanagimachi et al. 1992) in a 12^{o}C incubator. In experiments designed to determine sediment effects on embryo development sperm, eggs, and sediments were co-incubated for 2 h (this was the approximate time frame required for adhesive hardening). No-sediment controls consisted of eggs fertilized and treated like experimental cultures except that sediments were not present. After the predetermined sediment treatment time (15 min or 2 h), cultures were washed (3 x) in sediment-free16 psu seawater to remove unattached sediments and/or sperm. The initial 2 h period after eggs were dispensed into water was denoted as 0-2 hours post-fertilization (0-2 HPF). Embryos that were treated with sediment at all time periods after 0-2 HPF were fertilized in sediment-free water and then exposed to sediment from 2-4 HPF, 4-6 HPF, or 24-26 HPF, or for 2 hr at 5 days post-fertilization.

We scored fertilization at 2 hr post-fertilization (the chorion elevates and becomes translucent; Appendix I; Yanagimachi et al. 1992), however, to insure that spontaneous egg activation was not erroneously interpreted as fertilization, only embryos that progressed to the epiboly stage of development (circa 24 h after fertilization) were deemed fertilized.

For embryonic development experiments, dishes containing fertilized eggs were allowed to develop inside incubators at 12^{o}C until larval hatching occurred (6 -12 days). Cultures of developing embryos were monitored and provided with 100% daily exchanges of water. In most of the experiments sediment-free water was added daily. For the chronic sediment exposure experiments, 16 psu seawater containing the appropriate sediment concentration was added. Attempts to delineate the timing of developmental stages prior to hatching proved inconclusive; sediment particles adhering to embryos in the 0-2 HPF sediment treatments precluded accurate determination of developmental progression (Appendix I); although progression through embryonic developmental stages was monitored, larval hatch was deemed the best measure of successful embryonic development.

Beginning at first sign of hatching, larvae were removed with daily water changes and counted. Normal and abnormal larvae were scored; abnormal larvae were those that did not uncoil or straighten out after hatch or were scoliotic. Random aliquots of hatched normal appearing larvae (10 individuals per culture) were photographed for length and yolk-sac

measurements through a digital camera mounted on an Olympus stereo-zoom microscope (see *Microscopy* below).

For chronic sediment treatments (duration of embryonic development), eggs were added to tissue culture flasks and the flasks were capped and placed on a rocker table at 12^0C for 2 h. The cultures were then washed free of sperm after 2 hr and subsequently received water and sediment changes daily.

Sediment-Induced Egg Aggregation Experiments

During early experiments with Fuller's earth we observed a greater tendency for eggs to adhere to one another and form clusters (aggregate) in the presence of suspended sediments. To document this phenomenon we exaggerated egg-egg contact during egg settling. The water in culture dishes was swirled as eggs were dispensed; swirling was maintained until eggs began to adhere to the bottom of a dish (~5 seconds). We also allowed eggs to settle undisturbed onto Nitex screens through a 25 cm column of water in the 15 cm diameter 2 L glass settling columns. In both cases embryos were subsequently cultured as described above. To quantify egg aggregation we tested the hypothesis that increased aggregation could be measured as an increase in average area of egg or egg cluster "footprints". Average "footprint" were determined using NIH ImageJ software (see *Microscopy and Image Analysis* below).

Larval Culture

Larval age was measured in days post-hatch (DPH). Larvae designated as 1-DPH were those that hatched during the first 24-hour period after larvae were first observed in a culture. Ages of larvae that hatched after the initial 24 hour period were then designated as 2-DPH, 3-DPH, etc; thus, 2-DPH larvae were larvae that had hatched 24-48 hr after first observation of larval hatching. Larvae from 0-2 HPF sediment treated embryos began hatching one day before no-sediment controls (see results), therefore in experiments on larval size, growth, and survival we utilized larvae that hatched on the same calendar date in order to compare larvae of the same post-fertilization age.

Approximately 200 larvae were pooled from replicate embryo dishes. Larvae were transferred into clean 1 L rectangular glass dishes containing 400 ml of sediment-free 16 psu seawater at a final density no greater than of 200 animals per 400 ml and cultured as described by Griffin et al (2004). The larvae were kept at 12^oC and, following daily water exchanges of 50%, larvae were fed 0.5 ml of a concentrated rotifer solution (300-500 rotifers). Mortalities were removed and recorded from cultures daily. Aliquots of live larvae were removed and photographed for measurement of larval growth and development (total length and yolk-sac diameter) as described (under *Microscopy and Image Analysis*).

Microscopy and Image Analysis

Eggs, embryos and larvae were assessed and images collected through an Olympus SZH stereo-zoom microscope equipped with bright-field and dark-field optics. Images of embryos and larvae were collected using a PixelLink CCD camera using PixelLink capture software. Spatial distribution of embryos in cultures was analyzed by determining the average area of embryo "footprints" using ImageJ software (National Institutes of Health). Images of embryo cultures were cropped, threshold adjusted and average cluster size or embryo "footprint" measured with ImageJ software using the area measurement tool

(Appendix II). Larval length and yolk-sac area were measured with ImageJ software measurement functions and calibrated against collected images of a 2 mm stage micrometer.

Oxygen Consumption Measurements

Oxygen measurements were made in a jacketed RC 350 respiration cell (Strathkelvin Instruments, Glasgow, U.K.) with a chamber volume of 1.25 ml at constant temperature (13^{o} C $\pm$ 0.5) using a flow through system that supplies cold water around the jacketed respiration cell. Measurements were made using a micro-cathode O_2 electrode (model 1302) with a Strathkelvin Model 781 oxygen meter. Data was downloaded directly to a computer and oxygen rates determined using 949 Oxygen System (Version 2.2) software (Strathkelvin Instruments). To insure equal O_2 distribution during data collection, a small stir bar was placed in the respiration cell and gently activated. All O_2 measurements were conducted in 16 psu seawater that had been saturated with O_2 via a diffuser at 13^{o} C (for at least 15 min); Oxygen concentration at saturation was determined with an Oxygen Solubility Table (based on temperature and salinity). Oxygen consumption in the control (no organisms) was measured after every four embryo or larval samples. Any decrease was as denoted as background oxygen consumption (i.e. bacterial) and was subtracted from embryo and larval O_2 consumption measurements.

Eighteen to twenty embryos and 2 to 4 larvae were used to make the oxygen consumption measurements. The organisms were placed in the test chamber with 1.25 ml of 16 psu seawater and the [O_2] changes measured. After 30 minutes, the test organisms were removed from the chamber, washed very briefly in distilled water and placed on aluminum foil weighting cups. The organisms were oven dried (60^{o}C) and then weighed. Total weights for all the organisms (for each experiment) were used in calculating the respiration rates.

Oxygen consumption was measured for developing embryos at three post-fertilization time points, day 1, 2 and 6 PF. Larval respiration rates were measured using 2 DPH larvae. Individual embryo and larval respiratory rates were calculated by dividing the total rate by the number of embryos or larvae used in each experiment. Absolute individual and normalized by weight rates were calculated.

Statistical Analysis

Data were collected on Excel spreadsheets (Microsoft Corporation) and analyzed using SIGMASTAT (Jandel Scientific) or SYSTAT (SYSTAT Software, Inc) software. Unless otherwise identified, data in Figures are presented as means $\pm$ standard deviations (minimum n = 5). Where data was presented as percentages, arcsine square root transformation was performed to achieve normality before statistical analyses were run. GLM (General Linear Models, SYSTAT) regression analysis was run on transformed data to identify relationships between dependent and independent factors. When relationships were indicated, a Student t-test (SIGMASTAT) was employed to determine if there were differences between individual dependent and independent variables. To eliminate group effects on Student t-tests the significance level was set at $\alpha = 0.05 /k$, where k is the number of comparisons. Student t-tests that were not assigned to a GLM regression analysis utilized a significance level of $\alpha = 0.05$.

Survival Analysis (SYSTAT) of larval survival was used to compare no-sediment control and sediment treated cultures (Muenchow 1986). Data were presented as number of days post-hatch that larvae survived during 14 days of the experiment; larvae that remained

alive at experiment termination were denoted as surviving 15 days and were defined as right censored data. Mean survival times as well as Kaplan-Meier survival probabilities for individual post-hatch days were recorded. In addition, GLM ANCOVA regression analysis was employed on three segments of transformed data (0-5, 6-10, and 11-15 days post-hatch) to discern if decreased larval survival in sediment treated cultures was due to higher mortality during a particular time segment of the experiment. GLM ANCOVA was conducted after cumulative mortality data was converted to cumulative percentage mortality (based on 200 larvae per culture at experiment initiation) and subjected to arcsine square root transformation.

Results

Suspended Sediment Particle Interactions

Processing of SF Bay sediment was conducted to wash and reduce particle size. Washing, filtering, adjustment to 2000 mg/L, and storage yielded a sediment stock concentration with particle size at or below 40-50 μm after freeze-thaw in pH 8.0, 16 psu seawater (Fig 1A). Particles did aggregate into larger size clumps after positively charged solutes were added to the water; this was true for both Fuller's earth and SF Bay sediments. Particle aggregation due to increased positive solute concentration occurred after $[H^+]$ was increased (pH reduced to 4.0, Fig. 1B), or when 0.25% glycine, (Fig. 1C) 0.25% lysine (Fig. 1D) or 0.25% polylysine (Fig. 1E) were added to the water; negatively charged solutes (e.g. OH^- pH 8.0, see Fig. 1A or 0.25% bovine serum albumin, not shown) did not produce particle aggregation.

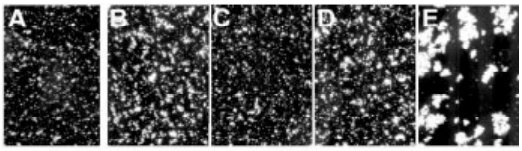

Figure 1. Sediments aggregate in presence of net positive charges. A. Sediments in pH 8.0 water retained filtered size of less than 40-50 μm. Sediments aggregated in water containing excess positive charge; B. pH 4.0. C. 0.25% glycine, D. 0.25% lysine, and E. 0,25% polylysine.

Suspended Sediment-Egg Chorion Interactions

Pacific herring eggs are surrounded by an extracellular adhesive envelope, the chorion, that adheres eggs and embryos to substrata (Hay and Miller 1982; Hay 1985; Grosse and Hay 1988 Gillis et al. 1990). When sediment particles contacted eggs or embryos during the first two hours after the eggs were dispensed into water they attached to the adhesive layer of eggs and remained attached. Eggs sank to the bottom of and adhered to glass culture dishes both in the presence and absence of suspended SF Bay sediments, and six hours later remained bound (Fig. 2). Eggs also adhered to Nitex mesh fibers (not shown). In no-sediment control cultures embryos were translucent and attachment plaques were evident (centralized circular regions in each embryo, Fig. 2A). In contrast, cultures treated with 500 mg/L of suspended SF bay sediment during the first four hours after first egg contact with water, 0-2 HPF and 2-4 HPF, possessed embryos that were opaque due to the attachment of sediment particles to chorions (Fig. 2B & C). Sediment particles also attached to the substrata in close proximity to embryos. No visible sediment attachment on

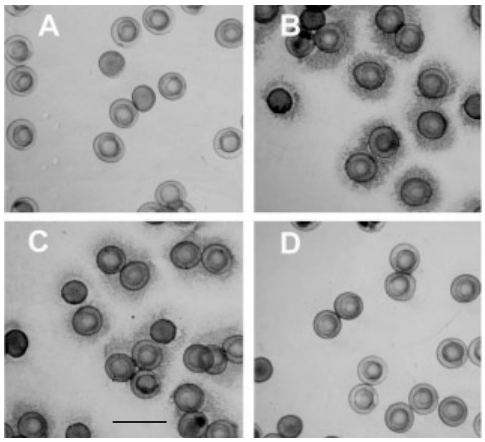

Figure 2. Suspended sediment particle adherence to egg chorions. Eggs and embryos were treated for a 2 hour period beginning at three different time points after eggs first contacted water and observed at 6 HPF. A. No-sediment control. B. Sediment treatment at 0-2 HPF. C. at 2-4 HPF. D. at 4-6 HPF. Scale = 2 mm.

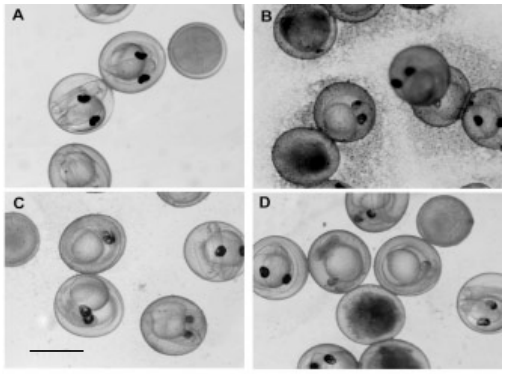

Figure 3. Retention of suspended sediment particles on chorions. A. No-sediment control embryos. Embryos that had been treated with 500 mg/L suspended SF Bay sediment at 0-2 HPF (B), at 2-4 HPF (C), and at 4-6 HPF (D) were observed at six days post-fertilization. . Scale = 1 mm.

embryos or substrata was observed when sediments were added after 4 HPF (compare Figs. 2B, C, D). Attachment of sediment particles was permanent only on 0-2 HPF treated embryos (Fig. 3). On day six of embryonic development, embryos that had been treated with 500 mg/L of suspended SF Bay sediments at 0-2 HPF (Fig. 3B) were still coated with particles while embryos that had been treated with suspended sediment at 2-4 HPF had lost the particles (probably during daily water exchanges) and those treated 4-6 HPF remained sediment free (Fig 3C, D). In the 0-2 HPF cultures sediment particles also remained attached to the substrata immediately surrounding embryos, but had disappeared from the 2-4 HPF cultures. The ability to retain suspended sediment particles that attached during 0-2 HPF correlated with the period of time the herring chorion remained adhesive or sticky.

Sediment treatment experiments provided an opportunity to follow expansion and hardening of the chorion's adhesive layer during the first two hours. After adhering to the bottom of glass dishes the adhesive layer appeared to gravitationally "flow" or expand around eggs and onto the dish. This produced a "zone" of adhesive material around eggs on the bottom of culture dishes that was marked by sediment particles around embryos that had been treated with suspended sediments at 0-2 HPF or 2-4 HPF (see Figs 2B, 2C, 3B).

Suspended Sediments and Spatial Distribution of Eggs/Embryos

During natural spawns, egg deposition in clusters or aggregations can occur when eggs settle onto and adhere to one another; the chances of this happening increase with spawn density or when eggs are released into the water column by spawning females (Stacey and Hourston 1982; Hay 1985; Haegle and Schweigert 1985). In the laboratory we observed a greater tendency for eggs to aggregate in the presence of suspended sediments. To quantify this phenomenon we artificially induced egg-egg contact by swirling the water in the dishes as eggs were dispensed. Swirling had little effect on sediment-free cultures, but induced egg aggregation when suspended SF Bay sediment was present (Figs. 4A & B). This was quantified by measuring the circularity and area (footprint) of eggs and egg clusters. In no-sediment cultures and cultures treated at 0-2 HPF in 65 mg/L of suspended SF Bay sediment aggregation was minimal; the average "footprint" was close to the area of an individual embryo, diameter circa 1mm, area circa 1.57 mm^2 (Fig 4 graph and APPENDIX II). Embryos that had been treated with 250 and 500 mg/L of sediment attached to the substrata in clusters and possessed "footprints" that were on average significantly larger than that of an

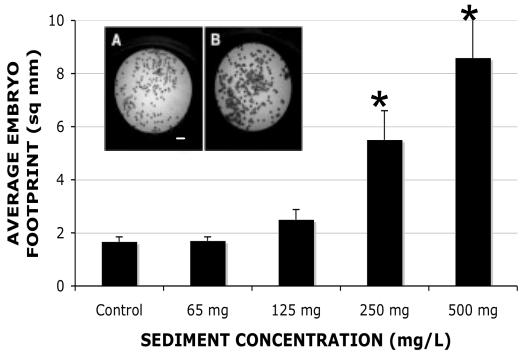

Figure 4. Suspended sediments induce aggregation of eggs into clusters. Eggs were treated with suspended SF Bay sediment (concentration from 0-500 mg/L) during fertilization and through the first two hours in water (0-2 HPF). Images of embryo cultures were cropped, threshold adjusted and average cluster size or embryo "footprint measured with Image J software using the area measurement tool. A. No-sediment control eggs that settled onto and adhered to bottom of culture dishes provided embryo "footprints "about equal to the size of an individual egg. B. Aggregated eggs that were co-incubated with 250 mg/L of sediment formed footprints larger than individual eggs. The 250 mg/L and 500 mg/L treatments yielded increased aggregation (t-test $p < 0.001$*). n = 5, with 20 measurements per n. Scale = 5 mm.

individual embryo; 500 mg/L of suspended SF Bay sediment produced an average "footprint" that was 8 times the size of an individual embryo (Fig 4 graph).

Fertilization and Embryonic Development

Analyses of the effects of suspended SF Bay sediments at concentrations up to 500 mg/L on fertilization and total larval hatch (completion of embryonic development) produced ambiguous results. Fertilization in the presence of suspended SF Bay sediment (65-500 mg/L) ranged from 58.6 % + 8.5 to 66.2 % $\pm$ 8.9 compared to 68.4 % + 8.6 for no-sediment control eggs. Regression analysis revealed an interaction between sediment treatment and fertilization (Fig. 5, $p = 0.023$), however, predictability of the interaction was low ($r^2 = 0.095$). When fertilization data across the range of sediment concentrations were individually compared to no-sediment control fertilizations they were not statistically different for any sediment concentration (t-test $p > 0.0125$). Effects of suspended SF Bay sediment on completion of embryonic development to larval hatch were even more ambiguous. Experiments conducted during the study examined the effects of suspended sediments on larval hatch (end point of embryonic development) as a function of one or more of three parameters; concentration of suspended sediments, short-term versus long-term sediment treatment, and short-term treatment at different developmental times. In the first set of experiments sediment concentration was varied from 65–500 mg/L and all exposures were short-term at 0-2 HPF. The second set examined effects of sediment treatment duration; eggs/embryos were treated with 125, 250, or 500 mg/L for the entirety of embryonic development (chronic exposure) and compared to no-sediment controls and treatment with 500 mg/L sediment at 0-2 HPF. In the third set of experiments embryos were treated with 125, 250, or 500 mg/L sediment for two hours at different embryo development time points. Table 1 presents percent total larval hatch (as a percent of fertilization) in the experiments that examined these three parameters. Regression analysis suggested there was an interaction between sediment concentration and total larval hatch in the Constant Short-term and Timing of Exposure (0-2 HPF and 2-4 HPF) experiments (Table 1). There was however, no interaction between treatment and total larval hatch in the Short vs Long-term nor in the 24-24 HPF and 5 DPF two-hour sediment treatment Timing of Exposure experiments (Table 1). As occurred in the fertilization experiments, low r^2 values implied high variability and little confidence in the regressions. Comparisons of within-experiment no-sediment controls and sediment treatments showed significant hatching declines for only the 250 and 500 mg/L treatments in the Constant Short-term experiments ($p = 0.004$ for 250 mg/L and $p = 0.005$ for 500 mg/L where $\alpha = 0.0125$).

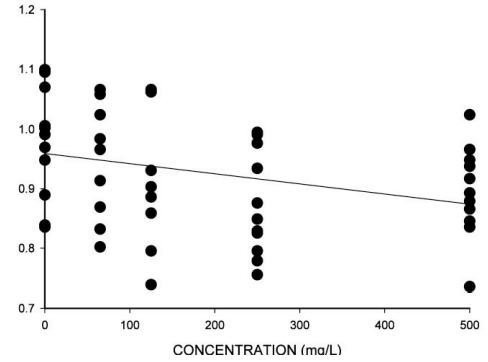

Figure 5. Regression analysis of fertilization in the presence of suspended sediments. Eggs were deposited into water containing sperm and suspended SF Bay sediment (0-500 mg/L). There was a weak interaction between fertilization and sediment concentration [GLM $F(1,53) = 5.515$ $p = 0.023$ $r^2 = 0.095$]. Individual comparisons of no-sediment control cultures and sediment treatments (t-test) did not yield differences ($p > 0.0125$). $n = 11$.

Table 1. Impacts of Suspended SF Bay Sediment on Larval Hatch

Sediment Exposure: Timing & Duration	Sediment (mg/L)	N	Mean Hatch (% of Fertilization)	Std Dev
1. Constant Short-term	GLM REGERSSSION: F(1,33) = 13.418 p = 0.001 r^2 = 0.289			
No Sediment	0	8	75.81	5.90
0-2 hour sediment	65	8	76.37	6.59
0-2 hour sediment	125	8	72.48	8.05
0-2 hour sediment	**250**	**8**	**66.61**	**3.07**
0-2 hour sediment	**500**	**8**	**66.74**	**3.64**
2. Short vs Long-term	GLM REGRESSION: F(4,40) = 1.359 p = 0.265			
No Sediment	0	9	64.56	7.61
0-2 hour sediment	500	9	59.06	6.53
Chronic sediment	125	9	62.39	7.92
Chronic sediment	250	9	60.28	3.53
Chronic sediment	500	9	59.94	8.02
3. Timing of Exposure	GLM REGERSSSION: F(1,58) = 5.688 p = 0.020 r^2 – 0.089			
0-2 hour sediment				
	0	15	62.00	9.63
	125	15	60.20	8.26
	250	15	53.73	8.87
	500	15	50.56	10.31
2-4 hour sediment	GLM REGERSSSION: F(1,58) = 4.731 p = 0.034 r^2 = 0.075			
	0	15	61.27	5.44
	125	15	60.07	6.84
	250	15	57.27	6.94
	500	15	56.87	4.76
24-26 hour sediment	GLM REGERSSSION: F(1,58) = 0.126 p = 0.723			
	0	15	60.13	8.18
	125	15	59.13	8.63
	250	15	58.40	6.05
	500	15	60.93	7.81
2 h at 5 day sediment	GLM REGERSSSION: F(1,58) = 0.074 p = 0.787			
	0	8	61.78	7.19
	125	8	60.89	6.39
	250	8	63.56	7.67
	500	8	62.11	8.87

Regression analyses were run on arcsine square root transformations of total percentages of fertilized eggs that hatched. Treatments in **Bold** were significantly different from controls (t-test $p < 0.0125$, significance set at $\alpha = 0.05/k$, where k = the number of comparisons).

SF Bay and Fuller's Earth Comparison

Fuller's earth, a control sediment, was utilized in preliminary experiments to establish protocols and sediment concentration ranges to be used with SF Bay sediments. As a result of lack of statistical clarity regarding effects of suspended SF Bay sediment on total larval hatch we compared 0-2 HPF suspended sediment treatments using SF Bay sediment and Fuller's earth to elucidate whether there was a difference in the experimental outcomes of the two sediments, possibly due to sediment type or contaminants associated with SF Bay sediment. Figure 6 depicts regression analyses of transformed data for fertilization (Fig. 6A) and total larval hatch (Fig. 6B). Fertilization did not differ as a result of sediment source (GLM ANCOVA interaction of sediment source & sediment concentration, $p = 0.393$, Fig 6A). Likewise, total larval hatch was not significantly different as result of sediment source (GLM ANCOVA interaction of sediment source & sediment concentration, $p = 0.566$, Fig 6B).

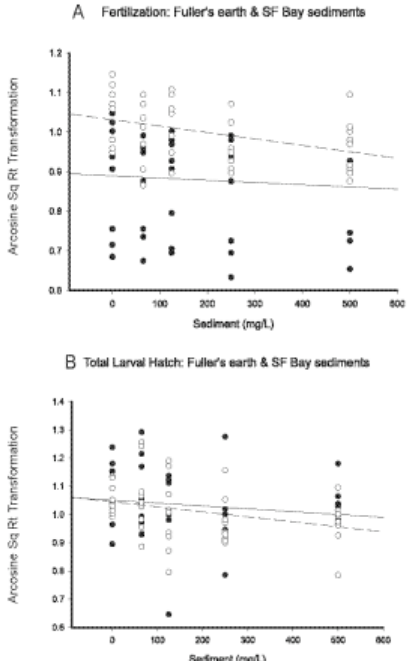

Figure 6. Comparison of effects of Fuller's earth and SF Bay sediments on fertilization (A) and total larval hatch (B). Regression analyses comparing Fuller's earth (● ——) and SF Bay (○ ·····) sediment treatment revealed no differences based on the combination of sediment source and sediment concentration for fertilization [GLM ANCOVA $F(1,86) = .736$ $p = 0.393$] or total larval hatch [GLM ANCOVA $F(1,86) = 0.332$ $p = 0.566$]. n = 9.

Sublethal Sediment Effects on Embryo Development

Treatment of eggs with suspended SF Bay sediments correlated with several sublethal effects; the percentage of hatched larvae that were morphologically normal, duration of embryonic development (time from fertilization to hatching), larval size at hatching, post-hatch larval growth, and larval survival all declined with suspended SF Bay sediment treatment of eggs at 0-2 HPF. Abnormalites that were observed included scoliotic larvae and larvae that did not straighten from their pre-hatch tightly coiled posture (APPENDIX III). There were additional abnormalities that precluded hatching; instances of larvae that did not completely exit the chorion and died during hatch as well as embryos that had developed to the larval stage, but did not hatch. These non-hatch abnormalities were not included in the abnormal larval count. An increase in larval abnormality (decrease in normal larvae) was linked to suspended SF Bay sediment concentration (Fig. 7 GLM ANCOVA $F(1,66) = 23.198$ $p = 0.001$, $r^2 = 0.778$). The percentage of normal larvae that hatched from embryos treated with 250 or 500 mg/L of suspended SF Bay sediment at 0-2 HPF significantly declined from total hatch and from the no-sediment control percentages (t-test $p < 0.0125$). In no-sediment controls 68.7% $\pm$ 0.43 of the total hatch was normal while normal hatch after exposure to concentrations of 250 mg/L was 45.3 % $\pm$ 3.9 and 37.3 % $\pm$ 4.1 with 500 mg/L treatment at 0-2 HPF (Fig 7).

Larvae from sediment treated eggs also hatched earlier than did no-sediment controls. Larvae from both 0-2 HPF and chronic suspended SF Bay sediment treated cultures hatched one day before no-sediment control larvae. In two sets of experiments sediment treated larvae began hatching on day 6 PF and control larvae commenced hatching on day 7 PF. In a third set sediment treated cultures produced hatched larvae beginning on day 7, while controls did not begin to hatch until day 8 (APPENDIX IV). Figure 8 depicts the pooled data from these experiments. Instead of grouping larval hatch by day post-fertilization we designated the first day that no-sediment control larvae hatched as day 1; thus, day 1 equated to day 8 PF for the third set of experiments and day 7 for the other two. There was a strong interaction between hatch day and sediment treatment (Fig. 8 GLM ANCOVA $F(10,144) = 11.104$ $p < 0.001$ $r^2 = 0.767$). On day -1 the number of larvae that hatched from both sediment treated cultures was greater than that from the control cultures (t-test $p < 0.001$, $\alpha = 0.01$). On day 1, when control larvae began to hatch, and on day 2 there were no differences in number hatched between sediment treatments and controls ($p > 0.01$). On day 3 both sediment treatments produced fewer larvae than did no-sediment controls and on day 4 only hatching from the chronically treated culture was below that of controls ($p < 0.001$). Although sediment treated larvae began hatching earlier, total hatch in these experiments did not differ between treatments. Total mean hatch was 118.11 larvae $\pm$ 13.06 in 0-2 HPF sediment treatment cultures and 119.89 $\pm$ 16.03 in chronic sediment cultures compared to 129.11 larvae $\pm$ 15.22 ($p = 0.119$ and $p = 0.229$). In both sediment treatments there was a longer hatch period, five days as opposed to four days in controls.

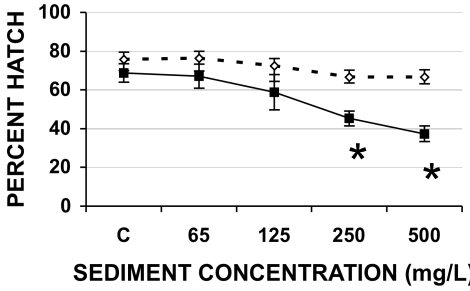

Figure 7. Suspended sediment impacts on total (◇) larval hatch and normal (■) larval hatch. Herring eggs were treated with suspended SF Bay sediments at 0-2 HPF and larvae were counted at hatching and expressed as a percent of fertilization. Total larval hatch differed from normal larval hatch (GLM ANCOVA $F(1,66) = 23.198$ $p < 0.001$ $r^2 = 0.778$). Percent normal larvae were decreased over both no-sediment controls and total hatched larvae in both 250 and 500 mg/L treatments (t-test, $p < 0.0125$ *). Graph = Mean $\pm$ std dev. n = 8.

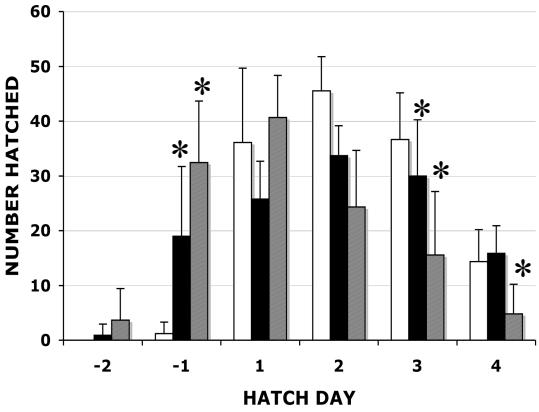

Figure 8. Daily larval hatch. Embryos were incubated in 500 mg/L suspended SF Bay sediment at 0-2 HPF (■), treated chronically with sediments throughout embryonic development (⊠), or were no-sediment control cultures (□) . Hatch Day 1 was designated as the first day for which the hatch per day of no-sediment control larvae was greater than 10% (~ 20 larvae) of embryos in the culture. There was an interaction between hatch day and sediment treatment (GLM ANCOVA $F(10,144) = 11.104$ $p < 0.001$ $r^2 = 0.767$). * Significantly different from no-sediment controls on same day (t-test $p < 0.001$ *). Graph data = mean $\pm$ std dev, n = 9.

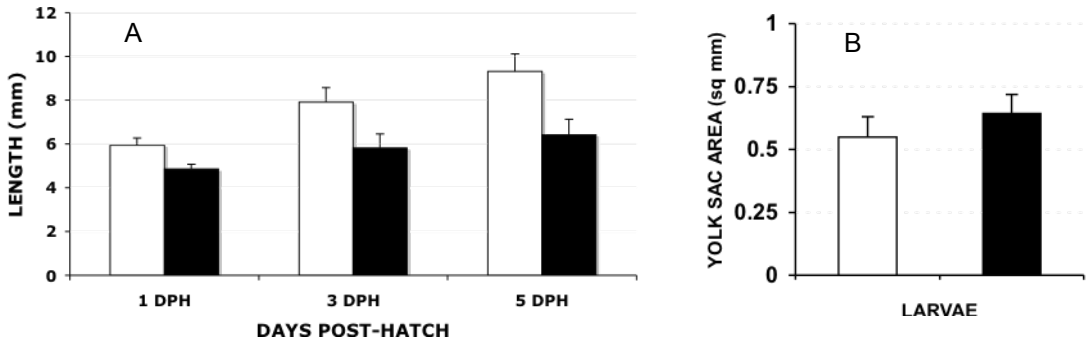

Figure 9 Larval size measurements. A. Hatched larvae from embryos that had been incubated in 500 mg/L suspended SF Bay sediment (■) and no-sediment controls (□). Sediment treated embryos produced shorter larvae (A) with larger yolk-sacs (B). Sediment treated larvae remained shorter through 5 days post-hatch (A). ($p < 0.003$, t-test for all sediment/no-sediment comparisons. Sediment treated larvae did not increase length by day 3 (t-test $p > 0.05$), but had done so by day 5 (t-test $p = 0{,}009$). n = 5 with 10 measurements per n.

At hatch, normal appearing larvae from 500 mg/L sediment treatments were shorter (total length) with larger yolk-sacs and grew at a slower rate over the first 5 days post-hatch (Fig. 9 and see APPENDIX III). No-sediment control larvae averaged 5.74 mm $\pm$ 0.33 total length at hatch and grew to 9.32 mm $\pm$ 0.80 by day 5 PH. Larvae from embryos that were treated with 500 mg/L suspended sediment at 0-2 HPF averaged 4.85 mm $\pm$ 0.23 at hatch and grew to 6.42 mm $\pm$ 0.72 total length by day 5 PH. Total lengths of control and sediment treated larvae were significantly different for all three post-hatch time points (Fig. 9A, $p < 0.001$). Sediment treated larvae also possessed larger yolk sacs at hatch (Fig 9B). Yolk sac area of no-sediment treated larvae averaged 0.55 mm^2 $\pm$ 0.08 while those of sediment treated larvae averaged 0.64 mm^2 $\pm$ 0.08 at hatch, again a significant difference ($p < 0.001$). Early hatch, shorter total length and larger yolk-sac indicate precocious hatch in sediment treated larvae (Williams et al, 2003).

Larval survival was reduced when eggs and embryos were treated with 500 mg/L suspended SF Bay sediment at 0-2 HPF and this decrease was due to early post-hatch mortality (Fig 10). Survival analysis (SYSTAT) revealed a mean larval survival time for no-sediment controls of 14.64 days PH and 11.86 days PH for sediment treated cultures (Fig. 10A). The Kaplan-Meier probability of survival to day 5 PH was 0.955 in no-sediment controls and 0.819 in sediment treated cultures, indicating that survival difference began during the early post-hatch period. The overall Kaplan-Meier probability of survival to 14 days was 0.744 for no-sediment controls and 0.496 for sediment treated cultures. GLM ANCOVA analysis of cumulative mortality after separating the data into three discrete blocks of time (2-5 days PH, 6-10 days PH, and 11-14 days PH) supported survival analysis (Fig. 10B). Cumulative mortality in sediment treated cultures for days 2-5 PH was greater than that in no-sediment controls (GLM ANCOVA $F(1,66) = 21.79$ $p < 0.001$ $r^2 = 0.812$). After day 5 post-hatch, difference in cumulative mortality between controls and sediment treatments was not linked to sediment treatment (GLM ANCOVA days 6-10: $F(1,104) = 0.016$ $p = 0.899$; GLM ANCOVA days 11-15: $F(1,86) = 0.319$ $p = 0.574$). Cumulative mortality in cultures where larvae were treated post-hatch with suspended SF Bay sediment for 2 hrs (at day 1 and at day 10 post-hatch) tracked that of no-sediment controls throughout the experiment (Fig. 10B). Mean cumulative mortality in the day 1 PH sediment treatment was 55.1

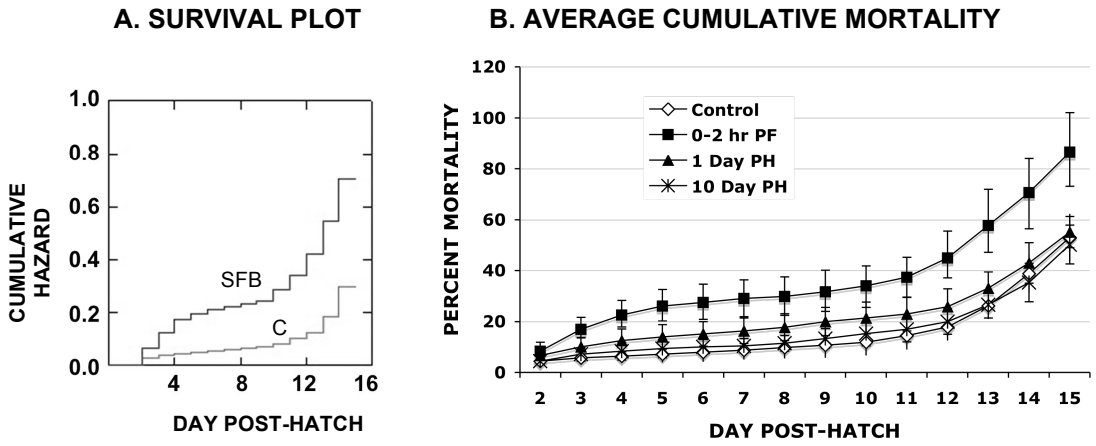

Figure 10. . Larval mortality as a function of sediment treatment of eggs at 0-2 HPF. A. Survival plot of cumulative hazard comparison for larvae from eggs treated with 500 mg/L of suspended SF Bay sediment at 0-2 HPF (SFB) and larvae from no-sediment control eggs (C). B. Average cumulative numbers of post-hatch larval mortality in no-sediment control cultures (◇) and after treatment with 500 mg/L of suspended SF Bay sediment at 0-2 HPF (■). Larval mortality in 0-2 HPF sediment treated embryo cultures was higher than in no-sediment controls on days 2-5 post-hatch (GLM ANCOVA $F(1,66) = 21.79$ $p < 0.001$ $r^2 = 0.812$). Between days 6 -10 and days 11-15 there was no difference in new mortalities between 0-2 HPF sediment treated and no-sediment cultures (GLM ANCOVA, Days 6-10: $F(1,86) = 0.016$, $p = 0.899$. Days 11-15: $F(1,86) = 0.319$, $p = 0.574$). Mean $\pm$ std dev. n = 9.

larvae $\pm$ 6.9 and was 50.2 larvae $\pm$ 7.6 for the day 10 PH treatments, while mortality averaged 53.0 larvae $\pm$ 9.2 in the no-sediment controls. This compared to a mean cumulative mortality in 0-2 HPF treated cultures of 86.5 larvae $\pm$ 6.5.

Embryo and Larval Respiratory Rates

Oxygen consumption rates for embryos and larvae obtained from eggs that had been treated with SF Bay suspended sediments at 0-2 HPF were recorded. Absolute respiration rates for individual no-sediment control embryos measured at 24 HPF ranged from 0.08 µg/h to 0.13 µg O_2/h while normalized rates varied between 0.018 µg O_2/h/mg and 0.044 µg O_2/h/mg. As the embryos developed (day 6 PF) the absolute respiration rate increased to a range of 0.38 µg O_2/h to 0.56 µg O_2/h while normalized consumption rates varied from 0.028 µg O_2/h/mg to 0.12 µg/h/mg over the 6-day period. Embryos treated at 0-2 HPF with 500 mg/L of SF Bay suspended sediment were statistically compared to no-sediment control embryos at the three stages of embryonic development. At 24 HPF and at 2 days PF there was no statistically significant difference between controls and sediment treated embryos ($p > 0.05$). However, at 6 days PF O_2 consumption was significantly reduced from that of no-sediment control embryos ($p < 0.001$).

Larval respiration values ranged from 0.35 µg/h/mg to 3.7 µg/h/mg. There were no statistically significant differences between treatments (normalized and absolute) at the 250 mg sediment exposure level ($p = 0.286$). At the 500 mg exposure level there was a statistically significant difference between controls and larvae produced by embryos that had been treated with SF Bay sediment at 0-2 HPF ($p < 0.001$). However, the analysis was complicated because there were statistically significant differences between some controls.

Discussion

The most relevant findings in the current study are that sublethal developmental effects result when Pacific herring eggs and embryos are exposed to what were previously reported no-effect concentrations of suspended sediments during the first two hours of egg/embryo contact with water and that these effects can have lethal outcomes. If suspended sediments were present at concentrations of 250 or 500 mg/L during this period of time it was found that: a) particles attached permanently to eggs; b) eggs could aggregate into clusters; and c) even in the absence of egg aggregation, significant developmental effects occurred that included precocious hatching of larvae, increases in larval abnormalities, and a decrease in survival of yolk-sac larvae after hatching. The literature concerning suspended sediments and herring reproductive biology appears to contradict these findings, collectively suggesting that embryos and larvae tolerate higher concentrations of suspended sediments up to 1000 mg/L (Boerhlert et al. 1983; Morgan and Levings 1989; Kiorboe et al.1981). Previous studies, however, did not test the effects of suspended sediments during these initial two hours and either did not examine sublethal effects or, if they did so, did not follow the outcome. In prior studies eggs were fertilized in sediment-free water, on glass slides or Nitex mesh, and then transferred to sediment containing water after periods of time ranging from three to several hours (Boerhlert et al. 1983; Morgan and Levings 1989; Kiorboe et al.1981). Results from our experiments in which embryos were treated with suspended SF Bay sediment after the initial two hour time period generally agree with these previous studies; when embryos or larvae were incubated in suspended sediments at time points after 0-2 HPF, regardless of exposure time, sediment particles did not permanently bind and at concentrations of up to 500 mg/L there was no significant effect on embryonic development, larval hatching, or larval survival. Conversely, if sediments were present during the first 2 hrs following egg contact with water (0-2 HPF), suspended sediment concentrations had to be below 250 mg/L for there to be no effect.

Suspended sediments increased the aggregation of eggs into clusters or multiple layers. The Pacific herring egg, approximately 1 mm in diameter, is negatively buoyant and is contained within a thick (~50 μm) extracellular envelope, the chorion (Hay and Miller 1982; Grosse and Hay, 1988). Most of the surface of the chorion is adhesive (Gillis et al. 1990); the only portion that is not is the micropyle region at the animal pole of the egg. Gillis et al (1990) described a two-step hydration that took place after eggs contacted seawater, during which eggs "became strongly adhesive" and debris and sperm attached to the outer surfaces of chorions. The full development of this adhesive property of the egg chorion requires approximately two hours, the time period during which sediment particles attached to eggs and embryos.

Mechanisms by which suspended sediment particles adhere to herring eggs and embryos were not investigated, but our observations indicate two, not mutually exclusive, possibilities. First, there could be a charged based attraction. Sediment particles appeared to be negatively charged and did attract to one another when positively charged solutes were increased in water. This behavior has been previously reported (Hunter and Liss, 1979; 1982). If egg chorions possess a positive charge, particles could attach to their surface by the same charge-based mechanism. In support of this charged based attraction mechanism, we have observed that eggs will aggregate when adhesive development is prevented (in ½ CaMgF PVA) if sediments are present (data not shown). The second mechanism would

involve particles becoming cemented to the adhesive layer as it develops and hardens. Particles would then become permanently attached to the chorion. This would be the mechanism that we observed during 0-2 HPF.

The adhesive layer and its ability to bind herring eggs to solid biotic or abiotic substrates throughout the embryonic period is essential to successful reproduction because eggs that do not attach to substrates or embryos that are dislodged can be transported out of shallow, protected nursery areas by currents and tides, thus loosing the developmental benefits of those nursery areas. The spawning behavior of female herring facilitates attachment of eggs. Spawning begins with some males releasing milt into the water at a spawn site, providing a pheromone that triggers both females and other male fish to spawn (Hay, 1985; Carolsfeld et al. 1997). Females and males exhibit a characteristic behavior involving substrate selection and substrate spawning which for females culminates in the release of eggs in ribbon like fashion onto or in close proximity to selected substrata (Stacey and Hourston 1982; Haegle and Schweigert 1985; Carolsfeld et al. 1997). Females, however, may swim away from substrata during spawning and release eggs into the water column (Stacey and Hourston 1982; Aneer et al. 1983; Hay 1985). When this occurs the negatively buoyant eggs then settle and attach to substrata or onto other eggs leading to aggregations in multiple layers or clusters. Layers greater than ten eggs thick are rare, but as layers increase in thickness so does anoxia, microbial growth and retardation of embryonic development (Stacey and Hourston 1982; Hay 1985). According to Stacey and Hourston (1982) water column spawning and egg aggregation can be exacerbated by crowded conditions in large spawning schools. In the current study we showed that suspended sediments, if present in the water column as eggs descend, enhances egg aggregation.

Whether or not sediment particles that attach to herring egg chorions alter the strength of adhesion of eggs and embryos to adhere to substrata is not known. In San Francisco Bay, embryonic development requires 8-10 days, a time frame during which embryos must be resistant to being dislodged from substrata to which they are attached (Eldridge and Kaill 1973; Hay et al. 1993; Griffin et al. 1998). Surprisingly little information is available regarding sediment effects on adhesion strength of aquatic eggs or embryos to substrata, regardless of the species. Auld and Schubel (1978) examined the effects of suspended sediments on eggs of several species, including the American Shad and Alewife. These authors, as in studies with herring, reported that sediment concentrations as high as 1000 mg/L had no effect on embryo development, however, the eggs of these species are not adhesive to the extent of Pacific herring eggs and may not bind sediment particles as do herring eggs. Wilbur and Clarke (2001) have reviewed the literature, but describe no studies on suspended sediment effects on adhesion. Chapman and Fletcher (2002) reported that the development of embryos of the marine algae, *Fucus*, can be impacted by sediments. Although not directly addressing adhesion, these authors suggest that sediments can result in embryo removal through burial and scouring processes. Similar to Pacific herring, the sturgeon (*Acipenser spp*.) egg chorion possesses an outer layer that facilitates egg attachment to the substratum at spawning (Cherr and Clark 1985). Although no studies have been published regarding suspended sediment effects on egg adhesion, techniques using suspended sediments to prevent sturgeon egg adhesion in aquaculture settings have been published (Kowtal et al.1986). Sediment presence either in suspension or settled onto substrata could be hypothesized to negatively effect egg-substratum adhesion strength. This deserves quantitative study because a reduction in adhesive strength could make eggs more

susceptible to dislodging from tidal or wave action. Thus two sediment effects on egg adhesion, induced egg aggregation (current study) and changes in egg-substrata adhesion strength (hypothesized), could predispose fertilized eggs to reduced developmental success.

Fertilization as a function of suspended sediment concentration has not been examined in previous studies. There was an interaction between sediment concentration and fertilization, however, this interaction explained only a small portion of the variability (low r^2) and we could find no sediment level where fertilization was statistically lower than no-sediment values. The functional morphology of eggs and sperm/egg interaction would argue against a suspended sediment effect on fertilization. The region of the chorion through which sperm gain access to the egg is not adhesive. The micropyle region, a 150 μm diameter region of the chorion containing a narrow channel or canal (termed the micropylar channel) that is about the same diameter as a sperm, is the only access to the egg plasma membrane and thus fertilization (Gillis et al. 1990; Yanagimachi et al. 1992; Griffin et al. 1996). Although the micropyle is not adhesive it does contain sperm activating molecules, herring sperm activating peptides (HSAPs) and sperm motility initiating factor (SMIF), that induce sperm motility and function to keep sperm located in the micropyle region thus enhancing chances for fertilization (Yanagimachi et al. 1992; Pillai et al. 1993; Griffin et al. 1996; Vines et al. 2002; Cherr et al. 2008).

Interpretation of SF Bay sediment effect on total larval hatch was problematical because of high variability; regression analysis of total hatch did suggest there was a decline in total hatch with sediment treatment, however, low predictability of the regressions suggests other factors were involved in total larval hatch declines (Table 1). Inherent variability has been reported previously in herring embryo studies, including sediment studies. Suggested causes include quality differences in eggs from different females reproductive condition of females, fungal infections in cultures, and sediment related anoxia (Kiorboe et al. 1981; Alderdice and Hourston 1985; Hay 1985; Laine and Rajsilta 1999). Another possible source of variability could be contaminants associated with the SF Bay sediment. SF Bay sediments used in our experiments did contain contaminants. However, concentrations of all but one measured contaminant (chromium) were low and amphipod bioassays for biological toxicity showed no effect (USACE 2005). Furthermore, our statistical analyses suggested no difference in total hatch due to sediment type (control Fuller's earth and SF Bay sediment). There was a clear increase in the number of abnormal larvae that hatched from suspended SF Bay sediment treated eggs compared to no-sediment treated controls. Abnormal larvae and sublethal effects of sediment treatment on normal appearing larvae point to perturbations in development. Effects including precocious hatch, smaller larval size at hatch, and larger yolk sac size at hatch indicate stress during embryonic development and would support the possibility of sediment effects on total larval hatch.

Precocious hatch, smaller larval size at hatch, and larger yolk sac size at hatch may also have been causative factors in reduced larval survival. Stress from a variety of sources, including sediments, has been reported to initiate all of the sublethal effects that we observed, Temperature stress can lead to precocious hatch of reduced size larvae in herring (Ojaveer, 2006) and exposure to crude oil hydrocarbons (Carls et al.1999), Orimulsion 400® (Williams et al. 2003) and creosote (Vines et al. 2000) all produce precocious hatching. In the Orimulsion 400® study Williams et al (2003) reported that high concentrations produced larvae that hatched prematurely, were smaller and had larger yolk sacs; several deformities, including coiled larvae, and several varieties of spine flexion were also described as a result

of hydrocarbon induced stress (Vines et al. 2000; Williams et al. 2000; Incardona et al. 2004). Morgan and Levings (1989) reported an increase in time to hatch as a result of sediment exposure, most likely due to low oxygen, but most studies on stress including low dissolved oxygen effect investigations have reported precocious hatches (Morrison et al. 1991; Rankine et al. 1990). Geffen (2002) has shown that in the absence of stressors larval size at hatch and timing of hatch are linked. Within a cohort of embryos, those that hatch first are smaller with larger yolk sacs than the larvae that hatch several days later. Geffen (2002) observed increased growth rates after hatch, such that those that hatched early had caught up in length with those that hatched late. In our experiments not only did embryos treated at 0-2 HPF produce larvae that were smaller at hatch, they yielded larvae that grew at slower rate over the first five days post-hatch. Some authors have suggested that early hatch alone can have negative implications; these include reduced swimming ability which could lead to loss of position in nursery areas or to increased predation (Rosenthal and Alderdice 1976; Blaxter and Fuiman 1990; Batty and Blaxter 1992; Williams et al. 2003).

In fish, respiratory rates are low during early development, but increase between the time of larval formation and hatching, the time period when larvae increase in size and competency to survive outside of the chorion (Holliday et al. 1964; Collins and Nelson 1993; Finn et al. 1995). We were able to measure significant differences in oxygen consumption at 6 days PF, during larval formation and prior to hatching, between no-sediment controls and sediment treated embryos. This reduction in oxygen consumption could arise from one or both of two sediment linked sources: 1) Physical binding of sediment particles to an egg could hinder diffusion across the chorion during development; and 2) Sediment particles could deliver contaminants to embryos, and it could be the actions of the contaminants that leads to decline in oxygen consumption. Previous studies have equated herring egg aggregation with anoxia and low embryonic respiratory rate (due to anoxia) with precocious larval hatch. Mortalities of naturally spawned Atlantic herring embryos have been reported from hypoxia due to diatom capsule sedimentation (Morrison et al. 1991). Again with Atlantic herring, studies have shown that embryo aggregation into multiple layers produces larvae of variable length, indicating stress (Rankine et al. 1990). And, increased mortality and precocious hatching have been reported for herring and whitefish larvae exposed to hypoxia as embryos (Rankine et al. 1990; Czerkies et al, 2001).

The ranges of suspended sediments used in the current study were ecologically relevant to San Francisco Bay Pacific herring. Although suspended sediment concentration estimates are not available prior to anthropogenic disturbance of San Francisco Bay (which began circa 1850s), sedimentation rates due to input of suspended sediments were thought to vary spatially and temporally as they do presently. Sedimentation rates ranging from 0.7-2.6 mm per year have been hypothesized with delta flow rates that varied from 500-3000 m^3 /sec; the highest flows and sediment input were during the Winter/Spring, during the herring reproductive season (Cloern et al. 1983; Nichols and Thompson 1985; Ingram and DePaolo 1993; Conomos et al. 2004). Beginning in the 1850s placer mining and then subsequent additional anthropogenic disturbances to the Sacramento/San Joaquin River and local San Francisco Bay watersheds significantly altered the quantity and composition of sediment input into San Francisco Bay; input into the Bay increased from a pre-gold rush estimate of circa 1 x 10^6 metric tons/year, consisting primarily of sand and fine gravel to over 5 x 10^6 metric tons per year with a large concentration of fine silt & clays. We know from more

recent estimates that ambient suspended sediment levels routinely fluctuate from less than 50 mg/L up to 500-600 mg/L (Ruhl and Shoelhamer 1998; McKee et al. 2006).

The information garnered from the present laboratory study has elucidated a previously unreported interaction between particulates and Pacific herring eggs that can only occur during the first two hours of egg contact with water. Extrapolation of laboratory experimental outcomes to natural spawns should be made with caution. Significant sublethal and lethal impacts on embryonic development and larval survival were obtained, but taken individually, the percentage of embryos/larvae impacted were relatively small. Alternatively, taken together, these impacts across developmental stages (embryo-larval stages) could be additive and given the reported variability of embryonic success, may be important. Mortality during embryonic life stages is thought to be highly variable, ranging from 56-99% in British Columbia (Schweigert 1993). Such factors as multiple layered spawns, predation, wave action, and exposure to air during low tides can contribute to daily losses of 8% or more of spawn biomass during embryonic development. (Hay and Miller,1982; Hay 1985; Schweigert 1993). To elucidate the ecological impact of suspended sediments on Pacific herring embryo and larval success in the environment several questions should be addressed including the following: 1) Do suspended sediments produce egg aggregation during natural spawns? 2) Do coatings of sediments on embryos reduce adhesion between embryos and substrates to which they are attached? and 3) What are the respective contributions of particles and contaminants contained on particles to observed developmental effects? Lastly, there is a need to understand how anthropogenic input of suspended sediments (through dredging or shoreline development) adds to not only total sediment loads, but also to contaminant loads.

Acknowledgements

The authors thank the San Francisco Bay LTMS Environmental Windows Science Work Group and U.S. Army Corps of Engineers for commissioning the study. The project was funded through a contract (# W912P7-05-P-0020) from the U.S. Army Corps of Engineers, San Francisco. We thank Peter LaCivita, U.S. Army Corps of Engineers, for his consultation and supervision during the project. The Port of Redwood City dredged sediment was supplied by Charles Anderson, U.S. Army Corps of Engineers. We thank Michael Donnely and Neil Hedgecock, U.S. Army Corps of Engineers, for providing Port of Redwood City sediment toxicity test information. Ryan Watanabe, Ken Oda, and Tom Moore of the California Department of Fish and Game provided ripe herring from their adult surveys. Karl Menard, Bodega Marine Laboratory, provided gonads from fish collections made by the Bodega Marine Lab oratory Aquatic Resources Group. Herring gonads also were generously supplied by Dr. Paul Dinnel, Shannon Point Marine Center, Western Washington University. We thank Dr. William N. Brostoff (USACE) for his critical reading of and suggestions on a draft manuscript. Lastly we thank Dr. Steven Morgan, Dr. Vic Chow, Dr. Will White, and Suzy Jackson for their assistance with statistical analyses, as well as editorial input.

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APPENDIX I.

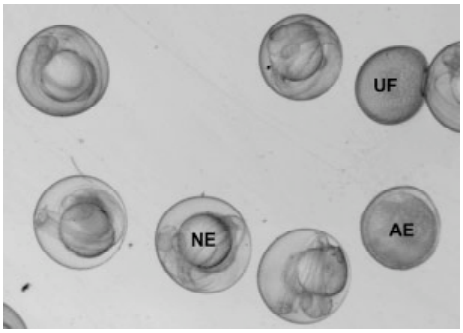


Figure 1. Pacific herring eggs and embryos at 4 days post-fertilization. Unfertilized eggs (UF) fail to round-up and remain opaque. A fertilized egg ceased development prior to epiboly and is dead (AE) and embryos that were normal embryos (NE). Fertilized eggs and viable embryos are more translucent than unfertilized eggs and dead embryos.

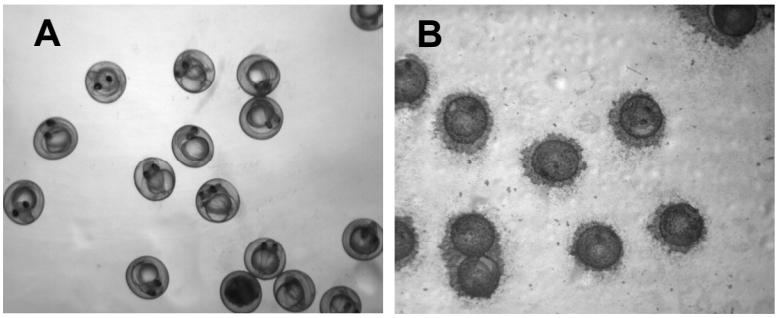
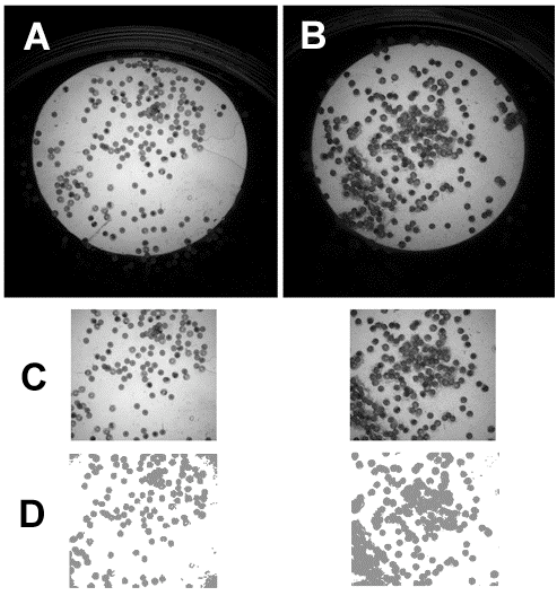
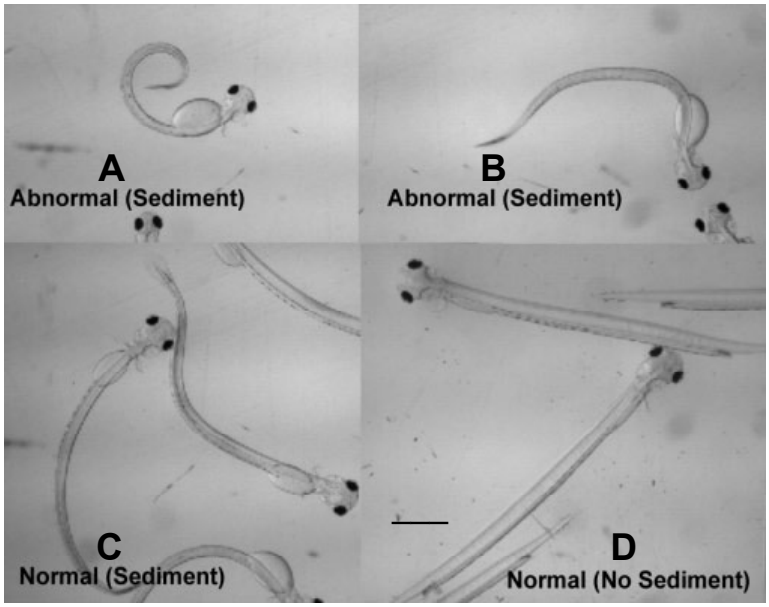


Figure 2. Suspended SF Bay sediment particles attach to egg/embryo chorions when present at 0-2 HPF and preclude accurate determination of developmental progress. A. No-sediment controls embryos imaged at 6 days PF. B. Embryos imaged at 6 days PF that were treated with 500 mg/L of suspended SF Bay sediment at 0-2 HPF.

APPENDIX II. Aggregation of eggs in the presence of suspended sediments was digitally recorded and enhanced for quantitative analysis. Eggs were added to water with or without suspended sediments, swirled for 5 secs and allowed to settle. A. Sediment-free control embryos. B. Embryos incubated in 250 mg/L suspended sediment for two hours beginning at first egg exposure to water. After cropping (C) and thresholding (D) images, average area of embryo footprints was determined (see Fig. 4).



APPENDIX III. Abnormal and normal larvae. Abnormal larvae from eggs/embryos that were treated with 500 mg/L suspended sediment at 0-2 HPF included: (A) Larvae whose tails did not completely uncoil from around the head and yolk-sac; and (B) Scoliotic larvae with one or more kinks in the spinal column. Normal appearing larvae from the same sediment treatments (C) were shorter and possessed larger yolk-sacs than did larvae from no-sediment controls. Scale =1 mm.



APPENDIX IV Cumulative hatch of larvae from embryos incubated in 500 mg/L PRC suspended sediment was delayed in both 0-2 hr PF (❁) and chronic throughout embryonic development (▲) sediment treatment compared to non-sediment controls (◊). * Hatch in chronic and 0-2 hr PF sediment treatments was significantly greater than non-sediment controls on these days ($p > 0.05$, t-test). By day 10 PF total hatch had equalized in all treatments. Mean ± std dev (n = 3).