

***Mytilus galloprovincialis* Development and Copper Toxicity Test**

Adapted from Pacific EcoRisk S.O.Ps

Introduction

This method was obtained from the standard operating procedures of Pacific EcoRisk, Fairfield, California in December 2007. These procedures follow the U.S. EPA Guidelines described in Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms (EPA/600/R-95-136) and meet the requirements of ASTM Method E 724-89.

Methods

Ordering & Holding of Test Organisms

1. *Mytilus galloprovincialis* is one of the most sensitive estuarine species to copper bioavailability and so should be used for these toxicity tests
2. Approximately 30 - 50 animals should be ordered in anticipation of damage from shipping and to increase chance of good quality spawn. The following is a list of companies Pacific EcoRisk ordered from:
 - a. Tomales Bay Oyster Co. 415.663.1242
 - b. Carlsbad Aquafarms 619.438.2444
 - c. Proteus Sea Farms 805.798.2505
3. Until use, store mussels in aerated filtered saltwater at 12°C. Replace water everyday. Mussels may need to be stored like this for 1 – 2 days before use in test. See Figure 1 for photos of mussel-storing.

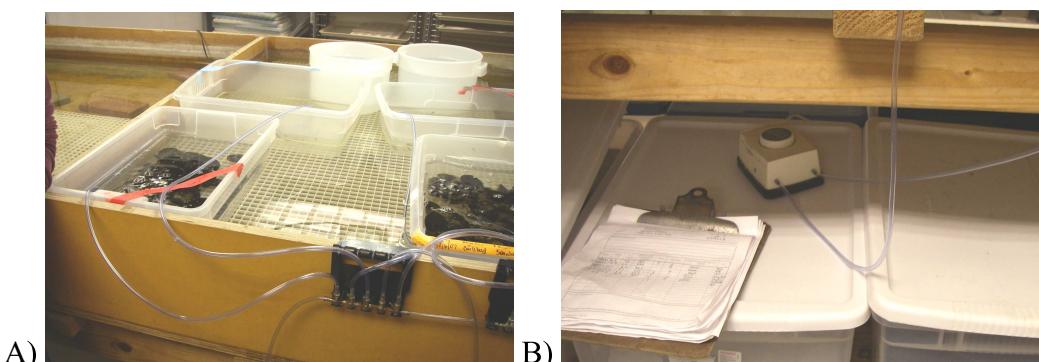


Figure 1. A) Buckets of filtered seawater for mussel storage before use in toxicity tests. B) Pump for aeration system.

Collection and Holding of Water Samples

1. Upon arrival, water should be stored at 4°C until used. This water comes sand-filtered and so further filtration before use may be necessary. Filtration can be done using 0.2 µm and 0.45 µm porous filters in a Buchner funnel or a peristaltic pump, tygon tubing, and a medium-size dispose-a-filter (Figure 2).

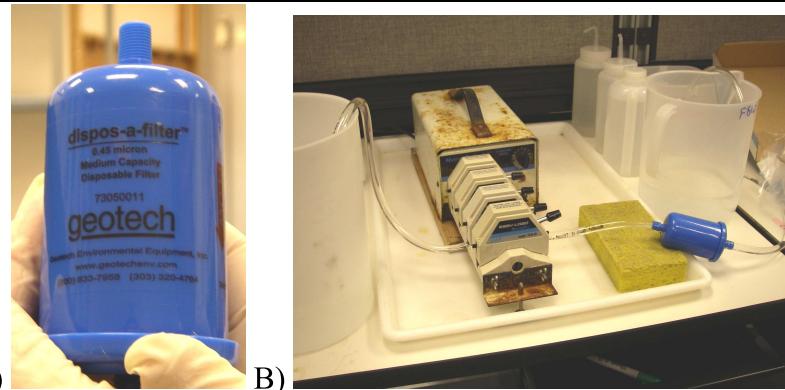


Figure 2. A) Medium-size dispose-a-filter. B) Saltwater pumping through filter into a clean beaker.

2. The water samples should be used within 72 hours after collection.

Test Solution Preparation

1. Prepare test solutions in 200 mL beakers and measure initial water chemistries (pH, salinity, CRS, DOC, FEEM) for each test concentration and the control. Additional replicates should be prepared for each so there is enough to measure final water chemistries.
2. Glass scintillation vials and lids must be cleaned in 10% nitric acid and rinsed thoroughly. Then soak the vials overnight in DI water, allow them to dry completely, and then rinse them several times with filtered saltwater before use.
3. Place 10 mL of appropriate sample into a 20 mL glass scintillation vial. Three replicates should be done for each test and two additional tests for final water chemistry measurements. Additional control vials should be done to monitor the progress of development during the test. This can be seen in Figure 3.

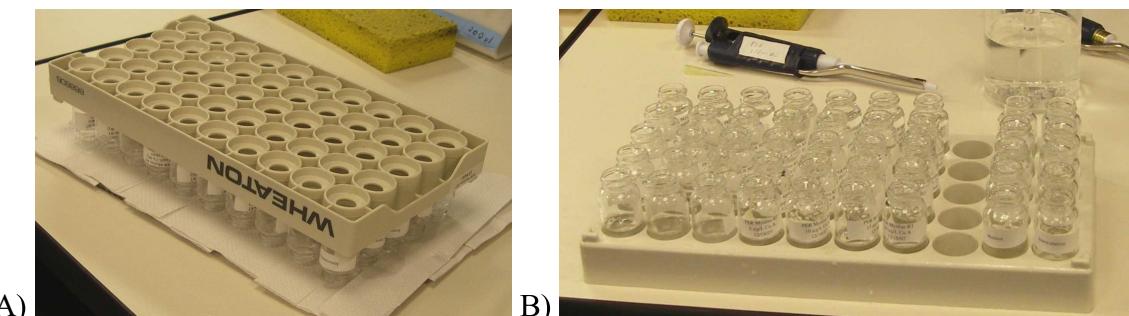


Figure 3. A) Clean, dry glass scintillation vials. B) Five runs of each sample are performed. The first three are to test the toxic effects and the last two are for final water chemistry measurements, assuming 20 mL is enough to measure everything again afterwards. The last two columns on the far right are extra controls to monitor larval development.

4. Place solutions in a water bath at 15°C or 18°C ± 1°C to adjust to test temperature.

Spawning and Fertilization

1. Clean detritus off 8 – 12 animals. Use the dull edge of scissors to scrape off detritus and cut off all byssal threads. Larger mussels are more likely to spawn. Discard of any mussels with cracked shells. Once cleaned, put in filtered saltwater at 4°C until spawning bath is ready. Images of this are in Figure 4.



Figure 4. The mussels were thoroughly cleaned of any debris and all the byssal threads were cut off. This was done to ensure no contamination from unwanted microscopic organisms in the spawning trays.

2. Fill the pyrex spawning tray and several 250 mL beakers with filtered saltwater (Figure 5) and warm to room temperature (~20°C). Trays do not need to have filtered water.



Figure 5. Spawning trays and individual beakers containing filtered saltwater at 20°C.

3. Once warm, roughly place (drop from inches above water) mussels into the spawning dish. Ensure mussels are completely submerged (Figure 6).



Figure 6. Clean mussels in spawning trays. These ones were separated because the test being conducted was on mussels that were shipped on different days.

4. Monitor animals for spawning activity. It usually takes 40 min – 2 hours for spawning to commence. It is typically characterized by an active pumping of water and finally the appearance of whitish stream of gametes, seen in Figure 7.

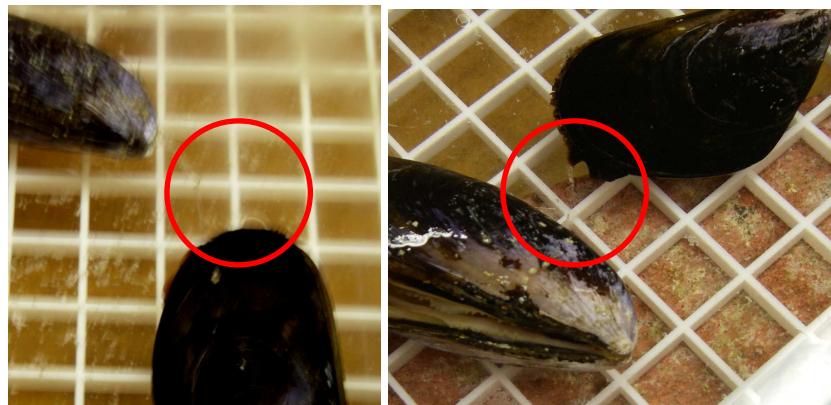


Figure 7. Gametes pumping out from mussels. A) Stream of sperm. B) Stream of eggs. Sperm is frequently a whitish colour while the eggs may appear blue, orange, or yellow.

5. As the mussels begin to spawn, quickly but gently stick a disposable plastic pipette into the bivalve opening to prevent it from closing, rinse it off thoroughly (be more thorough with females), and transfer mussel into one of the beakers.
6. The sperm from an early-spawning male can be pipetted into the incurrent flow of non-spawning mussels to stimulate spawning. Picking them up and dropping them back into the tray may stimulate spawning as well (causes physical distress). If it doesn't work, inject 0.5 mL of 0.5 M KCl into the posterior adductor muscle and use a pipette tip to keep the shell open while injecting. Wait 20 minutes before trying each spawning technique.
7. Repeat spawning-mussel collection (step 5) until an adequate quantity of both sperm and eggs are obtained. About 2 – 3 good spawning males and females should be obtained.
8. Gametes should be inspected under the microscope for sperm motility and egg quality. Eggs should be round and uniform in size; sperm should be active. Reject females with small, vacuolated, or abnormally-shaped eggs and males with

poorly motile sperm. Reject hermaphrodites as well. Over concentrated sperm samples may be mistaken for immobile sperm – add a drop of water to the slide and swirl around to dilute it and check again. Figure 8 displays the method. Figure 9 – 13 are examples of eggs and sperm viewed at 100x magnification.

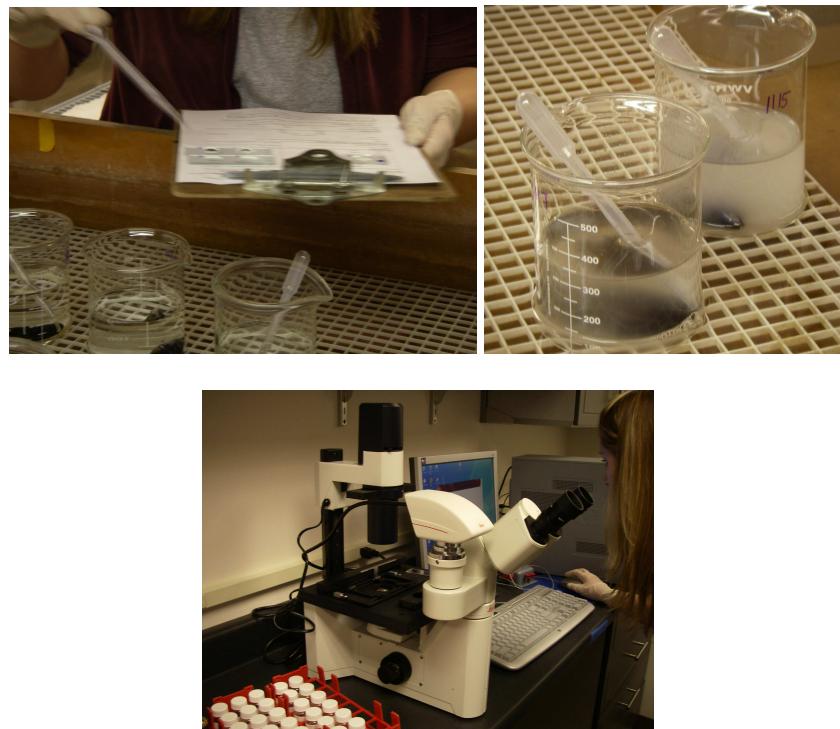


Figure 8. A small droplet of the eggs and sperm are placed on a concave slide to look for abnormalities.

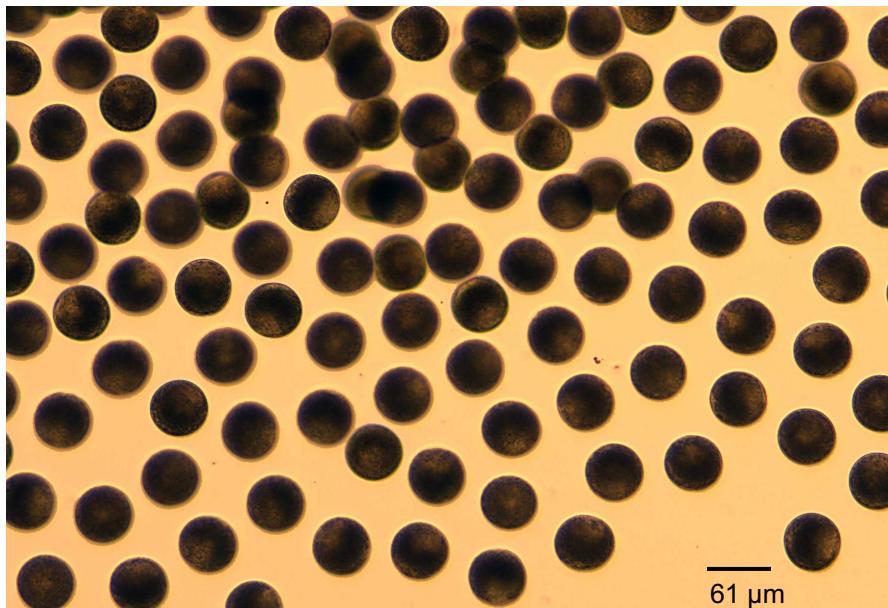


Figure 9. Good *Mytilus* eggs. They can be either round or egg-shaped with small to no vacuoles present (lighter shade within egg). If there are a small number of abnormal eggs, it may still be ok to use – abnormalities may produce good embryos.



Figure 10. If there are too many abnormal eggs, or unfertilizable eggs (<90% good eggs), then they cannot be used. Oblong (circled) or completely deformed (within square) are abnormal.

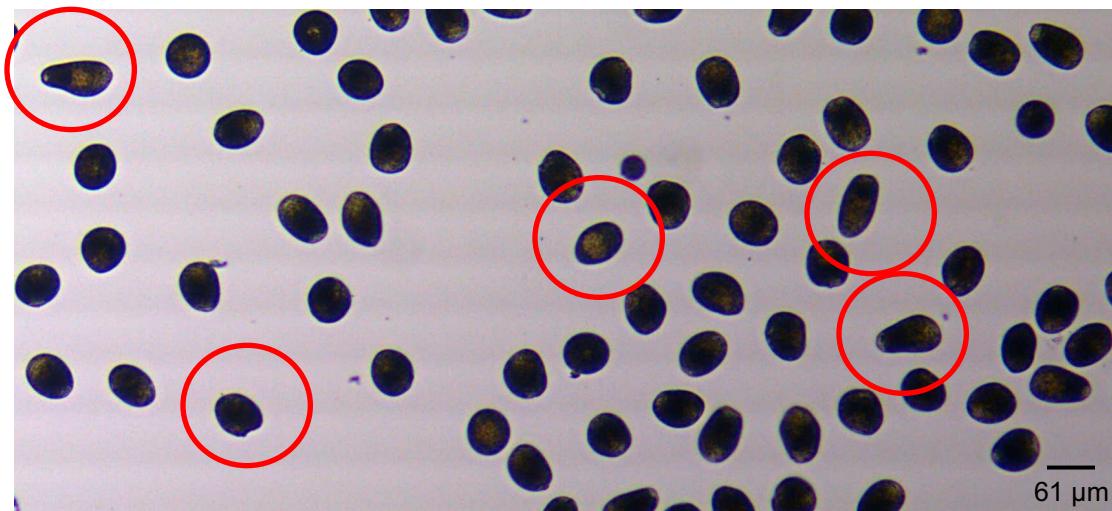


Figure 11. More examples of deformed or highly vacuolated eggs.

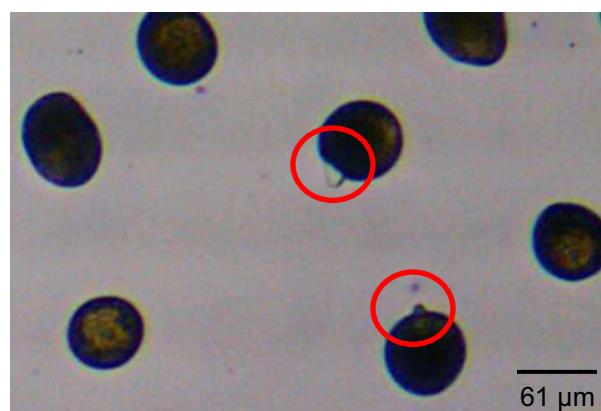


Figure 12. Eggs that have already been fertilized cannot be used. This can be seen by a small clear circle attached to the egg (circled). This would have occurred if the female mussel wasn't cleaned thoroughly enough before being transferred from the spawning tray into a beaker.

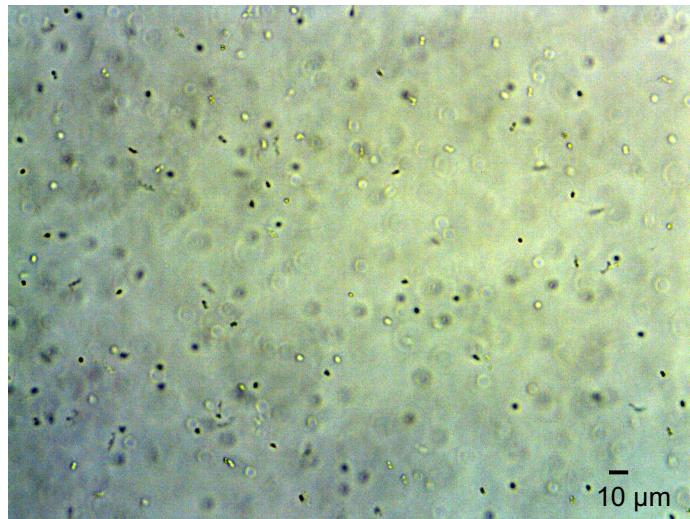


Figure 13. Good, mobile sperm. They are too small to notice any deformities, so only mobility is observed to characterize "good" sperm.

Gamete Fertilization and Embryo Production

1. Within 1 hour of spawning, prepare an egg stock suspension. This is done by pipetting concentrated eggs from the beaker(s) with the good quality eggs (settled on the bottom of the beaker) into ~250 mL filtered saltwater. When stirred thoroughly (gently), the water should look almost opaque, seen in Figure 14.



Figure 14. Egg stock solution. Left: eggs that have settled on the bottom. Right: eggs in suspension. The solution must have that appearance when diluting.

2. While the stock is thoroughly mixed, pour a small amount into 1 L of filtered saltwater. This will be used to prepare an egg suspension of ~1000 eggs/mL and can be seen in Figure 15. Store the stock solution at 18°C until the test is completed.

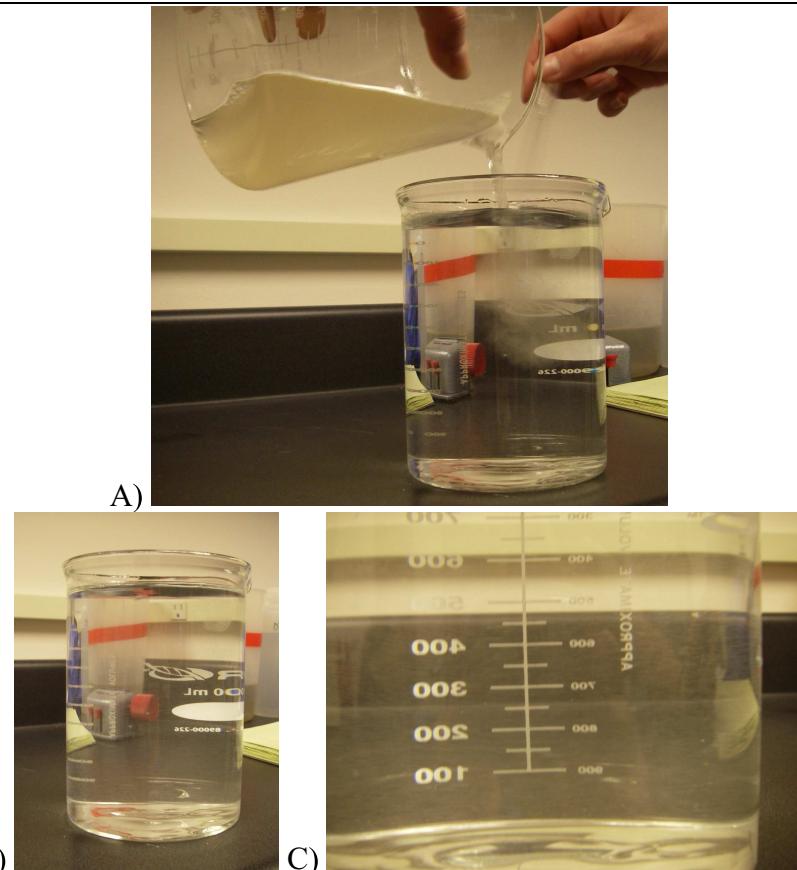


Figure 15. Preparation of the diluted egg solution. A) With the eggs all in suspension in the stock solution, a small amount is poured into 1L of filtered saltwater while stirring. B) & C) show what a ~1000 egg/mL should look like.

3. While stirring the diluted egg suspension continuously with a perforated beaker plunger (Figure 16), take a 100 μ L aliquot, inject it onto a concave microscope slide, and count the number of eggs. There should be about 100 eggs present. If not, dilute the stock and try again. Count the number of eggs in four aliquots of 100 μ L and take the average (see Figures 17 – 18). Multiply this by a dilution factor of 10, which gives the total number of eggs/mL. It should be approximately 1000 eggs/mL. Note: cut the tip of the pipette off beforehand – the tip hole may be too small and could damage the eggs. Cut the tip when transferring the embryos as well.



Figure 16. Perforated beaker plunger.

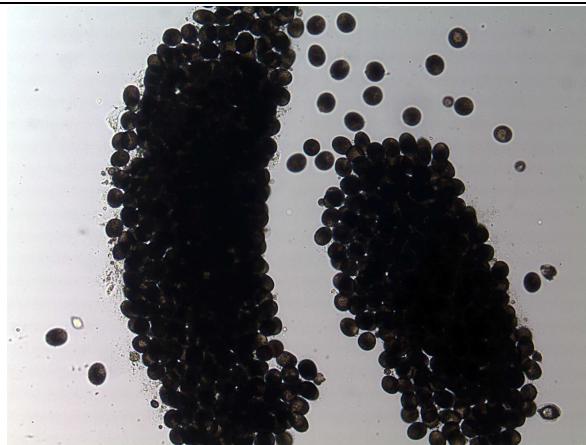


Figure 17. Clusters of eggs must be discarded if seen within the aliquot for counting. Stir the egg suspension and take another sample to count.

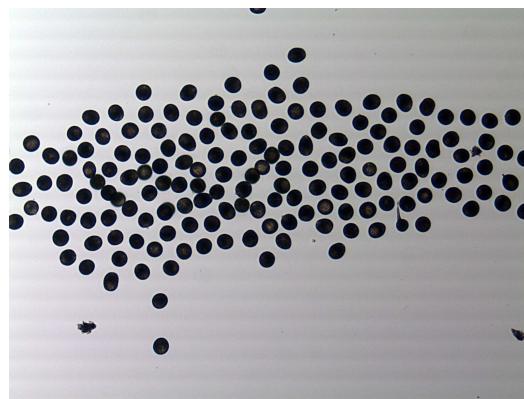


Figure 18. By gently rattling the concave slide back and forth while under the microscope aligns the eggs in a straight line. This allows for easier counting.

4. Divide this egg suspension into four 100 mL samples.
5. Obtain a small amount of concentrated good quality, mobile sperm from the beaker(s), seen in Figure 19.



Figure 19. Sperm stock. A very small amount is required and very rarely will there not be any good sperm available.

6. Pipette 0.025 mL, 0.05 mL, 0.1 mL, and 0.2 mL into the four beakers of egg suspension, stir immediately, and store it at 18°C for 1.5 – 2 hours, stirring every 20 minutes. This is done to achieve 90 – 95% fertilization in one of the egg batches. Store the sperm at 4°C until the end of the test.

7. After this time, examine and quantify the embryos to determine the percent fertilization. Fertilized mussel embryos are distinguished by the presence of one or two tiny polar bodies. Some embryos may have advanced to the 2- or 4-cell stage (Figure 20). Use the eggs with the lowest amount of sperm and the highest rate (up to 95%) of normal embryo development. Egg batches that are 100% fertilized may be polyspermic (Figure 21) and should not be used.

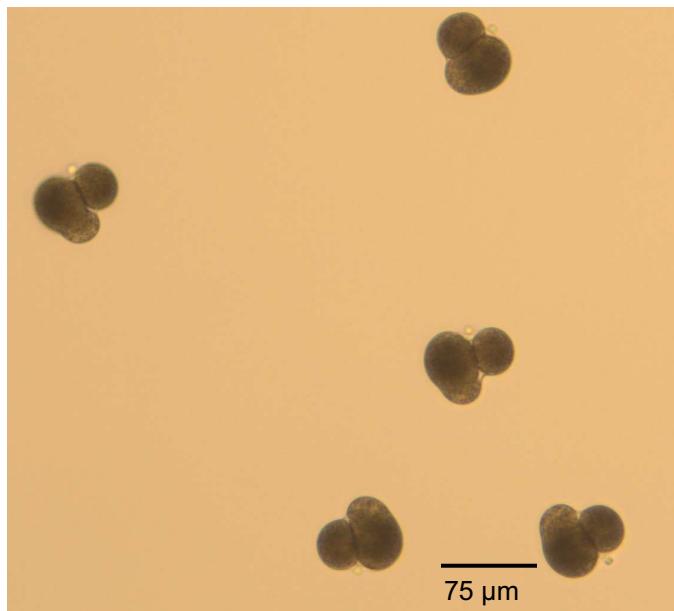


Figure 20. Embryos in the 2- and 4-cell stages. Once they are in this stage of development, they should be used in the toxicity test. If >10% are seen in the 8-cell stage, they cannot be used.

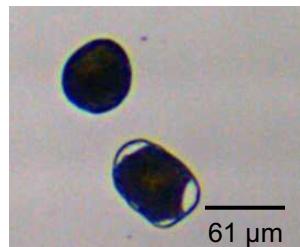


Figure 21. An example of polyspermic fertilization. The three clear zones are three sperm attached to one egg.

8. A fertilization rate of at least 75% is needed to proceed. If it is near 75%, mix gently with the plunger then allow the embryo suspension an additional 30 minutes and reexamine. If it still does not reach the 75% requirement, start over. The eggs or sperm may not be viable so if fertilizing a second time does not work, collect gametes from different mussels.
9. Double check the egg density of the embryo suspension to be used in inoculation.

Test Innoculation

1. Determine the volume required to pipette 200 embryos into each glass scintillation vial containing the 10 mL copper solutions (see **Test Solution Preparation** above). This volume should be between 100 – 400 μL . Despite the

requirement of 90 – 95% fertilization, the number of eggs/mL = number of embryos/mL for this calculation.

$$\text{Eg. } \frac{200 \text{ embryos / vial}}{1000 \text{ embryos / mL}} = 0.2 \text{ mL / vial}$$

2. Transfer the calculated amount into each of the vials, as seen in Figure 22. Include several control “monitoring vials” to check the progress of development during the assay. The test should be inoculated within four hours of embryo fertilization. It is ideal to have the embryos at the 4-cell stage.



Figure 22. Transferring of embryos into each vial. The pipette tip should be cut so as not to damage any of the embryos during transfer. Keep the embryos suspended in solution by constantly mixing with the perforated plunger while transferring.

3. Cap the inoculated vials and place in a temperature chamber at 16°C – 18°C for 48 hours (US EPA requires temperature to be at 18°C while ASTM methods state 16°C).

Test Termination

1. After 48 hours, check the developmental progress of a control monitoring vial. Most embryos should be at the D-shell stage (Figure 23); if not, continue the test until complete control development but no longer than 54 hours.

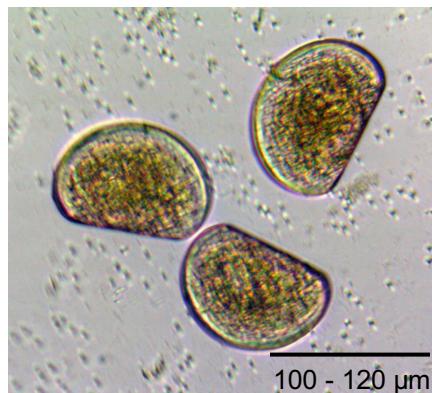


Figure 23. Normal development after 48 hours is observed as D-shaped calcified shell formation as well as good, consistent, brown flesh formation.

2. Preserve the embryos by adding 1 mL of 5% gluteraldehyde solution. The vials can now be stored for later analysis.

Test Enumeration

1. After fixation, the larvae should have settled to the bottom of the vial. If using an inverted microscope, remove the cap and place the vial under the objective lens. Otherwise, with a 1 mL pipette, gently scrape the bottom of the vial while slowly filling the pipette. Transfer the collected 1 mL onto a Sedgewick-Rafter counting chamber (Figure 24) and place on the microscope stand.

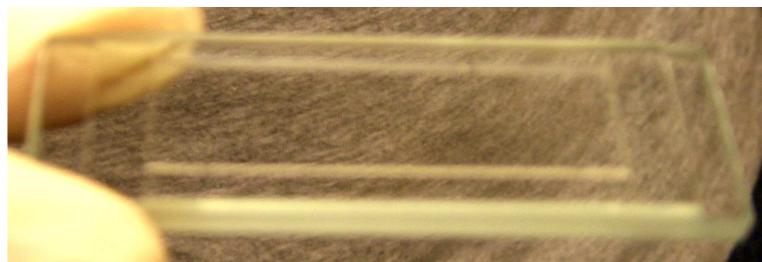


Figure 24. Sedgewick-Rafter counter. This chamber holds exactly 1 mL of solution and typically has a grid etched on the underside of the chamber.

2. Examine the contents of the counting chamber. Ensure all embryos are examined.
3. In each vial, count the number of “normal”, “abnormal”, and “dead” larvae. See Figure 25 for examples. Count at least 100 larvae.
 - a. Normal: Completely developed D-shell larvae. Larvae that have misshaped or otherwise malformed shells are also considered normal provided development has been completed.
 - b. Abnormal: Any embryo that has not developed to the D-shell stage by the test termination, even if it may have died. Shells may appear round, if developed. Ciliated blastula stage is most affected, causing these abnormalities. Eggs are not counted as abnormal development.
 - c. Dead: Completely developed D-shell larvae that appear to be empty (without the brown flesh).

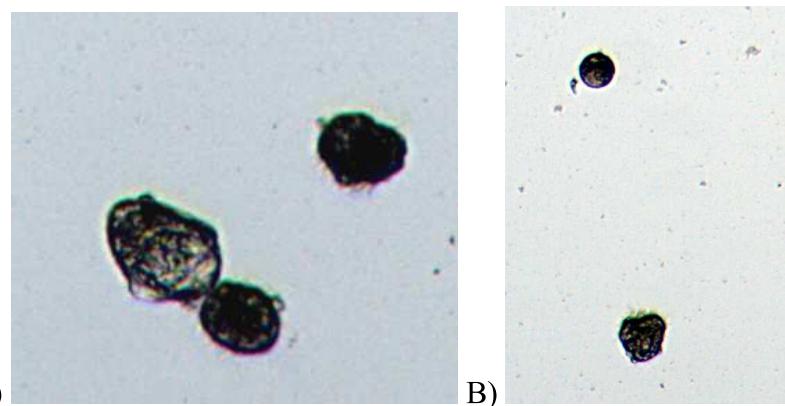


Figure 25. A) One dead D-shell (leftmost) and two abnormal larvae. B) One abnormal larva (bottom) and one unfertilized egg (top).

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- 4. If the number of larvae appears low in relation to the inoculation, this indicates either mortality and dissolution, or incomplete transfer to the counting chamber. Inspect the vials for evidence of the latter – some larvae may adhere to the walls of the vial
 - 5. If it is suspected that embryos died and disintegrated during the test, then mortality should be estimated from initial embryo counts prior to inoculation.
 - 6. The percentage of larvae that did not survive and develop normally is calculated for each treatment replicate.
 - 7. If the percentage of “normal” embryos in the control treatment is <90% or mortality is >30%, the test is invalid.

Reference Toxicant Testing

To ensure the organisms being used in the test are responding to chemical stress in a “typical” manner, a reference toxicant test is run using filtered saltwater from Granite Canyon. The results from tests using actual samples will be compared to the reference data.

Test Acceptability Criteria

The test acceptability is $\geq 90\%$ normal development, $\geq 50\%$ survival in the control treatment, and $\leq 25\%$ minimum significant difference relative to controls.