Master Experimental Protocol

Ahead of time:

1. Make 30, 50, and 70% EtOH
2. Make sieves: large 80 µm
3. Make dewar lid

Day 0

1. Materials
	1. 2 large beakers (eggs and sperm)
	2. 12 Falcon tubes (2 per treatment), pre-soaked in SW
	3. Transfer pipettes
	4. P1000 pipette and tips
	5. 48 fertilization containers (small beakers or dishes)
	6. Rafter slide
	7. VWR pen
	8. Calculator
	9. Squirt bottles
	10. 1.5 mL eppendorf tubes
	11. 4% PFA in SW
	12. filtered SW
	13. 30, 50 and 70% EtOH
	14. cuvettes for spec
	15. m-cresol purple (2 mmol/L)
	16. HgCl2
	17. Pyrex bottles for chemistry (6)
	18. Hemacytometer
	19. Scalpel
	20. Shucker
	21. Microscope
	22. Glass slides
	23. counter
2. Fertilize each treatment simultaneously – 6 dishes per treatment. Aim to fertilize ~60,000 eggs per container. Let sit static for 24 h.
	1. Dilute sperm 1:100 and count using hemacytometer. Amount of sperm used for number of eggs should be less than 50 sperm per egg.
3. Fertilize Falcon tubes, timed ½ hour apart (~5000 eggs per Falcon)
4. At 1 hour post fertilization (hpf) of Falcon tubes, gently homogenize tubes and filter out 2 aliquots of 500 µL of eggs + water from each Falcon tube (n=8). Add 1 mL of 7.5% MgCl2 (for 5 hpf only). Wait until all larvae are on the bottom (check back periodically). Draw off liquid and add 4% PFA in FSW and let stand 24h. Rinse 1 or 2x with 1x PBS, letting stand 15 minutes between each rinse (at this point the larvae can be stored in the last rinse of PBS for a short period of time at 4C). Rinse with increasing concentrations of EtOH (30, 50, then 70%), letting stand 15 minutes between each rinse. Store in 70% EtOH.
5. Repeat IV for 5 hpf.
6. Chemistry
	1. Initial DIC, TA, and pH (spec) of cooler water

Day 1

1. Materials
	1. P200 pipette and tips (ends cut off)
	2. Falcon tubes (36)
	3. Squirt bottles
	4. 1.5 mL eppendorf tubes
	5. 4% PFA in SW
	6. filtered SW
	7. 30, 50, and 70 % EtOH
	8. 20 µm large sieve + holding container
	9. Vortex
	10. Plate map
	11. 95% EtOH
	12. Transfer pipettes
	13. Inverted microscope
	14. cuvettes for spec
	15. m-cresol purple (2 mmol/L)
	16. HgCl2
	17. Pyrex bottles for chemistry (18)
	18. counter
2. For each larval container, decant entire volume onto mesh sieve. Rinse into 50 mL Falcon tube. Gently homogenize tube and remove 2 aliquots of 100 uL into one well of a welled plate. Fill out plate map with container and treatment information. Check density of larvae in 200 uL aliquot and adjust accordingly for the rest of sampling (target is >50 larvae). When density is determined, remove volume approximately equal to 50 larvae to fix in formalin as described Day 0.
3. Wash each container in vortex, fresh water, and salt water before returning larvae. Carefully rinse all larvae back into containers and connect to system so that they are flow through.
4. Count all dead larvae in the wells. Drop larvae with 95% EtOH and count totals for each well.
5. Chemistry
	1. TA and pH of cooler water
	2. DIC, TA, pH of containers A and B of each treatment

Day 2

1. Materials
	1. cuvettes for spec
	2. m-cresol purple (2 mmol/L)
	3. HgCl2
	4. Pyrex bottles for chemistry (18)
2. Start feeding larvae
3. Chemistry
	1. TA and pH of cooler water

Day 3 – Same as Day 1 except chemistry done for containers C and D

1. Materials
	1. SEM
2. Protocol?

Day 4

1. Materials
	1. cuvettes for spec
	2. m-cresol purple (2 mmol/L)
	3. HgCl2
	4. Pyrex bottles for chemistry (6)
2. Chemistry
	1. TA and pH of cooler water

Day 5 – Same as Day 4

Day 6 – Same as Day 1, chemistry for A and B

1. Materials
	1. Dewar
	2. Liquid N2
	3. Screw cap vials (2 mL)
	4. Centrifuge
	5. Box for storage in -80C freezer
	6. SEM
2. RNA samples will be taken from containers A and B from each treatment. As described for Day 1, the containers will be emptied and sampled for counting and fixing. Instead of being put back in the larval containers, the larvae will be put on the mesh again and rinsed into a 2 mL vial. The vial will be spun down immediately (5,000xg for 1 minute), the supernatant/water will be drawn off, and the larvae will be flash frozen in liquid nitrogen. The vials will then be transferred to the -80C freezer for storage.