ZIRC NURSERY

Introduction

An auto-nursery is a rack system that delivers water to individual nursery tanks and allows water to exit via appropriately sized mesh drains. Several different auto-nursery systems are available through commercial fish rack systems manufacturers. Because commercial auto-nursery systems are usually only compatible with that particular company's racking system, it is often not feasible for an existing facility to incorporate a production model system into a pre-existing system made by a different manufacturer. Many facilities have developed their own auto-nursery systems using materials readily available at home improvement stores at a fraction of the cost of a commercial unit.

The ZIRC nursery racks are tied into the facility's recirculating water system, which significantly reduces labor in the nursery because hand siphoning and cage cleaning are no longer necessary. Fish can also be fed earlier without the concern of diminished water quality.

Embryos are maintained in Petri dishes at a density of 50 fish per dish in 0.5 x E2 medium (Nüsslein-Volhard & Dahm, 2002), which is better buffered (pH) than standard fish water and therefore offers a more stable environment. With 0.5 x E2, the osmolarity is also close to the range of standard embryo medium and fish water. Methylene Blue is also added to the E2 solution to suppress fungal outbreaks. Petri dishes are best maintained at the standard 28.5° C either in an incubator or in a specialized room. Larvae enter the auto-nursery at day 4 post-fertilization.

There are three feeds used in the nursery: Paramecia, Brine Shrimp, and the 'ZIRC Nursery Mix' which is made from Zeigler AP Larval Diet and freeze dried rotifers. Detailed instructions on how to make each of these foods are covered in the **Food Recipes and Productions Instructions** section.

ZIRC NURSERY PROTOCOL

Overview

Feeding:

- 5 to 9-day-old fish are fed concentrated paramecia
- 10 to 21-day-old fish (and older) are fed ZIRC Nursery Mix and brine shrimp, both 2x per day

Water Changes:

- 0 to 4-day-old fish are kept in Petri dishes with E2 embryo medium
- 4-day-old fish are transferred to nursery tanks (static system water)
- 5 to 9-day-old fish are maintained in static system water
- 10 to 21-day-old fish (and older) receive water for 24 hours (constant)

Detailed procedures for feeding the nursery

Day 0-4:

- Fish are maintained at a density of 50 fish per Petri dish in 0.5 x E2 embryo medium, E2 contains Methylene Blue to suppress any mold or fungal outbreaks.
- During this time the Petri dishes can be stored in an incubator at 28.5° C. Incubators with a glass front and/or programmable light cycles are best suited for this application.

Day 4:

- Transfer larvae to an auto-nursery cage, with 300 ml of system water

Day 5-9:

- Maintain larvae in static system water
- Feed 40 ml concentrated paramecia (see recipe section), 2x per day.

Day 10:

- Discontinue paramecia feedings
- Switch to constant (24-hour) water flow
- Feed larvae 5 ml ZIRC Nursery Mix (powdered flake food prepared from Zeigler and freeze dried rotifers), twice per day
- Feed 5 ml concentrated brine shrimp, twice per day

Day 10 - 21:

- Continue feeding ZIRC Nursery Mix and brine shrimp, each twice per day
- Water flows 24 hours a day

Day 21:

- Larvae are usually ready to be moved from the nursery to the grow-out section of the main facility
- Continue nursery care if larvae are not large enough to be transferred out of the nursery

Day 21 - 80:

- Transfer larvae to the grow-out section. To prevent larvae and juveniles from escaping, cover the drains in these tanks with a fine mesh.
- Feed larvae and juveniles brine shrimp and a juvenile powder mix prepared from the Zeigler larval diet and Golden Pearl.
- Once juveniles are large enough, by approx. 2 - 3 months of age, the mesh juvenile drains can be replaced with adult drains and adult food mix can be fed. The juveniles can now be moved from the grow-out racks to the standard adult racks.

Stage definitions for Embryo, Larvae, Juvenile:
Parichy, D.M., Elizondo, M.R., Mills, M.G., Gordon, T.N., and Engeszer, R.E. (2009) Normal table of postembryonic zebrafish development: Staging by externally visible anatomy of the living fish. Dev. Dyn. 238(12): 2975-3015.

Embryo Media E2 Recipe

E2 medium ingredients (1x strength):
15.0 mM NaCl, 0.5 mM KCl, 1.0 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.7 mM $NaHCO_3$. We use half-strength (0.5 x) E2 as a working solution to better match the conductivity and buffering of the system water.

Stock Solutions. Prepare three stock solutions: E2A, E2B and E2C, which are then mixed to make large volumes of 0.5 x E2.

E2A (100x):
Dissolve:

140.0g NaCl
6.0g KCl
19.2g $MgSO_4$
3.3g KH_2PO_4
1.1g Na_2HPO_4

- add millipore water to a final volume of 1600 ml
- shake and stir to dissolve the reagents
- autoclave
- stir over night to dissolve any precipitation that has formed during autoclaving
- store at 4 ^{0}C

E2B (500x):
Dissolve 11.0 g $CaCl_2$ (or 14.6g $CaCl_2$ x $2H_2O$)
- add millipore water to a final volume of 200 ml
- shake to dissolve completely
- autoclave
- aliquot into 20 ml portions (in 50 ml Falcon tubes)
- store at -20 ^{0}C

E2C (500x):
Dissolve 6.0g $NaHCO_3$
- add millipore water to a final volume of 200 ml
- shake to dissolve completely
- autoclave
- aliquot into 20 ml portions (in 50 ml Falcon tubes)
- store at -20 ^{0}C

E2 WORKING SOLUTION
For 20 L of 0.5X E2, mix:

100 ml 100x **E2A**
20 ml 500x **E2B**
20 ml 500x **E2C**
10 ml Methylene Blue Stock Solution

- add Reverse Osmosis (RO) water to 19 liters
- adjust pH to 7.0-7.2 (with concentrated HCl or concentrated NaOH)
- add RO water to 20 liters
- store at room temperature

Methylene Blue

Stock Solution: Dissolve 1g Methylene Blue (Sigma, MB-1) in 1 liter (0.1% w/v)

FOOD RECIPES AND PRODUCTION INSTRUCTIONS

ZIRC PARAMECIA PROCEDURE

List of Ingredients and Materials:

Starter Cultures

- Sterile containers or petri-dishes, at least 150x20 mm (large surface area is ideal)
- Nanopure, reverse osmosis, or deionized water
- Nutritional brewer's yeast tablets (crushed and/or powdered)
- Autoclaved, dry, whole-wheat kernels

Cultures

- 200 to 2000 ml plastic containers with large surface area, or Petri dishes, at least 150x20 mm
- Dechlorinated filtered tap water, Nanopure, reverse osmosis or deionized water
- Nutritional brewer's yeast (crushed and/or powdered)
- Autoclaved dry whole-wheat kernels

Other Items

- Measuring spoons, 0.05g
- Measuring spoons, 1 tbsp, 1/8 tbsp and 1/4 tbsp
- Warm room with medium light (24-28°C)
- Strainers/sieves made from 105 and 10 μm polyester filter cloth (Figure 2)
- Paramecia collector (for description, see Figures 3-4)

List of terms:

Starter Cultures

Stage 1 of the paramecia production process. Sterile paramecia cultures grown in **sterile** containers or large Petri dishes (150x20mm) using **Nanopure, reverse osmosis or deionized water.** These are used primarily to make new cultures for feeding larvae.

Cultures

Stage 2 of the paramecia production process. Clean, but not sterile, paramecia grown in **Nanopure, reverse osmosis, or deionized water**. Cultures are inoculated using the starter cultures. The paramecia grown in these cultures are fed to the fish larvae.

Additional Information:

- Culture containers should hold 200 to 2000mls of water, have a large surface area, be easy to wash, and ideally able to withstand high temperatures so that they can be cleaned using a cage washer or high temperature dishwasher. A large water surface to air ratio is vital in growing dense paramecia cultures.

- Both starter cultures and cultures will require approximately 1-4 weeks to reach optimal density.

Making Cultures

Introduction

When culturing paramecia, you are essentially creating an ecology in which microorganisms thrive. There are a host of other organisms besides paramecia that thrive in the same conditions, so it is important to monitor your cultures to make sure you haven't introduced any unwanted organism(s).

Starter cultures at ZIRC are routinely monitored for the presence of opportunistic organisms. On occasion, we have seen a small percentage of vorticella rotifers in our colony. Rotifers are a known food source for zebrafish. While rotifers do not harm paramecia cultures, we have performed serial dilutions on the ZIRC starter cultures in order to reduce rotifers to undetectable levels. In your own facility, a serial dilution can be performed on established cultures at any time and will ensure the cleanliness of your colony if contamination occurs.

If you have questions regarding anything you see in your cultures or observe in your colony, please feel free to contact us at fish_requests@zebrafish.org.

Preparation

1. Autoclave dry whole-wheat berries once and store in sterile container.

2. Start with a sterilized work surface. This will reduce the risk of contamination.

3. Bring 1 tbs of the whole-wheat berries to a rolling boil for 10 minutes using Nanopure, reverse osmosis or dechlorinated water.

4. Prepare 5, 10, or 20 sterile petri-dishes (containers) at least 150x20mm in size.

5. Fill each dish with 125 ml Nanopure water.

6. Add approximately 0.01 grams of powdered brewer's yeast to each dish.

7. After boiling (step 3 above), remove the wheat berries from the hot plate, pour off the excess liquid and wait a few minutes to allow the wheat to completely cool.

8. Add 5 wheat berries to each Petri dish.

Inoculation

9. You need approximately 5 paramecia starter culture dishes (800 ml) to inoculate 20 new Petri dishes. The starter cultures should have a density of at least 100 cells/ml.

10. Strain starter cultures through a kitchen (or tea) strainer to remove wheat berries. Use a squirt bottle filled with Nanopure, reverse osmosis or dechlorinated water to rinse and remove any paramecia attached to berries. Collect flow-through in a sterile glass bottle (or 1L beaker).

11. Divide starter culture equally between the prepared petri dishes, adding at least 40 ml of inoculate per petri dish. Gently mix the inoculating culture repeatedly since paramecia tend to concentrate on the surface.

12. Cover each petri dish and label it with the date of the inoculation.

13. Maintain paramecia cultures between 24 and 28 ºC. Avoid temperature fluctuations; cultures grow best in constant conditions.

14. To reach optimal density, allow the cultures to reproduce and grow for approximately 1-4 weeks.

Notes:

- The inoculated paramecia cultures have a relatively long "shelf-life" that is ideal for adapting to changes in nursery feeding requirements.
- Dishes can be used for feeding as early as 1 week after inoculation and up to 4 weeks. After 4 weeks, the paramecia populations reach a stationary phase and tend to decline in density. Optimal feeding density is between 2 and 4 weeks.

Harvesting Paramecia from Cultures for Larval Feeding

Concentrated Paramecia Preparation

Filter and rinse paramecia thoroughly before concentrating and feeding to the nursery.

1. Pour each paramecia culture through a 105 μm strainer (See Figure 2).
2. Rinse petri dishes through the strainer with a squirt bottle filled with Nanopure water.
3. Collect the flow-through (paramecia) in a 5L pitcher. Discard the debris collected in the strainer.
4. Rinse the strainer with Nanopure water (squirt bottle) between dishes to accelerate the filtering process.
5. Make sure that both valves of the paramecia collector are closed (See Figure 3).
6. Fill the paramecia collector half-way to the top with system water.
7. Pour the filtered paramecia into the collector (See Figures 3-4) and top off with fish water.
8. Wait 20 minutes. This will allow the paramecia to gather at the water surface.
9. Open the top valve and collect paramecia in a 10 μm strainer. Discard the flow-through. This will remove all contaminants (such as ammonia) from the water.

10. Close the top valve and refill the collector with fish water.
11. Repeat steps 8. – 10. until you harvest virtually no (or very few) paramecia. This usually takes 2-3 cycles with the collector.
12. After the majority of the water has been drained, use fresh fish water to rinse the paramecia and remove remaining ammonia.
13. Re-suspend collected paramecia in fresh system water. Dilute to feeding concentration (100-150 cells/ml).
14. After the last collection, the collector can be emptied and the remaining liquid discarded by opening the bottom valve and allowing it to drain.

Notes:

- The paramecia are too large to fit through a 10 μm filter. Unlike the 105 μm strainer, the 10 μm strainer retains the paramecia and the liquid flow-through can be discarded.
- Feeding concentration: After rinsing, pour the paramecia into a clean container. Fill the container with clean fish water until a concentration of 100 to 150 paramecia/ml fish water is achieved.
- Before feeding the paramecia, check the ammonia level with a test kit. If it shows any trace of ammonia, repeat the 10 µm mesh straining process. To prevent the mesh strainer from clogging with debris, rinse using a high-pressure spray.

Materials and Figures:

Culture Containers/Petri Dishes

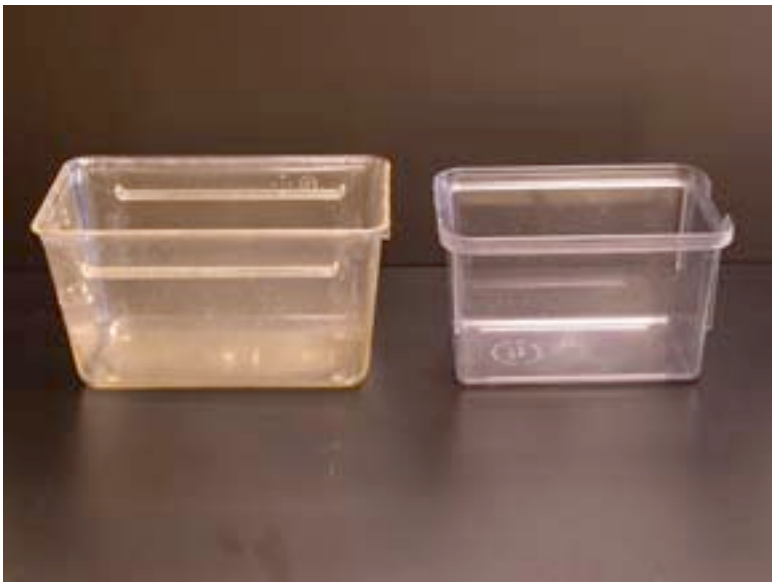

Figure 1. Examples of Culture containers ranging in size from 500 to 2000 ml. The container on the left is a polycarbonate Nalgene animal cage (24x14x13 cm). It has a volume of approximately 2 liters. The container on the right is a Thoren brand crossing cage and holds approximately 1 liter. Sterile petri-dishes at least 150x20mm are an alternative.

Micron Mesh Strainers/Sieves

Figure 2. Strainers used in the cleaning and concentrating of paramecia. The strainers are hand made by taking a large plastic beaker and cutting it into cylinders. Polyester filter or bolting cloth is then fixed to one side using hot glue. 10 μm and 105 μm mesh sizes.

Paramecia Collector

Figure 3. Paramecia collector. The paramecia collect at the top of the carboy and are then removed by the pipefittings near the top. The collector is made from a carboy with a spigot at the bottom and a modified suction drain (see Figure 4). A four-gallon carboy is recommended for larger facilities, while a 2-gallon carboy will work well for those needing only small amounts of paramecia.

Pipe Fittings for the Paramecia Collector

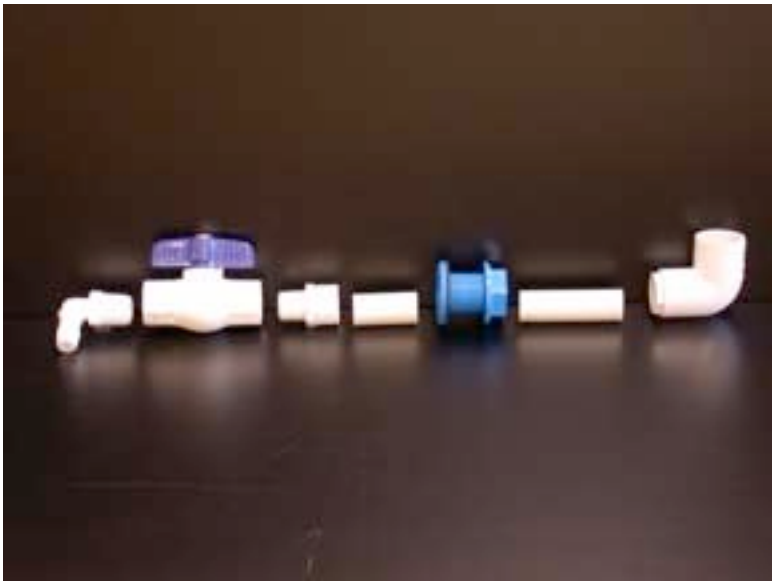

Figure 4. Paramecia suction drain. The suction top drain is made using (from right to left): 90° tubing adapter (1/2" id, 1/2" threaded), 1/2" PVC ball valve, 1/2" male adapter, a 2" length of 1/2" PVC pipe, 1/2" bulk head fitting, 1/2" PVC pipe, 1/2" 90° elbow. The length of the second piece of PVC pipe is determined by the type and size of container. The elbow needs to sit directly below the center of the opening in the carboy. Cut the PVC pipe to accommodate this. We recommend press fitting the pieces together as opposed to PVC gluing them. This makes dismantling easier when the unit needs to be cleaned.

ZEIGLER POWDERED FOOD RECIPE AND FEEDING INSTRUCTIONS

Larval Mix (Nursery; up to 21 dpf):

Ingredients

- 100-150 micron Zeigler Larval Diet, AP 100
- 150-250 micron Zeigler Larval Diet, AP 100
- Freeze Dried Rotifers,

Directions

- Mix equal parts (by weight) of Freeze Dried Rotifers, 100-150, and 150-250 μm Zeigler Larval Diet powder.
- Date opened canisters and store in food freezer
- Suspend 1 g of Larval Mix in 1 L of fish water
- Feed each tank 5 ml/50 fish.

Juvenile Mix (Grow-out section; 21 to 90 dpf):

Ingredients

- 150-250 micron Zeigler Larval Diet, AP 100 = 500g
- 250-450 micron Zeigler Larval Diet, AP 100 = 500g
- 200-300 micron Golden Pearls = 250g

Directions

- Mix Golden Pearls, 150-250, and 250-450 μm Zeigler Larval Diet powder (by weight).
- Date opened canisters and store in food freezer
- Feed each tank 20 mg/20 fish
- Feed juvenile tanks at least 2x per day

BRINE SHRIMP

We recommend Great Salt Lake brine shrimp cysts for larval and adult zebrafish. If smaller brine shrimp species are available we recommend using those for larvae/juveniles. We strongly recommend decapsulating brine shrimp before feeding.

Decapsulation Procedure

Background

Decapsulation of brine shrimp is a procedure that removes the cyst shell prior to hatching by soaking them in a bleach/lye solution. The brine shrimp are then stored in a saturated salt solution and refrigerated. Cysts remain viable in this state for more than 6 weeks.

There are several benefits for decapsulating brine shrimp prior to use. They include:

- Decreased chance of fish ingesting cyst shells, which can impact the digestive system
- Air does not have to be removed to collect brine shrimp for feedings. This decreases the number of brine shrimp that die and start to spoil in the cone because of repeated collections. It also decreased the time involved daily in preparing brine shrimp for feedings.
- Allows for more accurate feeding dilutions, due to the fact that you can drain a set amount from the cone without having to remove the air.

Materials

- 15 oz can of dried Artemia cysts (approximately 430 g)
- 4.3 L ~6% laundry grade bleach (e.g. Clorox), chilled to 4ºC
- 1.25 kg Rock Salt (NaCl)
- 125 ml 40% Lye (NaOH) solution (w/v)
- 30.0 g Sodium thiosulfate ($Na_2S_2O_3$)
- 16 L Hatching Cone with aeration
- 125 μm mesh bag (Aquatic Eco-Systems PMB3, 125 micron x 18")
- Several 3-5 L beakers
- (1-2) Squirt bottles - squeeze type

Solutions

Prepare all solutions in advance and chill to 4 ºC before use.

1. Bleach, ~6% laundry grade
   - Chill a large bottle of bleach (need 4.3 L) in the refrigerator overnight at 4 ºC

   25 ppt Salt Solution
   - Combine:
     - 50 g Rock Salt (NaCl)
     - 2.0 L with tap water
   - Stir to dissolve completely.

- Refrigerate overnight at 4 ºC

3. 40% Lye (NaOH) solution (w/v)
   - Combine:
     - 200 g Lye (NaOH)
     - 500 mL with tap water
   - Stir to dissolve completely
   - Store in refrigerator over night (4 ºC)

4. Buffered Salt Solution
   - Combine:
     - 2 L 25 ppt Salt Solution, prechilled to 4 ºC
     - 125 mL 40% Lye Solution, prechilled to 4 ºC

5. 1.0% Sodium Thiosulfate
   - Combine:
     - 30 g sodium thiosulfate
     - To 3.0 L with tap water
   - Stir to dissolve

6. Saturated Brine
   - Combine:
     - 1.2 kg Rock Salt (NaCl)
     - Fill up to 4.0 L with tap water
   - Stir to dissolve

Decapsulation Method

1. Cyst hydration:
   - Hydrate a full can of dried cysts in 5 L of tap water in an aerated hatching cone for 1 hour at 28 ºC.
   - Check the progress of the cysts under a dissection scope every 15 min.
   - Examine the cysts under a dissection microscope with incident light before proceeding. Dry cysts are dimpled, resembling a deflated basketball, whereas fully hydrated cysts are completely spherical. The cysts must be fully hydrated before decapsulation.
   - If cysts are not completely spherical after 1 hour, continue the hydration process (for a maximum of 1.5 hours)

2. Filter and rinse cysts:
   - Collect the hydrated cyst in a 125 um mesh bag and,
   - Rinse with cool tap water

3. Transfer cysts back to the cone with chilled Buffered Salt Solution
   - Keep a filled squirt bottle of salt solution to help transfer cysts to cone
   - Aerate

4. Decapsulation:
- Combine Lye (125 ml) and Bleach (4.3 L) solutions in a 5 L pitcher
- Add the chilled bleach/lye mix to the cone
- Continue aeration
- Watch the cysts turn from brown to grey to orange
- Stop the reaction quickly by collecting the cysts in a 125 μm mesh bag and rinsing well with cool tap water (Stop reaction, when approximately 90% of the cysts are orange)

5. Neutralization of residual chlorine:
- Transfer the mesh bag to a clean 4 L beaker
- Pour the 1.0% Sodium Thiosulfate (3 L) into the bag
- Soak the cysts in the sodium thiosulfate solution for ~1 min
- Rinse the cysts with dechlorinated tap water

6. Dehydration for long-term storage:
- Transfer the cysts back to the cone with 4 L of saturated brine (keep a full squirt bottle of saturated brine solution to help transfer cysts)
- Aerate for 18-24 hours
- Add granular NaCl as needed to keep the solution saturated during the dehydration process
- Transfer dehydrated cyst to 1 L bottles and fill with fresh saturated brine
- Store in refrigerator
- The decapsulated cysts are stored in saturated NaCl in 1 L bottles with 100 ml markings. When cysts settle in the bottle, make sure you have at least 100 ml of cysts per bottle. Distribute cysts per bottle accordingly.

Cone Set Up and Feeding Instructions

1. Cyst Hatching
- Fill 5 L cone with dechlorinated tap water (4.5 L per cone)
- Add 1.5 Teaspoons of Sodium Bicarbonate (ca. 8 g)
- Shake each bottle well to distribute cysts evenly
- Split the contents of one bottle in two 5 L cones (add 500 ml per bottle or add 50 ml cysts per cone)
- Aerate overnight in cone

2. Cyst Collection
- Do not remove the air stone (when using decapsulated brine shrimp, the cone does not need to settle before it is collected for feeding)
- Remove 1200 ml of hatched brine shrimp from the cone (via spigot at the bottom). (Note: For adult feeding remove 3200 ml)
- Rinse brine shrimp in a 105 μm sieve
- Add the rinsed brine shrimp to (a) 1 L squirt bottle(s) and fill with fish water

3. Feeding Instructions/Decapsulated Brine Shrimp

We use squirt bottles to feed brine shrimp, the amount of brine shrimp varies, depending on the opening diameter of the squirt bottle, the pressure, and the duration of the pressure on the bottle.

We feed ca. 9 ml brine shrimp from the squirt bottle per 20 fish (1-gallon tank).

The average dry weight of brine shrimp in 9 ml is approximately 85 +/- 2.7 mg.

Non-Decapsulated Method

We do not use this method at ZIRC, because hatched brine shrimp still contain a considerable fraction of unhatched and empty cysts. These tend to cause digestive problems, particularly in larvae, and in adult cysts can be trapped in the gills. Nevertheless, this method is useful sometimes, when decapsulated brine shrimp are not available.

Ingredients

- Dechlorinated Tap Water
- Rock Salt
- Baking Soda
- Brine Shrimp Cysts

The amounts of each ingredient depends on how much brine shrimp is being hatched and the size of the cone used. The following recipe is for a 16 L cone and can be altered depending on your facility's needs:

- 16 L Dechlorinated Tap Water
- 400 ml Rock Salt
- 1.5 tsp. Baking Soda
- 50 ml Artemia Cysts

Other Supplies

- Small air pump for each cone, or one pump large enough to supply both cones
- Brine Shrimp Hatching cones, available for most aquaculture equipment suppliers
- 105 μm polyester filter cloth mesh basket (see Figure 2, Paramecia Instructions)

Directions for Setting up Brine Shrimp Cones

Ideally, brine shrimp cones are maintained in a room with 24-hour light and a temperature of approximately 24-28 ºC. Small cones can also be kept in light boxes if they are set up inside a fish room.

Set up cones and add cysts approximately 18-24 hours prior to use. The late morning or early afternoon is usually the best time to do this, so that the brine shrimp are ready for the first feeding by the next morning. Because of the time needed to hatch, two cones are used. This allows one cone to hatch, while the other is still being fed to the fishes during that day.

The cones need to be aerated. To achieve this, connect flexible air tubing to the pump outlet and a piece of rigid air tubing that is long enough to reach the bottom of the cone to the end of the flexible tubing.

- Set up the first cone with water, rock salt, baking soda, and brine shrimp cysts.
- Adjust the air pump so that the cone is staying mixed, but not bubbling over.
- The brine shrimp will hatch and be ready for use approximately 18-24 hours after the cysts were added, depending on factors such as light intensity and room temperature.

Collection and Feeding Instructions for Non-Decapsulated Brine Shrimp

- Remove the air stick from the cone and allow brine shrimp to settle for approximately 5 minutes.
- Use the spigot at the bottom of the cone to drain off 100mls of brine shrimp, once they have settled.
- Collect brine shrimp in a 105 μm mesh basket
- Rinse the brine shrimp gently with fish water to remove all salt in the 105 μm mesh basket.
- Pour the collected brine shrimp into a 1-liter squeeze bottle and fill with fish water.
- Each tank of 50 babies should receive a 1-second squirt of this dilution, 2x per day