

Rotifer High Density Culture, Harvesting, and Fish Feeding

Overview

The lab uses rotifers^{i,ii} (*Brachionus plicatilis*) as a food for larval zebrafish. As the entire research program depends on efficient rearing of zebrafish through the larval period, it is absolutely critical that a constant supply of high quality rotifers is available at all times. The following instructions must be followed exactly, without deviation. Comments and suggestions are welcome but must not be implemented until they are considered and formally incorporated into the protocol used by everyone in the lab.

Rotifers are raised in high density culture tanks (aka “factories”) containing “rotifer water” that is made by adding Instant Ocean salts to water purified by reverse osmosis filtration.ⁱⁱⁱ We use marine rotifers because the cultures are more robust than those of freshwater rotifers, and the marine rotifers live in our standard zebrafish larval water.^{iv}

We use RO water as our starting point for making rotifer water and larval water because even dechlorinated Seattle tap water is toxic to the cultures.

Water quality in the rotifer factories is maintained by: partial, daily water exchanges; aeration; a protein skimmer to remove excess organic material; and supplemental dosing with a solution of ChlorAm-X to ensure ammonia is removed and sodium bicarbonate (baking soda) to control pH. Rotifers are fed a preserved algae paste while they are in the factories. Both ChlorAm-X/bicarb and algae paste are delivered automatically by peristaltic pumps throughout the day and night; the two together determine the number of rotifers produced each day.

Healthy, well-fed rotifers are reasonably nutritious for larval fish. However, we increase the nutritional value of harvested rotifers by feeding them Rotimac—which is high in vitamins, omega fatty acids, and other nutrients or Algamac, a thyroid hormone-free rotifer supplement— about 45 minutes before providing them to the fish.

This protocol covers care and maintenance of the rotifer cultures and systems, harvesting rotifers, and feeding of the harvested rotifers to fish. Each of these tasks is detailed separately and a protocol for integrating all of the tasks is included at the end.

Daily feeding and monitoring of the rotifer factories

Factories must be checked daily to verify culture health, algae and ChlorAm-X/bicarb dosing, mechanical components, and water quality. If there is any doubt about culture health or system functions, immediately consult with Dave (cell: 206 734-7331) or other senior lab personnel. At each check:

- 1 Record in the lab spreadsheet the current date and time (must be in 24 h format). Correct entry of this information is essential for automatic calculations that will be performed on data to be gathered.
- 2 Verify that the rotifers have eaten and that the cultures are grossly healthy. Healthy rotifers will be fast moving with attached eggs. Examine the color and turbidity of the cultures: healthy cultures are a relatively clear green/brown color. Turbid green implies overfeeding and possibly a crash in rotifer numbers. Clear brown to red implies inadequate feeding and starving rotifers, and also a potential crash. Take a pipette-full of rotifers and assess motility and egg production; record your observations for each factory in Parichy Lab Spreadsheets.
If there are few rotifers in the cultures, check with senior lab personnel about how much to harvest. Also check to see if the protein skimmers are producing foam at a normal rate. Do not increase the algae drip to feed more to the rotifers, as this can easily cause the cultures to crash if the filtration is inadequate.
3. Count rotifers to estimate total population. To adequately maintain both our larval fish and the viability of the rotifer cultures themselves each factory should contain at least 1.6×10^8 rotifers. To estimate total population, make serial dilutions from each rotifer factory into a 24 well plate and count whole, healthy rotifers. To do this, gently stir rotifers within each factory using a length of dedicated PVC pipe, so as to ensure the rotifers are well-dispersed throughout the culture. with Using a wide-bore 3 ml disposable plastic pipette (not a micropipette) remove ~1 ml of rotifers from just beneath the surface of the culture at a point halfway between the culture vessel edge and the center filter. Deposit the rotifers in well of the culture plate. From this sample, gently transfer 100 μ L into 900 μ L rotifer H₂O using a yellow-tip micropipette with a tip that has been trimmed to a wide bore to ensure that rotifers are not sheared during dilution. Gently pipette up and down or stir to mix. Take 100 μ L of this solution and dilute similarly in another 900 μ L of rotifer H₂O. Repeat this entire process twice more so that you have 3 independent samples from each rotifer factor and 3 different pools of twice-diluted rotifers to count for each factory. After all dilutions have been made, add 1-2 drops of vinegar to each final dilution to immobilize rotifers. Count all intact rotifers within each final dilution and record on the lab spreadsheet your individual counts, as well as the dilution factor used (typically, 2). If the coefficient of variation (CV) calculated in the spreadsheet indicates too much variability (CV>15), repeat the counts with new samples drawn from the rotifer factories. To obtain final rotifer population sizes, estimate the total water volume in each factory by measuring the water level against the dedicated measuring stick. Under normal circumstances, the water level should be at 60 L (green tape). The spreadsheet will automatically calculate the average density (rotifers/mL) and total population. These counts must be performed twice daily, in the morning and afternoon.
Accurate estimates of population sizes are essential for calculating short and long-

term population growth rates and stability, and dictate how many rotifers can be harvested and, therefore, how many larval fish can be supported. Careful attention to counts is critical.

4. Check aeration. All three air diffusers in the rotifer tanks should be bubbling gently, with one air diffuser in each third of the tank demarcated by hanging filters; the aeration ring around the center filter should be bubbling as well. Overall appearance of the culture should be a gentle “simmering,” not a vigorous boiling. Within the biological filter tank (that does not contain rotifers), however, the two air valves tank should be open fully and the diffusers bubbling vigorously.
Aeration in the rotifer tank should be sufficient to keep the culture suspended but not so vigorous as to damage the rotifers. Aeration around the center filter is essential to keep it free of debris; inadequate aeration can result in clogging, which will overflow the tank! We have one extra air pump should either of the two current pumps fail.
5. Verify that the peristaltic pumps and timers are working for algae and ChlorAm-X/bicarb dosing. Turn off and on the powerstrips that control the timers to reinitiate a dosing cycle. Verify that algae and ChlorAm-X/bicarb drip into the rotifer tank and are not caught on air hoses, pipes, etc.
Since the cultures are run at their carrying capacity based on algae and ChlorAm-X/bicarb dosing, any problems with the pumps, timers, or feed lines can quickly cause the cultures to crash! Note that we have one extra peristaltic pump should any of the four current pumps fail. Normally, a 10/4 interval /duration is sufficient. Do NOT change the timer without first receiving approval from Dave!
6. Record the solution volumes remaining in the algae reservoirs. Algae should be immediately refilled to 100 mL after recording. Verify that the ChlorAm-X/bicarb reservoir is sufficiently filled to last ~2 days, given current rates of depletion; refill if necessary. When adding algae or ChlorAm-X/bicarb check that mold or other debris have not accumulated in the bottles and swap-out the bottles if necessary. Make sure the algae is sufficiently submerged in ice. Ice buckets should be refilled twice daily in the morning and evening..
If the ChlorAm-X/bicarb solution has run-out or if the pumps have failed, check the pH of the rotifer tank and adjust with sodium bicarbonate powder if necessary (typically ~half tablespoon sodium bicarbonate per 0.2 pH units). Normal pH should be 7.5–8.5.
7. Check the water levels in the tanks. Water lines in the rotifer tank and biological filter tank should be at the same level. Use the graduated PVC pipe to determine the total volume in each factory.
If the water level in the rotifer tank is higher than it should be, if the water level in the biological filter tank is lower than it should be, or if both are occurring, this means that water is not being returned properly to the biological filter tank. The

most likely explanations are: (i) clogging of the center mesh filter in the rotifer tank: close valve #3 to isolate the rotifer tank, remove the current filter and replace with the spare; (ii) too rapid flow from the protein skimmer into the rotifer tank: close valve #1 to further restrict the flow; (iii) clogging of protein skimmer apparatus, which reduces overall flow to the skimmer and will push it to the main tank; (iv) some combination of the above.

8. Check for escapee rotifers in biological filter tank. Take a 3 ml sample from the tank and inspect (in the pipette). Record escapee numbers in the spreadsheet. If more than 1-2 escapees are found, a hole in the center filter or some other problem is likely; the issue should be identified and immediately fixed.
Escapees can indicate a major problem with potential to rapidly deplete the cultures, particularly since the vast majority of escapees will not be visible as they are removed by the protein skimmer.
9. Check the protein skimmer water level, turbulence and suction, and the waste collection buckets. The water level should be just below the bottom of the clear portion of the skimmer and there should be plenty of foam being produced. For the skimmer to work a strong suction must be generated by the black venturi valves; verify the presence of a strong suction by placing your thumb over the hose leading to the valve. The waste buckets should not be overflowing.
Be sure the water level is stable and not too high. Open and close valve #2 to change the water level in the skimmer; the valve is very sensitive: small changes (~1 mm) dramatically affect the pressure and resulting water level: too much can cause the system to overflow through the skimmer into the spill palettes.
Important: It is critical that skimmers are producing foam. If they are not, the cultures may crash owing to protein and bacterial accumulation. Likewise, if suction is low, it is probably time to clean the factory. Under these circumstances, you should alert the next two people on the rotifer factory cleaning schedule and also consult with Dave or other senior lab members about any necessary changes in algae drip or harvest to avoid overloading the culture with biological material under these conditions.
10. In the event of a major spill or other mechanical problem that cannot be remedied as described above: assuming there are rotifers to save, isolate the rotifer tank by closing valve #3, unplug the yellow water circulation pump and leave the culture with aeration and oxygen only. Contact Dave immediately.

Harvesting rotifers

Rotifers are harvested once per day, typically beginning about 7–8 am. The harvest must be completed by 8:30 am so the rotifers can be fortified with Rotimac or Algamac for ≥30 min and fed to the fish by 9:00 am. The harvesting itself takes about 20 minutes

(with experience); note that filters are cleaned and mechanical systems checked and this requires about 15 minutes additional.

1. A typical full harvest is 6 L per factory (this is 10% of the volume in the factory). The spreadsheet will automatically calculate the suggested harvest based on total population and average growth over the previous few days. A lesser harvest may be suggested by the formulas if population size or growth rates are too low, or if there have been inaccurate counts or errors in data entry (e.g., date/time formatting), or problems in cutting and pasting formulas. Be sure to identify why a curtailed harvest is suggested and whether it is based on problem with the actual population sizes and growth rates, or a data collection or data entry problem; note that some formulas depend on data entered several days previously so a problem on one day can have cascading effects. If you cannot identify an easily resolved data collection or entry problem, check with Dave or another of the most senior lab members for advice: if cultures are dense and relatively stable (e.g., populations have been consistently $>1.8 \times 10^8$), it is likely they can be harvested though the source of the problem will still need to be identified; if there is a bona fide population problem then this will need to be solved as well. In either instance a decision needs to be made: a failure to harvest, when such harvest would be reasonable, will cause fish to starve; overharvesting will endanger the cultures themselves and thereby imperil fish as well.
2. After determining how much of each culture to harvest, gather the two 23 μm rotifer screen filters (one for each rotifer factory) and pre-wet them with tap water.
3. Close valve #1 (skimmer return to rotifer tank) and close valve #5 (biological filter isolation).
4. Remove the center filter from its hole in the rotifer tank. For cleaning, place the center filter and the hanging three filter pads in a clean bucket.
5. Gently stir the rotifers with the dedicated PVC pipe so they are evenly distributed then use a 4 L plastic beaker to collect the correct volume of rotifers from the factory. Collected rotifers should be poured very gently through the 23 μm mesh screen filter. Once the rotifers have been collected on the screens, they should be rinsed with rotifer water into the 5 gallon drink dispenser.
6. Place a clean dry center filter (from previous day) into the rotifer tank, after wiping off and affixing the center aeration ring. Make sure the center filter is properly seated in its housing.
7. Change-out additional rotifer water. To maintain good growth conditions, we aim to change ~25% of the total culture volume daily, inclusive of the harvest itself. For a 60 L total volume this will be 15 L total, so if 6 L have been harvested already, an additional 9 L of water (without rotifers) will need to be drained. To do

so, place the rotifer system hose end into the 23 μm rotifer screen filters in the sink. Open valve #6 (main factory drain). Open the hose valve and drain the water through the screen, watching for any evidence of escaping rotifers. Drain the appropriate amount by estimating volume with reference to the measuring stick in the rotifer tank. When the appropriate amount has been drained close the hose valves and close valve #6.

8. Wipe out the inside of the each rotifer factory with a clean piece of rotifer filter or clean sponge; be sure to clean off the sides both above and below the water line, the bottom itself, and the plastic air hoses and the airstones . This helps reduce bacterial build-up in the system. If needed, do the same for the smaller tank (without rotifers).
9. Clean the dirty center filter and hanging filters using a strong jet of hot tap water from the hose to flush out debris. Rinse pads with RO water and return them to the tanks; leave the center filters to dry for the next day. Make sure these items are cleaned before they have a chance to dry. Thoroughly rinse the 23 μm mesh screen filter
10. Refill the tank with rotifer water to the volume recommended in the spreadsheet (typically 60 L). Be sure to close the valves, and turn off the sump pump when finished to avoid burning out the motor.
11. Reopen valve #1 (skimmer return to rotifer tank) and reopen valve #5 (biological filter isolation). Re-coil the drain hoses and stow them out of the way.
12. Refill the rotifer water tank by adding the appropriate amount of RO water and salt; turn on the pump to mix.
13. Empty the skimmer collection buckets.
14. Inspect system for any leaks or problems.
Even slow leaks can completely empty the system overnight.

Feeding rotifers to the larval fish

1. Thirty to sixty minutes prior to feeding rotifers to fish, stir the daily harvest of rotifers in the large drink dispenser to disperse any settled rotifers. Fill the Algamac rotifer bucket to the specified level and add 5 mL of Algamac (labeled AR) solution to the rotifer. Drain the remaining portion of that feeding's "tick mark" of rotifers into a 4 L bucket and add 15 mL of Rotimac solution. At the end of the day, there should be 1 tick mark worth of rotifers remaining in the harvest bucket (to allow re-inoculating the rotifer systems in the event of a devastating culture crash overnight).. Add 4-5 drops of algae to the remaining rotifers in the daily harvest bucket. Add air bubblers to the rotifers eating Algamac and Rotimac

2. Thirty to sixty minutes after feeding Rotimac and Algamac to the harvested rotifers, they can be fed to the larval fish. Pour each treatment of the harvested rotifers through separate 23 μm rotifer screen filter. For Rotimac rotifers, wash them into a beaker and fill with ~250 ml rotifer water. Add 2 ml of 10% ChlorAm-X. For Algamac rotifers, check tape label on the bucket for appropriate resuspension volumes. This amount changes often because of the fluctuation in thyroid ablated fish population. Add a few drops of 10% ChlorAm-X.
Waiting for the rotifers to eat Rotimac or Algamac is absolutely essential, as these supplements contain essential nutrients for the fish. Think of the rotifers themselves as a package for delivering these nutrients; the rotifers must have enough time to feed but not so much time that they process the food. A quick re-screening of the rotifers keeps the larval fish water cleaner and helps to maintain a high quality rearing environment, thereby reducing fish mortality. We add ChlorAm-X to the rotifers to reduce ammonia levels in the larval fish tanks.
3. Determine how many fish tanks and fish beakers need rotifers and aliquot rotifers to them accordingly. Be sure to keep the rotifers suspended by gently swirling their container. Generally, tanks on static water should receive from 4–5 drops of rotifers (for newly hatched larvae) to as much as 1 ml (for older larvae). Try to disperse the rotifers across the tank. Tanks of fish should generally receive 1–8 ml of rotifers but all rotifer amounts should be scaled according to harvest volume and fish number. Fish that are on flowing water and receiving only rotifers should receive relatively large amounts (e.g., two full pipettes). While such small fish will only be able to eat a small number of the rotifers actually delivered, we try to maximize their encounter rates given the rotifers will die quickly in the system water and will be flushed out owing to the water flow. The same is not true for fish in static larval water, in which rotifers live longer and are not flushed out of the tank (necessitating care so as not to feed so much as to foul the water). Check with Dave for details. If there are left-over rotifers after the feeding and they are still healthy, they should be returned to the rotifer factories (typically split evenly between the two factories).

Daily protocol

Following is a suggested protocol for accomplishing all the required rotifer-related tasks. As written, it is easily integrated with daily Fish Maintenance (see **Fish Maintenance SOP**). While the exact timing can be changed according to one's schedule, please note:

- **Rotifers must be fed early enough so that larval fish can be fed by 9:00 am.** *Rotifers should be harvested and fed Rotimac/Algamac by 8:30am.* This deadline is critical for making sure the fish are fed at an appropriate time and because morning feeders should not be expected to wait around for rotifers that were not

prepared on time. As such, if you are on rotifer maintenance and you do not have the rotifers in Rotimac/Algamac by 8:30, it is now your responsibility to feed them.

- **Rotifers should receive their last check no earlier than 4 pm, and fish should receive their last rotifers no earlier than 4 pm.**

Additional daily tasks

1. After every evening shift, hose down the floor.
2. Take out the garbage as needed.
3. Double check that the spreadsheet formulas and formatting are correct.
4. Make sure to fill the rotifer water and RO water carboys before your week-long shift ends. Also be sure there is enough salt and Chlor/AmX and that the floors are clean.
5. Make sure a new Rotimac and Algamac (as needed) 50mL tube are thawing in the refrigerator for the next day. If there are 4 or fewer tubes of Rotimac or Algamac left, make a new batch.
6. Clean the counters and the refrigerator if necessary.
7. Refill rotifer water squeeze bottles.
8. The person responsible for overseeing and harvesting rotifer cultures typically also is responsible for supervising fish maintenance (see protocol **Fish Supervising SOP**). All animal housing areas (main fish room, quarantine room and incubator) must be inspected for to verify overall health of animals, normal system operation and environmental conditions, daily log entries for fish maintenance and feeders must be inspected to verify that that all tasks have been completed and all parameters are within normal limits, and all logs must be initialed. If problems are discovered during these inspections they must be corrected.

Notes:

ⁱ For more information see: Hoff F. H. and Snell T. W. 2004. *The Plankton Culture Manual, 6th Edition*. Florida Aqua Farms, Inc.

ⁱⁱ High quality starter cultures of marine rotifers can be obtained from Reed Mariculture (<http://rotifer.com>, <http://www.reed-mariculture.com>). On receiving the rotifers use the following procedure to avoid osmotic and other shocks (from the Reed Mariculture website):

- i. Open the box and store the bags of rotifers in a refrigerator (4-8 C) until you are ready to put them in the rotifer tank.
- ii. Measure the pH in the bag of rotifers (it will probably be about 7) and adjust the pH in your culturing tank to be the same. Otherwise the rotifers can be pH "shocked" and many of them can die. The pH will naturally return to its normal values within several hours and the rotifers will adjust without any problems. For information about adjusting pH click [here](#).
- iii. The rotifers will arrive at a salinity of roughly 20 ppt (1.015). Your water does NOT need to be adjusted to this salinity - they will go into water from 20 to 30 ppt (1.023) without any problems.
- iv. Add the recommend amount of ClorAm-X or Ultimate to the tank to buffer the water. Dosage rates can be found at www.cloram-x.com.
- v. Take the bag of rotifers from the refrigerator and put it in the rotifer tank for 10-15 minutes to allow it to slowly warm up. Then cut the bag open and release the rotifers into the tank.
- vi. Add enough Nannochloropsis or "Rotifer Diet" to the water to establish a green background.
- vii. Check the rotifer tank in 2 hours to see if more algae is needed. The rotifers will be hungry after their trip and will consume more than usual amount for the first few hours.

ⁱⁱⁱ Rotifer water is made by dissolving 280 g Instant Ocean salt per five gallons of RO water.

^{iv} Zebrafish larval water is made by dissolving 168 g Instant Ocean salt per 10 gallons of RO water.