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Guide to Zebrafish Larviculture using Amplifeed Replete and QuickStart Rotifer Cysts

Rationale:

Figure 1: Dedicated Rotifer Production Lab

Rotifers are a small (150 - 300µm) zooplankter critical for the first-feeding and early-feeding of many fish and invertebrate larvae (Hoff & Snell 2004, Wallace & Snell 2010, Snell et al. 2018). Rotifers have long been used effectively in the larviculture of zebrafish (Lawrence 2007), an important model in many fields of biology. Despite their importance in biological research, optimal zebrafish culture and management are still poorly understood (Lawrence 2011). Sustainable Aquatics has developed methods for rotifer mass culture, incorporating multiple innovations that produce more rotifers faster, of the right size for larval predators, and of the highest nutritional quality. A key to this method is QuickStart Rotifer Cysts for inoculating cultures and Amplifeed Replete for growing highly nutritious live feed. We recommend growing algae and rotifers in PhotoBioReactors (PBRs), which are disposable plastic bags with volumes of 5 – 1000 L. These allow labs to scale algae and rotifer production directly to their needs. We have found that the simplicity of these batch culture techniques allows most hatcheries to reliably mass culture rotifers with the highest nutritional qualities to produce the healthiest zebrafish possible. There is some evidence that rotifers may be able to replace Artemia and still meet goals for weaning and generation time (Lawrence et al. 2015). We believe that Amplifeed Replete enriched rotifers will perform even better.

There have been several approaches to using rotifers in zebrafish larviculture. The first uses the marine rotifer *Brachionus plicatilis* (240 – 300 µm long) reared at 15-20 ppt to feed zebrafish larvae at 1-2 ppt. This transfer to low salinity is clearly an osmotic shock to the rotifers and they typically stop swimming temporarily and fall to the bottom, where they are not available to fish larvae. A second approach is to raise *B. plicatilis* at 5-6 ppt and feed to zebrafish at 2 ppt which decreases the osmotic shock. Some *B. plicatilis* strains grow well at this low salinity compared to 15 ppt. Enrichment with Amplifeed Replete can be done for two days at 2 ppt, eliminating the osmotic shock upon transfer to the zebrafish larval rearing tank. A third approach is to grow the freshwater rotifer *Brachionus calyciflorus* (216 µm long) in freshwater and feed to zebrafish in freshwater. These rotifers swim longer in the larval rearing tank and are more available to zebrafish larvae. Some experiments indicate that zebrafish reared on *B. calyciflorus* have accelerated larval growth and about 30% shorter generation times, so that more generations can be obtained in a shorter period of time (Aoyama et al. 2015). *B. calyciflorus* also can be enriched with Amplifeed Replete and should perform even better. Others claim that zebrafish actually grow better and mature faster when reared in 2 ppt salinity water and fed *B. plicatilis* (Dabrowski and Miller 2018). We are confident that *B. plicatilis* enriched with Amplifeed Replete will be even better.

The following is a general guide for both lab-scale benchtop systems and 125 L reactor setup, rotifer inoculation, feeding, and harvest. Adjustments may need to be made depending upon rotifer and algae species/strains, reactor configuration, and culture conditions. These techniques are suitable mass culture of marine *Brachionus* species as well as *B. calyciflorus*. In freshwater, the alga *Chlorella* sp. is substituted for

Tetraselmis suecica. *B. calyciflorus*. QuickStart Rotifer Cysts are available from Sustainable Aquatics and Amplifeed Replete works just as well as in other brachionids.

Figure 2: Tubes with dried Rotifer Cysts

Suggested Use of QuickStart Rotifer Cysts:

To initiate rotifer cultures from diapausing egg inocula, a 1–100 L culture of live microalgae is grown to a density of about 10^5–10^6 cells/ml.

Many algae species are suitable, including *Tetraselmis, Nannochloropsis, Chlorella, Isochrysis*, and *Dunaliella*, grown at temperatures of 15–32°C and salinities of 15–35 psu.

Approximately 100 rotifer diapausing eggs/L are introduced into the algae culture where they hatch in about 24 hours. Diapausing egg hatchlings initiate rapid asexual reproduction and soon enter into log phase population growth.

After about 1 week, depending on temperature, the culture will contain a dense population of rotifers, free of contaminants, that is suitable for inoculating mass culture production tanks.

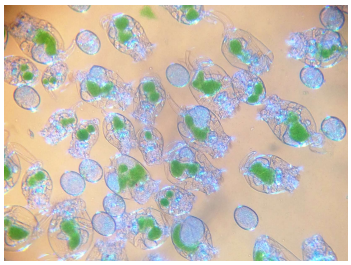
Figure 3: Live Rotifer Culture

We employ a lab scale bag culture system using 5 or 10 L bags for experimental work. This system is perfect for labs that do not need more than a few million rotifers at a time.

Lab scale algae and rotifer bag culture system - two sided

A compact culture system for growing microalgae and rotifers in plastic bags, is perfect for lab scale culture and experiments. The 2 foot unit can accommodate six 5 L bags (6X4X18”, 3 mil) and the 4 foot unit ten 10 L bags (7.5X6.5X20.5”, 3 mil). These 3 mil, square bottom, food grade bags are sterile and disposable. All models come with built-in LED lights and an air manifold system with valves and connectors. The rigid PVC frame is sturdy enough to support up to ten bags filled with water. Air pump needs to be supplied separately.

Helpful Tips for Setting up a Lab-scale Rotifer Production System

Storage:

- Rotifer cyst tubes should be stored in a freezer
- Live rotifers may be kept at room temperature or stored in a refrigerator for later use
- *Tetraselmis* should remain at room temperature

Inoculating cultures:

- Begin by preparing seawater at 6g/L for the *Tetraselmis*. Culturing at this low salinity will minimize osmotic shock when the rotifers are transferred to the zebrafish larvae tanks.
- Once the 5L bag is moderately green, you may inoculate with rotifer cysts.
- Wait until the rotifers graze down the algae (4-6 days), then start adding Amplifeed Replete.
- In two more days, rotifers harvests can begin. It is prudent to reserve about 10% of the culture to inoculate the next bag.
- A bag can be re-bloomed by adding more *Tetraselmis* and Amplifeed Replete.

Scaling for individual lab needs:

If additional algae production is needed to meet rotifer demand, we recommend deploying a second benchtop system, dedicated solely to algae production.

Starting a 125 L PBR:

Day 1:

*Set-up reactor cage in-front of LED light and place 125 L plastic bag into reactor (See Figure 4).
*Attach one airline to the bottom of each side of the bag and secure with a zip-tie or Velcro strap. Each bag will have 2 airlines (Figure 5).
* Fill each reactor with filtered seawater through a small hole created in the top of the bag (the "fill port" – Figure 6). *Tetraselmis suecica*, the preferred microalgae, can be cultured at salinities from 6-35 ppt.
*Add algae nutrients through the fill port (Figure 7).
*Add one 1.5-liter bottle of phytoplankton starter culture to each bag through the fill port (Figure 7).

Figure 4: Reactor Cage with LED Light and Plastic Bag

Figure 5: Airline Detail (notice Velcro strap at top)

Figure 6: Filling the Reactor

Figure 7: Adding Nutrients

Day 2-6:

*Allow algae to bloom for 5 days. During this time the bag will become a dark green color (Figure 9). If the bag does not darken or changes to a different color, the bag should be discarded and restarted.

Figure 8: Inoculation of Reactor with Algae

Day 7:

*Inoculate the bag with about 10 mg of dried QuickStart Rotifer Cysts (Figure 2). Each cyst hatches into a female who begins rapid asexual reproduction. The scale-up time starting from cysts is generally 2-3 days longer than live rotifers, but the advantage is that you can be confident of the purity of the rotifer species and that it is free from contamination.

*Alternatively, bags can be inoculated with approximately 0.5 million clean, filtered and rinsed rotifers (Figure 8) to through the fill port.

Day 11-13:

*Rotifers reproduce rapidly upon introduction, doubling *or more* every 24 hours at 25°C.

*Using the sampling port, take a 1mL sample daily and count under a microscope with about 10X magnification to assess rotifer health and population density in the reactor. A healthy rotifer population increases in density daily and has many egg-bearing females who swim vigorously (~1 mm/sec).

*At this time, it will be necessary to introduce supplemental food to the bag: Twice daily (morning and evening) add 0.5g Amplifeed Replete per million rotifers

Day 14:

*Perform a health check and density count, followed by addition of Amplifeed Replete

*Harvest reactor by filtering the entire reactor volume through a 50-70 um size mesh.

*Rinse rotifers and re-suspend in clean seawater and feed-out to larval tanks

*Remove bag from reactor and discard. Prepare a new reactor for reset (Beginning again with “Day 1”).

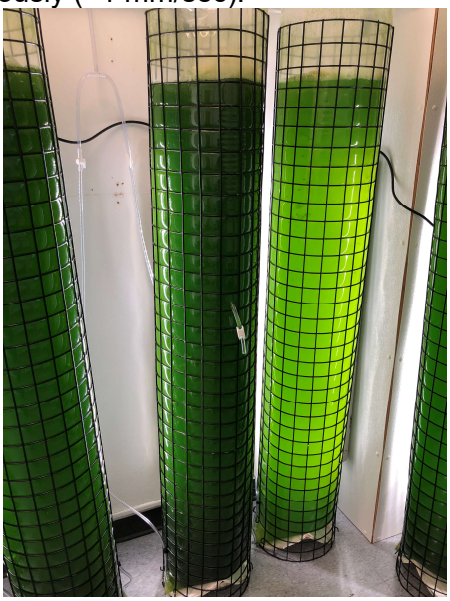

Figure 9: Dark green bag on left (notice sampling port in center of bag)

This bag culture system is easy to scale-up. In addition to the 125 L bags used in this example, Sustainable Aquatics also employs 1000 L bags for rotifer mass production during periods of intense rotifer use. Algae inoculation and inoculation with rotifer cysts need to be up-scaled proportionally.

Literature Cited:

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Brachionus life cycle video*:* https://youtu.be/_LLRKYAiqYQ

Guide to small-scale rotifer mass culture video: https://youtu.be/k712YcZyaF8