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The Cover: *Aphyosemion bitaeniatum* Lagos, male. Photograph by Anthony C. Terceira © 2006.

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A low cost and adaptable benchtop recirculating aquarium system for *Nothobranchius* fish

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Abstract

A recirculating aquarium system is described that can be set up on a laboratory bench. This system is adaptable to the needs of the researcher and can be up- or down-scaled as needed. It occupies little space. The system can be assembled at low cost by the researcher. It has been proven suitable for raising and spawning *Nothobranchius* for experimental purposes. Fry care and spawning are described in the context of the system. Disease and water quality management strategies are also discussed.

Introduction

Nothobranchius fish are gaining popularity as a model organism for aging research. A wealth of information has been gathered as to their natural history (Cellerino *et al.*, 2015) and advances in DNA technology has provided both insights into the genetics of *Nothobranchius* (e.g. Valenzano *et al.*, 2015) and means to genetically manipulate the fish (Harel *et al.*, 2015). Many researchers might be disinclined to explore this model organism for want of large fishroom facilities. In this article I describe a system that can be set up on a lab bench and which is undemanding to maintain.

The system is designed to be adaptable to the tank-space needs of the researcher as well as be space-economical. It requires very few pieces of equipment and any non-toxic water-tight container could be used to house the fish. The means to couple tanks is explained as well as to effectively filter the system and keep pathogens down to a minimum. Feeding and reproduction will be explained so far as it varies from the protocol of Genade (2005).

Materials and Methods

Tanks and filters

In my laboratory I am using USA standard 15 gallon tanks (56.7 L) of dimensions 0.6×0.3×0.3 m. These tanks can be subdivided (Figure 1) using 5 cm thick 45 PPI Poret foam into smaller compartments that can be used for different exerimental groups. The Poret

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Figure 1: Three aquaria, with Poret foam subdivisions, connected by siphons. The white U-bend tubing links the three tanks. Water is pumped from one end to the other. The water is filtered of ammonia as it flows through the Poret foam from subdivision to subdivision. The setup takes up 1.2 m of bench-space.

foam serves both as make-shift partition as well as a biological filter. Water can be pumped from one end of the aquarium to the other using an inexpensive aquarium pump (a Hydor Pico 200 in my setup).

Multiple tanks can be linked in series using siphons made from PVC plastic plumbing pipe (Figure 2). Aquarium-safe silicon sealant is used to bond the pipes and elbows and then the air is removed by inserting a length of plastic airline tubing up the siphon and sucking it out using a large volume syringe. PVC weld can also be used to bond the pipes but it is then difficult to dissessemble them to make adjustments. The silicon-joined pipes can be pried apart as needed. In my experience the silicon-sealed pipes are also more likely to be air tight. The tanks drain into each other by gravity. Water is pumped to the opposite end of the series of aquaria. Poret foam (10 PPI) was fitted over the entrance to each siphon to prevent fish moving from partition to partition. The diameter of the U-bends and overflows must be at least three times that of the inflow pipe from the pump to allow for efficient passive drainage by gravity.

Extra filtration is employed in the form of activated carbon and Seachem De-Nitrate to manage nitrate build-up[1]. A standard canister filter can be employed for this task. I have fashioned my own from recycled plastic juice bottles (Figure 3). A UV sterilizer (in my setup an Aquatop 10 W inline UV sterilizer) was employed to manage pathogen loads in the aquaria. Bacterial counts on LB-agar conducted on the effluent of the UV filter were zero. Ordinary shop lights were installed over the tanks and are turned on for 12 hours each day. Acrylic prismatic sheets were cut to size and used as hoods to limit evaporation and prevent fish from jumping out.

[1] The Seachem De-nitrate nitrate removal media is porous and the core of the pellets are anoxic. It is in this anoxic region of the filter material that nitrate is reduced to nitrogen gas using a carbon source to fuel the bacteria and accept the oxygen atoms of the nitrate. Any porous filter medium with a dark, anoxic zone will suffice for this task.

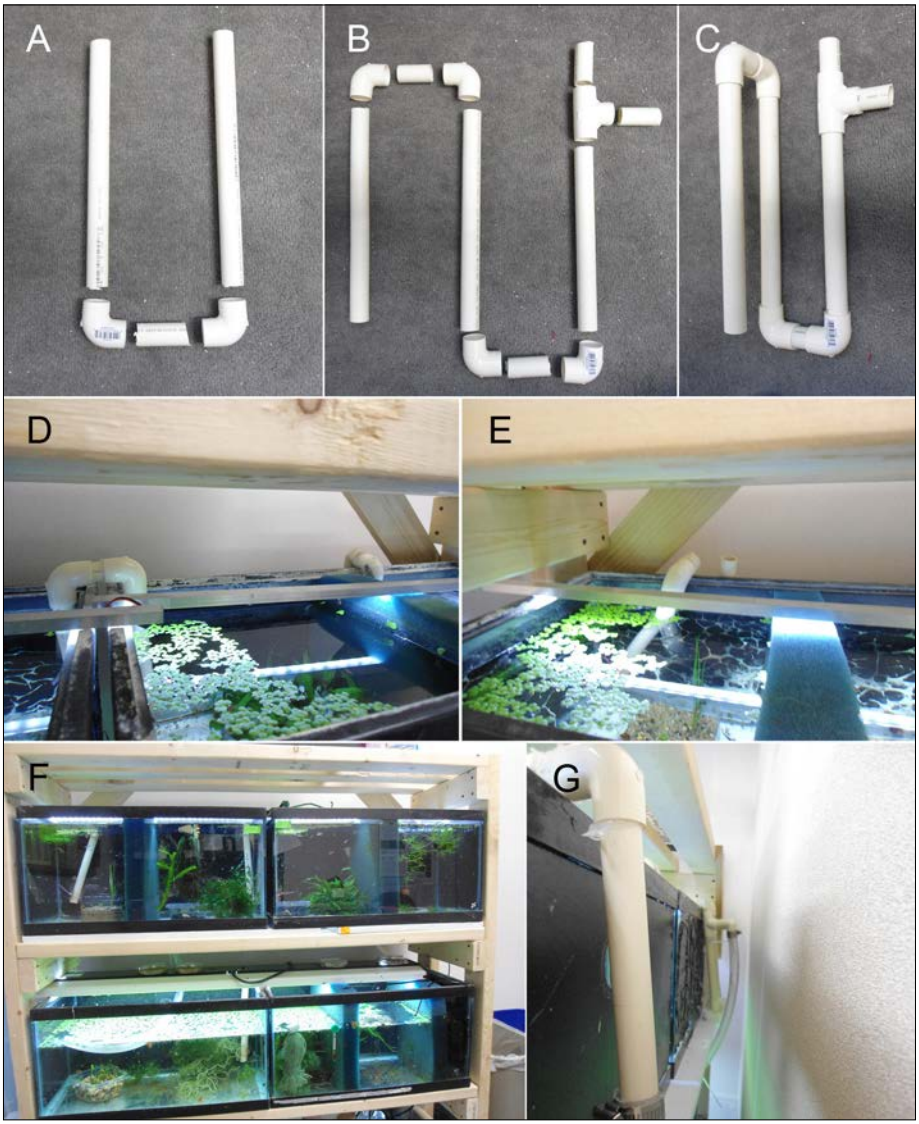

Figure 2: Connecting multiple tanks by means of PVC siphons. A U-tube constructed from PVC pipe (A) that links two aquaria (D) in a multi-tier aquarium system (F). The system is linked using gravity fed overflows (B,C) that prevent inadvertent drainage when the power is off, or overflow when the power is returned. The water depth is determined by the vertical placement of the horizontal portion of the overflow siphon (B, C, E, G). It is to this level that the tank will drain. The overflow will not siphon water below the level of the horizontal overflow pipe but, because of the double bend in the pipe, will also not break the siphon if the water level falls lower. The tank setup shown takes up approximately 1.2 × 0.4 m of floor space.

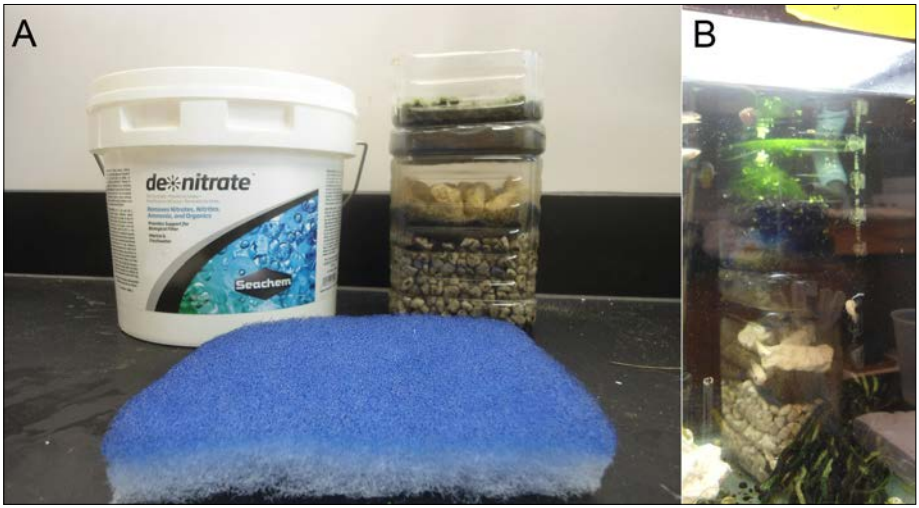

Figure 3: Home made canister filter. (A) The lower halves of juice bottles are stacked one on top of each other. The first layer is perforated and filled with activated carbon over some blue filter foam. The bottom layer is filled with Seachem De-Nitrate media with a layer of blue filter foam on top and a layer of crushed coral above that. (B) Water is pumped from one end of the series of aquaria and returned to the first compartment where it is directed into the filter. The carbon removes organic pollutants, the filter foam removes particulate matter, the crushed coral buffers the pH and the De-Nitrate media serves to both oxidize ammonia, reduce nitrate and manage nitrogen waste

Two 100 W heaters maintain the temperature at 25°C. One heater is placed in the first tank in the series (receiving filtered water) and the second midway to the end of the series. Such a setup is adequate to maintain at least four 15 gallon aquaria at the desired temperature.

Each tank is supplied with an airstone to improve oxygenation and water circulation. The airstone should be set to bubble slowly so as not to create too strong a current that might weary and stress the fish. Airstones are placed away from siphons to prevent air from entering the siphon.

Tank maintenance

Tanks are routinely siphoned of debris and uneaten food. The water is replaced using reconstituted RO water. I use 0.155 g of Seachem Cichlid Lake Salt as well as 0.142 g of potassium bicarbonate per liter of RO water to provide an ion constitution similar to the water catchment of the Mozambique flood plains (Chilundo *et al.*, 2008). The potassium bicarbonate aids in stabilizing pH.

Twenty-five percent of the water is changed at least once a week. Nitrate and ammonia levels are monitored and if need be the frequency of water changes and siphoning of debris is increased. Nitrate levels can be managed by means of aquarium plants (that also provide cover for fish) as well as by dosing the aquarium with small volumes of ethanol (Table 1) (Walton and Bjornson, 2008). Under

conditions of oxygen limitation certain species of bacteria can convert nitrate to nitrogen gas. The oxygen is extracted from the nitrate and used to oxidize carbon based compounds such as ethanol (Walstad, 1999). The dose of ethanol is escalated until the nitrate concentration is brought under control. The alcohol is rapidly metabolized to carbon dioxide and also serves to counter acidity (Uemoto *et al.*, 2014). The suggested 10 fish per 20 L is still encouraged (Genade, 2005).

Table 1: Ethanol dosing to manage nitrate concentration. Twenty percent Ethanol (e.g. vodka) is added at fixed volumes according to the schedule below. The dose escalation is stopped once nitrate concentrations are at the desired level. A 1 mL syringe works well for dosing. Dosing schedule based on values from Walton and Bjornson (2008).

Week	Ethanol dose mL per 10 L
1 - Day 1-3	0.021
1 - Day 4-7	0.042
2	0.148
3	0.254
4	0.360
5	0.466
6	0.571
7	0.677
8	0.783
9	0.889
10	0.995
11	1.101
12	1.206
13	1.312
14	1.418
15	1.630

Spawning and egg incubation

Fish are spawned using the method described by Wolfram (2012). In brief, granulated peat (e.g. sera super peat) is hydrated by soaking in several changes of hot water and then placed in shallow dishes in the aquaria. The fish spawn in the dishes and the eggs are retrieved using a kitchen sieve. The granulate peat and eggs are emptied into a bowl of water (freshly removed from the aquarium) and the peat vigorously stirred and shaken.

The eggs pass through the sieve and can be collected with a wide-bore plastic pipette. The eggs are then placed on a damp substance for incubation. In my experience damp sphagnum peat is the best substrate to use; see Genade (2005) for instructions on how to prepare the peat. Fine silica sand also works well provided it is kept damp by frequent wetting. Paper filters and cotton wool swabs have also been used but if an egg dies it is difficult to remove it from these substrates. Using the peat or sand, any dead eggs as well as surrounding medium can be easily excised to save the remaining healthy eggs. The eggs are routinely checked and dead eggs removed. The granulated peat is returned to the spawning fish. If the granulated peat is used for a different strain or species it should be sterilized by boiling in a microwave. The granulated peat can be used for a long time.

Petri dishes serve well as egg storage devices. These can be placed inside of another, larger, snap-fast container and floated in the aquarium or otherwise maintained at 25°C and high humidity. Under such conditions eggs may be ready to hatch in as little as 3 weeks. Eggs should be inspected using a dissecting microscope or magnifying glass. Eggs that show a gold

ring around the iris should be hatched as per Genade (2005). Eggs should be checked for development every three weeks and any developed eggs hatched or moved to cooler incubation conditions until hatching.

If slower development is desired the eggs can be incubated between 10 and 22°C when incubation can take as much as 6 months or more. Eggs that are incubated below 10°C will not exit diapause and will remain undeveloped for an undetermined length of time (perhaps years!). Subjecting the eggs to a temperature above 26°C will stimulate them to exit diapause and develop rapidly.

Fry care and feeding

Developed eggs are taken from the petri dishes and placed in a portion of peat sufficient to form a 5 to 10 mm layer at the bottom of a hatching container. Fresh water is added to the peat and eggs. I use RO water that is at room temperature. The peat with eggs is swirled, large clumps of peat broken up, and left to stand overnight (I normally hatch eggs in the evening). In the morning the fry are removed to a new container. To collect the fry the water of the hatching container can be poured into the fry-rearing container. Most fry will flow out with the water. The remaining fry can be spooned out of the shallow water of the hatching container. The hatching container is refilled and stirred. This is repeated three times over three days. On the fourth day the peat is redried. The same portion of peat is reused multiple times with developed eggs added to it and then inundated.

Once the fry are removed from the peat they can be fed. Feeding the fry over the peat can result in uneaten food dying in the peat, fouling it, and killing any remaining eggs in the peat. The fry are large enough to eat newly hatched *Artemia* nauplii and microworms (*Panagrellus* species). I have found that a mixed diet of fresh *Artemia* nauplii and enriched microworms or small Grindal worms is able to get the fish to sexable size in three to four weeks, and that growth on the mixed diet was faster than on *Artemia* nauplii alone (Figure 4). A diet of unenriched *Artemia* alone does not achieve this rate of growth. *Artemia* can be enriched using a product such as SELCO[2]. Microworms have high levels of fats, especially arachidonic and gamma-linolenic acid (Table 2) that are needed for growth and nervous system development. The use of microworms on their own is not recommended due to the low fiber content of the worms which could cause intestinal issues.

The microworms are cultured using a mixture of Gerber rice cereal with a generous supplement of spirulina powder (Figure 5). The rice cereal breaks down to a slurry and, if the medium becomes too liquid, more cereal can be added. These cultures last months before they begin to smell unpleasant. Microworms are easily enriched (Rouse *et al.*, 1992; Kumlu *et al.*, 1998) and spirulina was added with the goal of increasing the methionine and cysteine content of the worms. The amino acid methionine is needed to synthesize new proteins and as such is growth limiting. The amino acid cysteine is used in many metabolic processes as well as in protein

[2] http://www.brineshrimpdirect.com/c1/c8/selco-c47.html

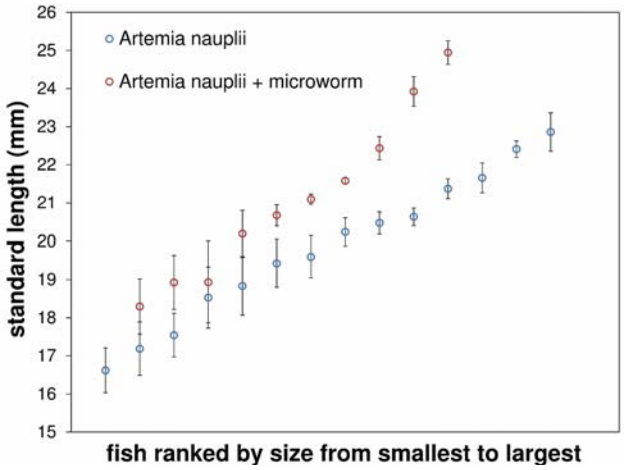

Figure 4: Fry provided with a varied diet grow faster than those fed with *Artemia* nauplii alone. Fry were fed either on *Artemia* nauplii alone or on a varied diet of *Artemia* nauplii and microworms and/or baby Grindal worms. To control against one group simply eating more than the other in this experiment both groups were provided with food in excess of their needs. Fry fed the varied diet were 7 percent larger at three weeks of age (p=0.009, ANOVA with Holm-Sidak). Standard length measurements of each group were taken from three photographs using ImageJ. Each data series is centered around the mean value of each group (19.8 vs 21.5 mm). Error bars represent standard errors for each fish measured three times. At two weeks there was no statistical difference in size between the groups.

synthesis but is especially needed by fish as a source of taurine - a physiologically important amino acid (El-Sayed, 2013). Similarly, Grindal worms (*Enchytraeus* species) are cultured on a bed of peat in a petri dish and fed Gerber rice cereal supplemented with spirulina powder. As long as the peat remains moist the young worms will crawl up onto the lid of the petri dish and are then easy to harvest and feed to fry. *Nothobranchius furzeri* fry have no difficulty swallowing the smaller grindal worms.

Fry are reared for the first three weeks in a medium-sized Lee's Kritter Keeper (approximately 6.6 L in volume) suspended in the aquaria (Figure 6). This ensures equal water temperatures between the fry and parental tanks. The tank is filled initially with water used to hatch the fry and then the volume doubled using mature water from the aquaria housing the parental fish. Each day the water volume is doubled until the tank is filled to capacity. Thereafter, the tank is given a daily 50 percent water change using the water from the parental aquaria. This is to prevent shock to the fry while at the same time ensuring that the fry are kept in water with diluted waste levels. Without large water changes growth of the fry will be stunted.

From the third week the fry can be given finely chopped *Chironomus* larvae (Bogut *et al.*, 2007). The adults can be fed *Chironomus* larvae their entire lives. Alternatively, "Repashy food formula N. furzeri v 1.1" can be used to feed the fish. The N. furzeri v1.1 formula is a gel food composed of black soldier fly larvae meal, fish and squid meal, and chlorella and spirulina algae powder. It is based on the hypothesis that in the wild the fish feed primarly on aquatic insect larvae and filter-feeding crustaceans (Reichard *et al*, 2010). It also has a nutritional profile comparable to that of *Chironomus* larvae (Table 2). A

Table 2: Percentage wet weight nutritional profiles for fresh *Chironomus* larvae, three concentrations (mass/volume) of the "Repashy N. furzeri v1.1", nematode worms and *Artemia*. Nutritional data for *Artemia* nauplii and adult taken from label of San Francisco Bay Brand Sally's Frozen Baby Brine Shrimp and Sally's Frozen Brine Shrimp. Nematode data is taken from Watanabe and Kiron (1994), based on 76 percent moisture content of live worms.

Food type	Chironomus larvae	Repashy N. furzeri v1.2			Microworms	*Artemia* nauplii
		14%	7%	3.5%	(White/Grindal)	(*Artemia* adult)
Crude protein	7.6	8.12	4.06	2.03	11.59 (14.06)	6.8 (4.7)
Crude fat	1.3	1.54	0.77	0.38	4.8 (6.67)	0.6 (0.5)
Arachidonic acid	0.00760	0.0077	0.0039	0.0019	0.306 (1.78)	0.0144 (0.0431)
Gamma-linolenic acid	0.01250	0.0154	0.0077	0.0039	1.362 (0.77)	0.1 (0.1)
Undigestable material (e.g. chitin)	0.546	0.364	0.182	0.091	? ?	0.5 (0.5)

7 percent mass of food in a final volume of 100 mL 7.5 percent gelatin is readily taken by the fish. They grow and spawn well on this diet. The gelatin solution is dissolved and then allowed to cool to 40°C whereupon the mass of food formula is added. I pre-cool metal baking dishes at -20°C and pour the food suspension into the cold dishes and leave it to set overnight at -20°C. In the morning the dish is inverted over a sheet of aluminum foil and then cut into smaller pieces for feeding. I prepare a volume of the gel food sufficient to form a 5 mm thick gel that can be cut into 1×2 cm pieces (1 mL volume). This is sufficient for ten fish. The food portion is finely chopped for feeding. Uneaten food must be removed 15 minutes after feeding as the gel dissolves, releasing the food to disperse in the water column where it will decay and foul the tank.

Managing disease

In a recirculating system contagious disease is a serious threat. The use of a UV sterilizer helps reduce the risk of serious infection. *Nothobranchius* are prone to infection by flagellate and worm parasites. Fish can be treated using metronidazole or flubendazole in their food (i.e. the Repashy gel food mentioned above). Metronidazole can be added to the food at a concentration of 10 mg/mL of gel food for five days (Francis-Floyd & Reed, 2009). Flubendazole can be administered at 10–35 mg/100 mL of gel food[3], fed once a day every second day for nine days (five doses)[4]. Flubendazole will kill both flagellates as well as intestinal worms.

Fenbendazole can be administered to the entire aquarium at 2 mg per mL. After three days 50 percent of the water

[3] Flubendazole is sold as a 5 or 10 percent powder and the dose added to the food needs to be adjusted according to the percentage powder. For example, 200–700 mg/100 mL if using a 5 percent solution.

[4] From http://www.versaquatics.com/treatmentandmethodology.htm.

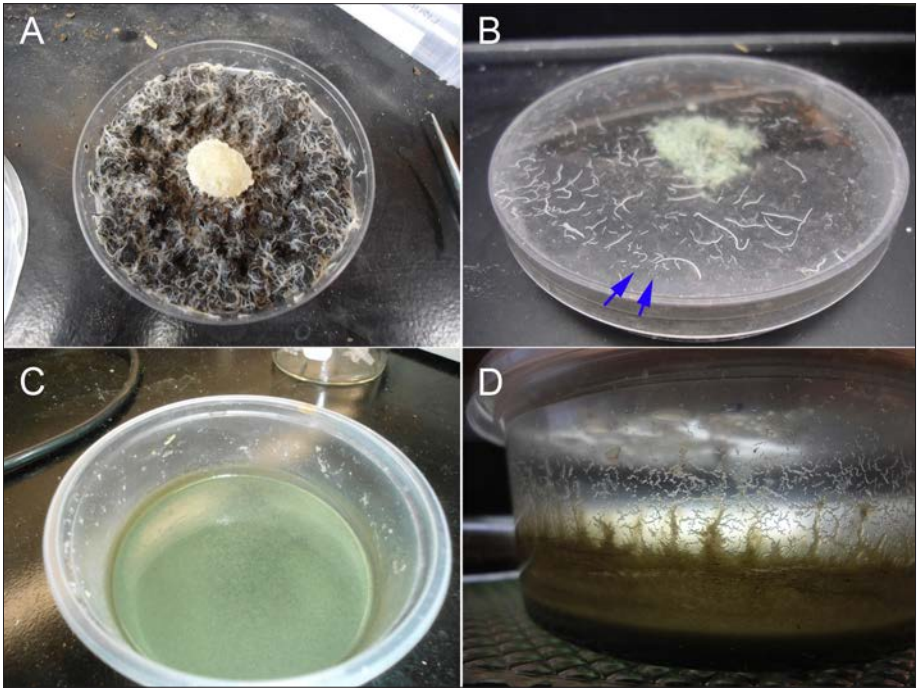

Figure 5: Worm cultures. (A–B) Grindal worms cultured in petri dishes on a bed of wet peat. Worms are fed a mixture of Gerber Single-Grain Rice Cereal with spirulina powder added. Worms can be easily picked off the peat or the lid of the petri dish. Blue arrows indicate baby Grindal worms suitable for small fry. (C–D) A microworm culture based on Gerber Baby Rice Cereal™ and spirulina powder. Worms are visible as a green haze on the inner surface of the plastic tub extending up the side of the container above the culture mixture. The worms are easily harvested by running a cotton swab along the inner surface of the tub and rinsing it into the fry tank.

is changed. The treatment is repeated twice, at weekly intervals. Alternatively the fenbendazole can be added to the food at 0.25 percent (Noga, 2010). In my experience *Nothobranchius* tolerate these treatments but fenbendazole (and levamisole, a commonly used deworming agent in aquaria) may cause sterility (Johnston *et al.*, 2006; Kent *et al.*, 2007). So, some caution is needed when using benzimidazole and imidazothiazole compounds; especially given the short reproductive cycle of killifish such as *Nothobranchius furzeri*.

Discussion

This system is highly adaptable. I am able to expand and contract the setup as necessary and in so doing optimize bench space. Tanks can be easily added to the system by adding U-bends. Using a suitably powerful pump, multiple tiers of tanks can be linked together without the need for expensive stands or complicated

plumbing that would necessitate drilling glass tanks. In this system a tank can be uncoupled from the system and removed to be washed or replaced.

Studies on the effect of ammonia and nitrate suggest that these two nitrogen pollutants have a synergistic toxic effect (Rubin and Elmaraghy, 1977). Because aquarium ammonia test kits are very insensitive, and nitrate can increase the toxicity of ammonia by a factor of two, it is important to keep nitrate concentrations as low as possible. This can be accomplished by large, frequent water changes but such water changes could stress the fish. As temperature changes affect longevity (Lu and Hsu, 2015; Valenzano *et al.*, 2006) water changes that affect the temperature of the aquaria could have a confounding effect on experimental outcomes. Seachem De-Nitrate is a porous substrate that permits colonization by anaerobic bacteria that can reduce nitrate to nitrogen gas but to do so the bacteria need a carbon source. Ethanol is a pro-denitrifying carbon source (Uemoto *et*

Figure 6: A medium-size Lee's Kritter Keeper suspended in an aquarium as a fry tank. Fry are housed in plastic tanks suspended in the aquaria. Water changes are easy to perform by exchanging water with that in the parental aquaria. The small tank enables high density feeding as well as preventing small fry from being sucked into filters. A small sponge filter is added to the tank and allowed to bubble gently. Fry should be large enough for the aquarium system by the end of the third week. A small mat of thread algae is placed with the fry to remove nitrogen waste as well as provide shelter for small fry. In the early stages the algae also supplies microorganisms as food for the fry.

al., 2014) that has no physiological effect at the very low concentrations it would be employed. The injection of volumes of ethanol into the filter system can help to manage nitrate levels. In addition, the inclusion of fast-growing plants such as duckweeds, *Ceratopteris*, *Salvinia*, *Elodea* or *Ceretophyllum* can help to remove nitrate and ammonia from the water column. Other plants such as Java moss can also work well but make counting fish difficult. Duckweeds can quickly become a pest. In my opinion green algae is beneficial in the aquarium as it is able to grow efficiently at low light and carbon dioxide levels and absorb nitrogenous waste.

The systems described here have been in operation for nearly three years without any overflow incidents. Disease was a problem until the UV sterilizer was included into the system.

This system can be employed in a pilot project at low cost compared to the installation of a specialized fishroom or sharing of facilities with, for example, a zebrafish laboratory that requires different temperatures and filter conditions. The initial outlay for equipment would be under $300 excluding the cost of aquaria and lighting (which may or may not be necessary).

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