

## Use of diluted acids and scrub pads in the mass culture of the freshwater rotifer *Brachionus calyciflorus*

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**Abstract.** The rotifer *Brachionus calyciflorus* is one of the principal live feed organisms cultured for the larval feeding of freshwater fish. In this study the addition of dilute hydrochloric acid (1%) and the commercial Acid Buffer™ (5%) from Seachem Laboratories were investigated to control the toxic levels of unionized ammonia in batch cultures of this rotifer species. The acids were added manually to the culture, three times per day to adjust to a neutral pH the culture medium. In a second experiment, the use of household cleaning scrub pads to manage the high organic load generated during the rotifer culture was also investigated. The rotifers for the experiments were placed in 400 mL polypropylene culture vessels, supplied with aeration, and the water temperature was maintained at 28°C. The rotifers were fed with the microalgae *Scenedesmus* sp. and exposed daily to 12-hour periods of indirect fluorescent illumination (< 300 lux), and darkness. Both experiments were run in triplicate and the data analyzed with ANOVA and Tukey tests ( $p = 0.05$ ). The results indicated the manual addition of either one of the diluted acids to the experimental rotifer cultures was enough to prevent the rise of unionized ammonia levels in the culture water, and favored increased population densities of the rotifers from an overall of  $1,094 \pm 67$  rotifers  $\text{mL}^{-1}$  in the culture medium, at the beginning of the trial, to above 2,000 rotifers  $\text{mL}^{-1}$  at the third day of the trial. The mean rotifer densities significantly increased from approximately  $445 \pm 6$  to  $2,679 \pm 64$  rotifers  $\text{mL}^{-1}$  at the fifth day of the trial when the scrub pads were used; which successfully trapped debris and living contaminants in the culture system. The methodology described in the study is inexpensive and easily implemented; and achieved rotifer densities significantly above those reported in the literature for the same species (e.g., 50 to 685 rotifers  $\text{mL}^{-1}$  in culture).

**Key Words:** live feed for aquaculture, appropriate technology, intensive culture, ammonia, pH.

**Introduction.** Rotifers, principally the species *Brachionus plicatilis* and *B. rotundiformis* have been widely used as live feed for fish larvae that are difficult to raise in saltwater and freshwater; due to their small size (typically 100 to 280  $\mu\text{m}$  in length), tolerance to wide range of salinity (5-60 ppt), slow-swimming nature, and relative ease of cultivation (Dhert 1996; Lubzens et al 2003; Kailasam et al 2015). In freshwater aquaculture, the predominantly mass-cultured rotifer species are *B. calyciflorus* and *B. rubens* (Dhert 1996; Lim & Wong 1997; Ogata & Kurokura 2012; Aoyama et al 2015; Torres-Valencia et al 2016). All these species are widely distributed worldwide, and favorable water temperatures for their cultivation range from room temperature (about 20°C) to 32°C (Arimoro 2006).

Although rotifers are easy to cultivate, when mass losses of the rotifers occur these are typically attributed to deterioration of water quality in the culture system, and its contamination with organic material and unwanted microorganisms (Dhert 1996; Lawrence et al 2012). Water quality is rapidly degraded by rising levels of toxic ammonia ( $\text{NH}_3$ ) formed from the biological processes and wastes in the production system (Yoshimura et al 2003). The addition of an acid to the culture system to maintain a neutral pH (7.0) has been an effective method to control the toxic ammonia (Yoshimura et al 1995, 2003). A neutral culture pH favors rotifer life while supporting an efficient rate

of conversion of ammonia ( $\text{NH}_3$ ) to ammonium ions ( $\text{NH}_4^+$ ) which are less toxic to the rotifers (Yoshimura et al 1995, 2003). The acid commonly used for this purpose is concentrated hydrochloric acid (e.g., 32%), which is typically dispensed by automation equipment to the culture system (Yoshimura et al 1995; Park et al 2001). Because of the high density of rotifers, their organic wastes, and debris from the feed, the culture system also becomes rapidly contaminated with a suspended floc material, which is formed mainly by the agglomeration of protozoa, bacteria, and fungi, that limits densities and negatively affects the overall health of the rotifers (Lubzens et al 2001). Experience with the mass culture of marine rotifers, has indicated the use of matts made of synthetic thin fibers (e.g., polyester fiberfill) which serve to trap unwanted microorganisms and debris (Lawrence et al 2012).

This study evaluated how the manual addition of diluted acids, and the use of regular household cleaning scrub pads, limit floc formation, affected the ammonia concentration, and densities in the experimental culture of the freshwater rotifer *B. calyciflorus*. This methodology could greatly simplify and facilitate the mass culture of freshwater rotifers to meet the high demand for live feeds required for the increasing number of freshwater fish species that are being cultivated, especially for ornamental purposes.

**Material and Method.** The rotifers were identified as *Brachionus calyciflorus*, isolated from plankton, and collected in ponds filled with freshwater for raising tilapia, in the municipality of Puerto Remolino in the state of Nariño, Colombia. The experiments were conducted in the 'Laboratorio de Fisiología y Productividad Primaria', of Universidad de Nariño in Pasto, Colombia during November/December 2016. Tap water from the city of Pasto was used to culture the rotifers. The water was left to aerate four days at room temperature and pretreated with a water conditioner (NovAQUA Plus, Kordon LLC). The experimental rotifers were maintained in culture inside a thermo-regulated room at 28°C and exposed daily to 12-hour periods of indirect fluorescent illumination (< 300 lux), and darkness. The experimental units consisted of polypropylene culture flasks with a volumetric capacity of 400 mL. The water was gently agitated with an air diffusing stone supplied by a low-pressure pump.

The first experiment investigated if the simple manual addition of dilute acids can increase the density of rotifers cultured, instead of using the typical concentrated acid solutions and automated processes. Two acids (treatment groups) were used: hydrochloric acid (HCl) at a 1-percent concentration, and a commercial buffer formulation available at a 5-percent concentration (Acid Buffer™, Seachem Laboratories). The acid solutions were added by dripping them directly into the experimental containers. The pH was adjusted to a neutral of 7, three times a day (08:00, 12:00, 17:00). A control group received no acid treatment.

The second experiment, used regular household cleaning scrub pads as floc traps to determine if they affected the ammonia concentration and density of rotifers in the culture system. Pads were laid inside the culture vessels and changed twice a day. They were washed by gently squeezing and rinsing them before each use. This time, however, in contrast to the first experiment, the water in the culture vessels was buffered by manually adding only the Acid Buffer™. All other conditions and procedures were the same for both experiments.

In the first and second experiments, the culture flasks were inoculated with  $1,094 \pm 67$  and  $445 \pm 6$  rotifers  $\text{mL}^{-1}$  in solution, respectively. Rotifers were fed with *Scenedesmus* sp. algae at a daily rate of approximately 4,000 algae cells per rotifer. Organism counts were performed daily from samples in volumes of 0.5-1.0 mL observed in a Sedgewick-Rafter chamber under a microscope. Water quality parameters of temperature, dissolved oxygen, pH, and ammonia were also measured daily using water quality meters (Hanna Instruments).

The experimental design was random, with three replicates per treatment for both experiments. Samples for count estimation were also taken in triplicate. Rotifer numbers were expressed as mean ( $\pm$  SD) abundance per mL in solution. To determine if there was a significant difference between the groups, an ANOVA with confidence level of 95% and

Tukey's tests were performed using the statistical software program Statgraphics Centurion, v16.1.18.

**Results and Discussion.** The rotifer *B. calyciflorus* is a cosmopolitan species relatively easy to find in natural freshwater ponds, and simple methodologies exist to quickly isolate them (Ogata 2017). Batch cultures with low ( $446 \pm 6$  rotifers  $\text{mL}^{-1}$ ) and high ( $1,094 \pm 67$  rotifers  $\text{mL}^{-1}$ ) rotifer densities of *B. calyciflorus* could successfully be established in small volume (400 mL) polypropylene culture flasks (Figures 1 and 2). In three to five days after initial inoculation, significantly higher densities from  $2,467 \pm 128$  to  $2,679 \pm 64$  rotifers  $\text{mL}^{-1}$  were achieved ( $p < 0.05$ ), depending on treatment type. On the third day, if the culture had no acid, nor the floc trap added to it, the number of rotifers began to decline or stabilized after reaching their peak (highest of  $1,814 \pm 200$  rotifers  $\text{mL}^{-1}$ ) (Figure 1).

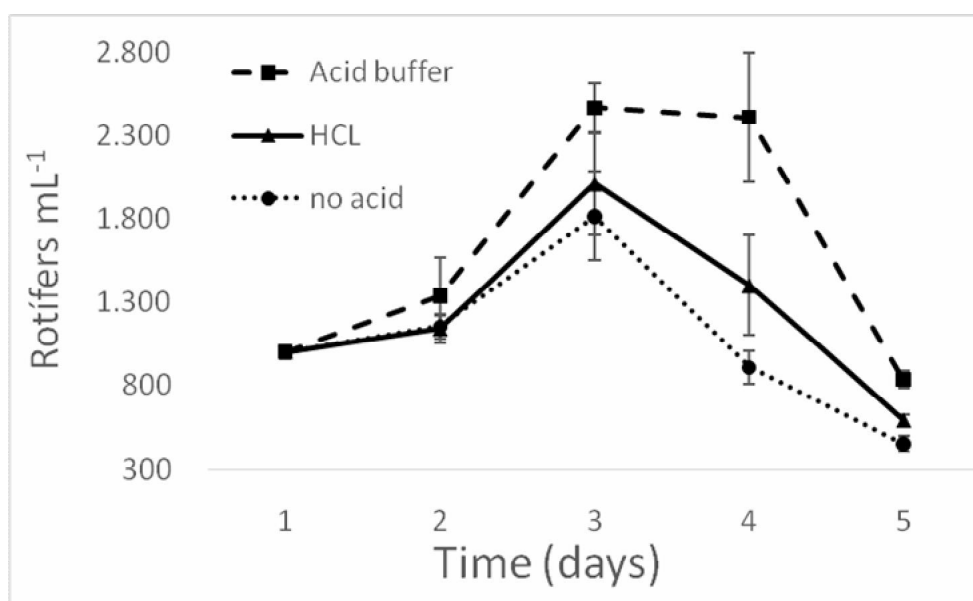


Figure 1. Effect of the addition of diluted hydrochloric acid (1%) and Acid Buffer™ (5%) on population growth of the rotifer *B. calyciflorus* in culture.

The data demonstrated that addition of dilute acids to the culture media sustained significantly higher number of rotifers than raising them without acid ( $p < 0.05$ ) (Figure 1). This was most apparent after rotifer densities had reached peaked values and their populations were crashing (Figure 1). The highest and significantly higher numbers of rotifers were obtained and sustained for three days when the Acid Buffer™ was added to the culture media, with or without the floc traps (Figures 1 and 2). The addition of dilute HCl also resulted in significantly higher numbers of rotifers ( $p < 0.05$ ) ( $2,008 \pm 264$  rotifers  $\text{mL}^{-1}$ ), which persisted for an extra two days (four and fifth days) when compared with no acid addition ( $907 \pm 100$  rotifers  $\text{mL}^{-1}$ ) to the culture media (Figure 1). However, the number of rotifers obtained with just the addition of dilute HCl ( $1,400 \pm 264$  rotifers  $\text{mL}^{-1}$ ) were significantly lower than those obtained with the Acid Buffer™ ( $p < 0.05$ ) ( $2,408 \pm 343$  rotifers  $\text{mL}^{-1}$ ), on the fourth and fifth day of culture (Figure 1).

The addition of a catch element or floc trap to the culture system resulted also in significant increases ( $p < 0.05$ ) in rotifer population densities compared to one without (Figure 2). By the third day in culture, the rotifer populations had significantly increased to  $1,337 \pm 105$  rotifers  $\text{mL}^{-1}$  from a density starting at an average of  $446 \pm 6$  rotifers  $\text{mL}^{-1}$  ( $p < 0.05$ ) (Figure 2). The most significant observation was that while the densities of rotifers in the culture system without the floc trap had peaked and started to decline on the third day ( $1,088 \pm 54$  rotifers  $\text{mL}^{-1}$ ), the densities of rotifers in the system with floc traps kept rising significantly reaching the highest densities of  $2,679 \pm 64$  rotifers  $\text{mL}^{-1}$ , on the fifth day in culture ( $p < 0.05$ ) (Figure 2).

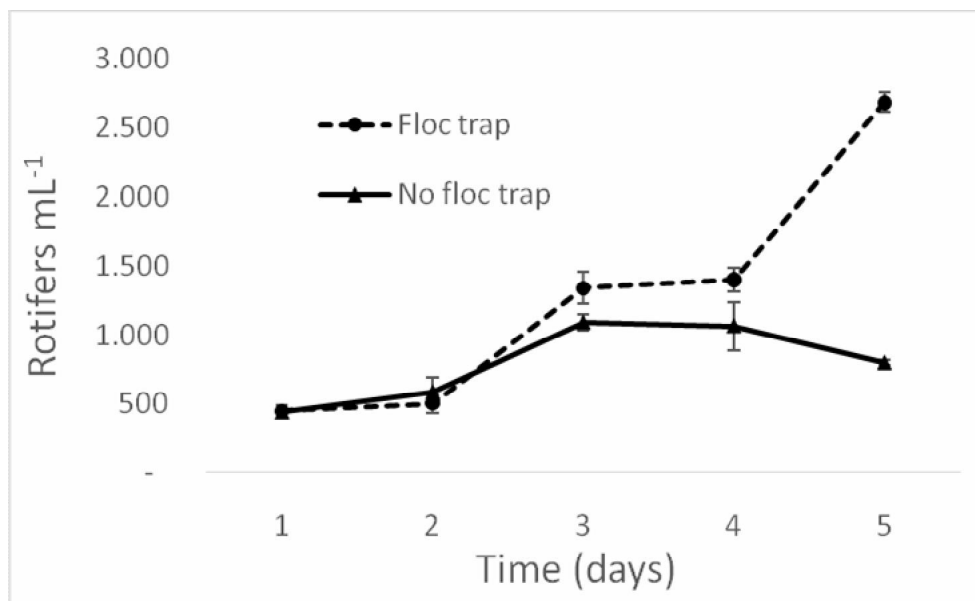


Figure 2. Effect of the use of a floc trap on the population growth of the rotifer *B. calyciflorus* in culture.

Water quality deterioration, especially from buildup of ammonia (Yoshimura et al 2003), and contamination from unwanted organisms, predominantly bacteria, yeast, and ciliates (Lawrence et al 2012; Lubzens et al 2001) are generally believed to be the major causes for rotifer die-offs. In this study, the addition of dilute acids to the culture media clearly had an effect in controlling or keeping at bay the amount of un-ionized ammonia in solution (Table 1). Perhaps also indicating the percent of un-ionized ammonia in solution is directly proportional to pH and may considerably affect the stability of the rotifer culture (Park et al 2001).

Table 1  
Daily concentrations of dissolved ammonia ions in the culture media of the rotifer *B. calyciflorus*

Day	Experiment 1			Experiment 2	
	Acid buffer (mg L <sup>-1</sup> ; NH <sub>3</sub> -N)	HCL (mg L <sup>-1</sup> ; NH <sub>3</sub> -N)	Control (mg L <sup>-1</sup> ; NH <sub>3</sub> -N)	With floc trap (mg L <sup>-1</sup> ; NH <sub>3</sub> -N)	Without floc trap (mg L <sup>-1</sup> ; NH <sub>3</sub> -N)
1	0.18-0.54	0.18-0.54	1.64	0.078-0.24	0.08-0.23
2	0.41-1.27	0.37-1.16	3.53	0.17-0.51	0.18-0.55
3	0.84-2.61	0.70-2.25	5.62	0.4-1.24	0.37-1.15
4	1.26-3.92	0.97-3.01	6.66	0.64-2.0	0.56-1.72
5	1.4-4.3	1.1-3.3	7.18	1.11-3.50	0.7-2.16

Experiment 1: with the addition of dilute acids. Experiment 2: with and without a floc trap. Control: ammonia concentrations without acid addition. The ammonia concentration ranges refer to the maximum pH values, 7.0 and 7.5, respectively, that were reached daily.

Unwanted debris and perhaps organisms other than rotifers also accumulated in the culture media (Figure 3). Such biological floc material may have caused water quality deterioration and significantly inhibited density of the rotifers, as deduced from the data in Figures 2 and 3.

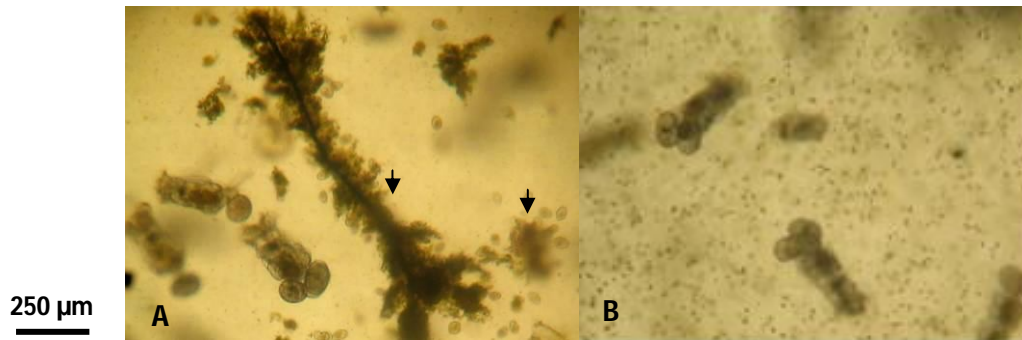


Figure 3 (A & B). Accumulation of unwanted debris and perhaps other organisms in the culture media of the rotifers *B. calyciflorus* (A), and its relatively clean appearance after the use of scrub pads or floc traps (B). Portions of the cultures were viewed under a conventional bright field light microscope at 10x magnification. Arrows indicate presence of floc.

Bacteria, protozoans, and fungi have been identified as the most common agents that contaminate rotifer cultures (Lawrence et al 2012, 2016; Lubzens et al 2001). Therefore, the use of floc traps can be a simple solution to increase rotifer densities, as demonstrated in this study. Based on this study, controlling the pH of the culture media combined with the use of floc traps can greatly increase rotifer population densities above those reported in the previous literature (Table 2).

Table 2  
Comparisons of population densities in batch culture of the rotifer *Brachionus calyciflorus* achieved by different investigators

<i>Rotifers mL<sup>-1</sup></i> (initial)	<i>Rotifers mL<sup>-1</sup></i> (final)	<i>pH regulation</i>	<i>References</i>
200	685±35	Without	Kabir et al (2010)
10-20	50-100	Without	Lim & Wong (1997)
8	73±9	Without	Khatun et al (2014)
0.66	163±19	Without	Xiang et al (2017)
20	213±9	Without	Odo et al (2015)
8	269±40	Without	Özdemir & Çiltaş (2010)
1,500	33,500	Automatic	Park et al (2001)
450	2,679±64	Adjusted (3x per day)	This study

**Conclusions.** *B. calyciflorus* can be raised in batches and maintained under laboratory conditions using the microalgae *Scenedesmus* sp. as the primary food source. Rotifer densities can be increased by controlling pH of the culture media and using floc traps to limit unwanted debris and other organisms.

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