

## The performance of a small-scale, high-density, continuous system for culturing the rotifer *Brachionus plicatilis*

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Received: 12.01.2009

**Abstract:** The performance of a high-density continuous system was evaluated as an alternative to conventional low-density batch-culture systems for culturing the rotifer *Brachionus plicatilis*. Rotifer densities ranging from 1700 to 3760 individuals/mL were achieved in 4 different trials that lasted 10-18 days. Problems associated with  $\text{NH}_3\text{-N}$  and pH were controlled by increasing the water exchange rate and periodic addition of ammonia remover. While  $\text{NH}_3\text{-N}$  and pH values were 0-8.8 mg/L and 6.9-9.3, respectively, the most important problem was the accumulation of TSS at concentrations of up to 85 mL/L within a very short time after start-up. Further studies are required to develop simple and cost-effective filters that reduce TSS concentrations. Use of continuous systems in combination with concentrated algae is potentially an effective alternative to existing batch-culture systems.

**Key words:** Rotifer, *Brachionus plicatilis*, high-density culture, continuous systems, concentrated algae

### Rotifer, *Brachionus plicatilis*, üretiminde küçük-ölçekli, yüksek-yoğunluklu, sürekli sistem performansı

**Özet:** Bu çalışmada rotifer *Brachionus plicatilis*, üretiminde kullanılan geleneksel, düşük yoğunlukta kesikli-kültür sistemlerine bir alternatif olarak, yüksek yoğunluklu, sürekli-kültür sisteminin performansı değerlendirilmiştir. On ile onsekiz gün arasında değişen 4 farklı denemede 1700-3760 rotifer/mL yoğunluğa ulaşılmıştır. Amonyak ve pH'a bağlı sorunlar, yüksek oranlarda su değişimi ve periyodik amonyak giderici kullanılarak kontrol altında tutulmuştur.  $\text{NH}_3\text{-N}$  ve pH değerleri, sırasıyla 0-8,8 mg/L ve 6,9-9,3 değişirken, en önemli sorun 85 mL/L seviyesine kadar ulaşan toplam çökebilir katı maddelerin birikimi olmuştur. Katı madde kontrolünü etkin bir biçimde azaltacak basit ve düşük maliyetli filtrelerin geliştirilmesi için çalışmalar gereklidir. Konsantre alg ile sürekli sistemlerin birlikte kullanımı mevcut kesikli-kültür sistemlerine göre etkin bir alternatif olabilir.

**Anahtar sözcükler:** Rotifer, *Brachionus plicatilis*, yüksek-yoğunluklu kültür, sürekli sistemler, konsantre alg

### Introduction

A major bottleneck in the economical production of marine finfish is inadequate nutrition during early growth stages. Despite improvements in

microparticulate diet technology, feeding larval fish with live food organisms, such as the rotifer *Brachionus plicatilis*, is still considered the best method for delivering essential nutrients to early

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larvae and results in higher growth and survival rates. As a result, rotifer culture technology improved significantly during the last 20 years and subsequent improvement in production technology receives much attention due to the ongoing problems in the mass production of rotifers. Today, rotifers are commonly cultured in batch systems, using baker's yeast or other commercial yeast-based products. However, rotifer densities in batch systems are inherently low due to poor water quality and rarely exceed 500-1000 individuals/mL (1,2). Sudden density crashes occur frequently and are a major obstacle to predictability, dictating the use of backup systems, which in turn increase overall production costs (3). In addition, yeast-fed rotifers lack the essential nutrients required by marine fish larvae and require enrichment before being used for feeding (4).

Alternative approaches to batch culture systems, such as continuous production of rotifers using recirculating systems, are reported to produce up to  $3-7 \times 10^4$  rotifers/mL (3). In recent years, attempts at developing ultra-high-density systems, with production rates ranging between  $2 \times 10^4$  and  $1.6 \times 10^5$  rotifers/mL, have also been reported (4-6). Although these studies show that high-density rotifer cultivation can be accomplished, critical aspects of production, such as accumulation of very high levels of solids and unionized ammonia ( $\text{NH}_3\text{-N}$ ), proliferation of pathogenic bacteria, excessive foam formation, and the complicated nature of the systems, remain major problems that need to be solved. Furthermore, in many studies in-depth technical details that would help other researchers to reproduce the results in order to obtain comparable data on the performance of different culture systems are not provided.

The aim of the present study was to test the performance of a small-scale, continuous high-density rotifer culture system. Performance parameters included rotifer density and the egg rate as indicators of population growth, and changes in  $\text{NH}_3\text{-N}$ , pH, and total settleable solids (TSS) as major indicators of water quality. Continuous high-density rotifer systems have been described (5,7) and are considered to have advantages over batch systems, with regard to maintaining water quality parameters. However, changes in physico-chemical conditions

significantly affect production and interactions between parameters further complicate predicting production rates. Therefore, determination of the relationships between the major parameters that affect the dynamics of rotifer production represents a major step towards optimizing the production of rotifers and the development of commercial-scale systems.

## Materials and methods

### Experimental setup

In total, 4 experiments were undertaken at 2 different commercial hatcheries. *Brachionus plicatilis* stock that was previously batch-cultured using a mixture of baker's yeast and algae was obtained from Akuatek Su Ürünleri A.Ş. (Şakran, İzmir, Turkey). The general setup of the experiments is depicted in Figure 1.

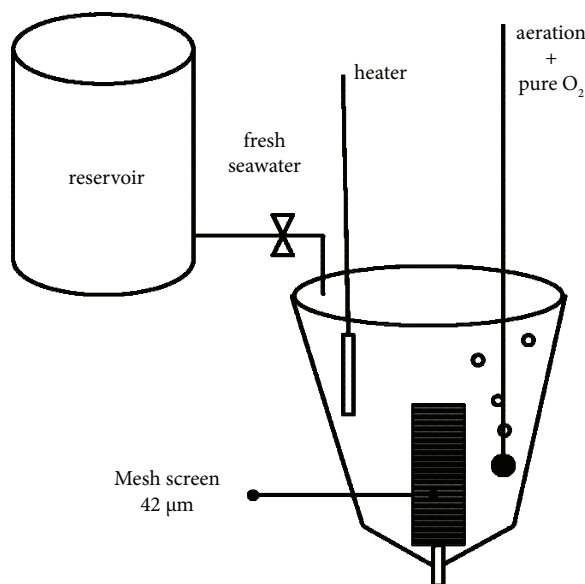


Figure 1. The general configuration of rotifer culture system used in the experiments.

### General configuration

In all the experiments water temperature was maintained at  $25 \pm 2$  °C using submersed heaters, except in experiments 2 and 4 in which the ambient temperature increased to 29 °C during summer. Salinity was ambient and ranged between 35 and 36 ppt in experiments 2 and 4, and between 26 and 27

ppt in experiments 1 and 3. Pure oxygen was used in each experiment in order to maintain adequate dissolved oxygen concentrations under high rotifer density conditions. Each tank was equipped with a central nylon screen to retain rotifers in the tank and facilitate water changing. Additional aeration was provided through an aeration collar located on the bottom of the screen in order to maintain homogeneous distribution of rotifers and algae in the water column, and to delay screen clogging. pH values were maintained using an NaOH solution and did not drop below 7.0. In order to reduce ammonia concentrations an ammonia remover was used (ChlorAm-X® [sodium hydroxymethanesulfonate], AquaScience Research Group, Inc., MO, USA) in all the experiments. Total settleable solids (TSS) concentrations did not exceed 50-60 mL/L, because our preliminary observations indicated that higher TSS concentrations interfered with routine maintenance of the system, feeding rotifers, and water quality. Therefore, once TSS concentrations exceeded this level, the system was given a complete cleanup. The total water volume in each experiment was adjusted to 150 L, except in experiment 4, in which the total volume was 100 L.

The general experimental setup was modified for each experiment, in terms of initial stocking density, water exchange rate, and duration of experiment, as summarized in Table 1. All the experiments were terminated when a predetermined amount of concentrated algae (10 L) was consumed by the rotifers. Additionally, in order to reduce the TSS concentration in experiment 3, a pair of nylon detritus filters was placed in the rotifer tank. In experiment 4, once rotifer density exceeded 2500 individuals/mL, rotifers in excess of the predetermined density were harvested.

#### Water quality

Temperature, DO, and pH were measured daily in all experiments. NH<sub>3</sub>-N concentrations were measured daily in experiments 1 and 3, and periodically in experiments 2 and 4. Due to technical failures, NH<sub>3</sub>-N and TSS concentrations could not be measured in experiment 4. NH<sub>3</sub>-N measurements were performed with water samples (20-100 mL) that were filtered through a GF/F filter (0.45 µm) and NH<sub>3</sub>-N concentrations were measured using a Hach DR 890 colorimeter (Hach Co., CO, USA).

TSS concentrations were measured volumetrically using an Imhoff cone (8). Briefly, a 1-L water sample from the rotifer tank was settled for 45 min, followed by gentle stirring of the sides of the cone with a glass rod. The sample was then allowed to settle for another 15 min and the volume of settleable solids was recorded as milliliters per liter.

#### Rotifer feeding

In all the experiments concentrated *Nannochloropsis oculata* (Reed Mariculture, USA) was used as rotifer food. One milliliter of the concentrated algae contains  $6.8 \times 10^{10}$  cells/mL. The rotifer feeding rate in each experiment was 1.5 mL per 1 million rotifers, as recommended by the manufacturer. In each experiment the daily ration was administered manually at 1-h intervals over the course of 12 h.

#### Rotifer growth

Rotifer densities were determined based on the mean of duplicate samples (1 mL) taken from the culture unit. A third sample was taken if there was >10% difference between the first 2 samples. Rotifers were fixed using Lugol's solution, and then were counted under a light microscope. The number of

Table 1. Differences in experimental conditions during trials of high-density rotifer culture.

Experiment no.	Tank shape	Tank volume	Initial rotifer density	Water exchange rate	Duration of experiment
1	Cylindro-conical	150	334	100%	18
2	Cylindro-conical	150	570	250%-300%	16
3	Cylindrical	150	622	350%-400%	10
4	Cylindrical	100	810	500%	13

females carrying eggs was also determined and reported as the egg rate. The specific growth rate (SGR) was calculated using the equation given by Øie et al. (9):

$$\text{specific growth rate} = (\ln N_t - \ln N_0)/t$$

where  $N_0$  is the initial rotifer concentration,  $N_t$  is the rotifer concentration at time  $t$ , and  $t$  is the culture period (days).

## Results

### Experiment 1

The temperature was stable and ranged between 24.8 and 25.7 °C (Table 2). However, pH and the  $O_2$  concentration fluctuated greatly, varying between 6.3 and 9.3, and between 0 and 20.5 mg/L, respectively (Table 2). The extremely low  $O_2$  concentration was a short-term (<1 h) problem due to mechanical failure of the pure oxygen delivery system on day 14.

Figure 2a shows the changes in rotifer density and the egg rate during the experimental period. Maximum rotifer density was 927 individuals/mL on day 14. The maximum egg rate was 52% (mean: 33%). On the first day of the experiment, when the egg rate was 2.7%, rotifer density dropped from 334 individuals/mL to 44 individuals/mL. The following day, the increased egg rate (50%) accompanied an increase in rotifer density. Similar results for rotifer density and the egg rate were observed on days 5-10 and days 13-15. The maximum specific growth rate was 0.30 (mean: 0.05) during the experimental period.

$NH_3$ -N and TSS concentrations increased as rotifer density increased (Figure 2b). Initially, the  $NH_3$ -N concentration gradually increased, reaching

4.9 mg/L on day 13. Similarly, the TSS concentration increased steadily, reaching 73 mL/L on day 12. Following a general cleanup on day 13, both  $NH_3$ -N and TSS concentrations decreased significantly. However, due to increased feeding rates,  $NH_3$ -N and TSS concentrations increased drastically during the 3 days following the general cleanup and reached 8.8 mg/L and 85 mL/L, respectively.

### Experiment 2

Maximum rotifer density was 1700 individuals/mL on day 15 (Figure 3). The maximum egg rate was 80% on day 11 (mean: 36%). Rotifer density increased considerably and reached 1000 and 1400 individuals/mL on day 5 and 8, respectively. Significant correlations between rotifer density and the egg rate were not observed during the experimental period, and rotifer density continued to increase irrespective of changes in the egg rate. The detritus filter was not very effective in removing suspended material; therefore, on day 6 the rotifer tank was cleaned thoroughly due to excessive TSS accumulation and clogging of the mesh screen. On day 9 the detritus filter was removed from the culture tank because a significant number of rotifers were observed between the fibers of the filter mat during rinsing. The rotifer tank was cleaned again on day 13 due to the accumulation of TSS. Following a drop in density on day 9, the rotifer population increased gradually until the end of experiment on day 16, reaching a density of 1500 rotifers/mL. The maximum specific growth rate was 0.18 (mean: 0.06) during the experimental period. Temperature ranged between 26 and 29 °C, the oxygen concentration fluctuated between 4.7 and 15.0 mg/L, and pH ranged from 7.2 to 8.1 (Table 2).

Table 2. Minimum-maximum concentrations of physico-chemical parameters during 4 trials. T: temperature (°C), DO: dissolved oxygen,  $NH_3$ -N: unionized ammonia, TSS: total settleable solids, ND: not determined.

Experiment no.	Physico-chemical parameters (min/max)				
	T (°C)	DO (mg/L)	$NH_3$ -N (mg/L)	pH	TSS (mL/L)
1	24.8-25.7	0-20.5	1.8-8.8	6.3-9.3	5-85
2	26.0-29.0	4.7-15.0	0.0-2.0	7.2-8.1	3-80
3	25.0-26.5	4.0-21.6	0.6-6.4	7.0-7.9	4-48
4	25.5-29.0	5.8-14.0	ND	7.2-7.9	ND

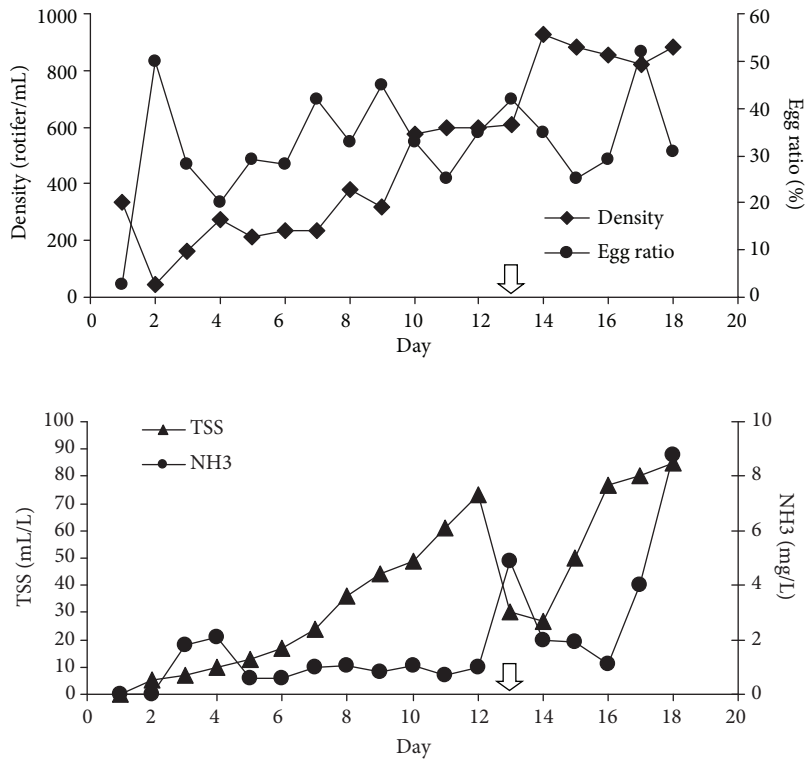


Figure 2. a. Daily changes in rotifer density and egg ratio during experiment 1. Arrow indicates system cleanup.  
 b. Daily changes in NH<sub>3</sub>-N and TSS concentrations during experiment 1. Arrow indicates system cleanup.

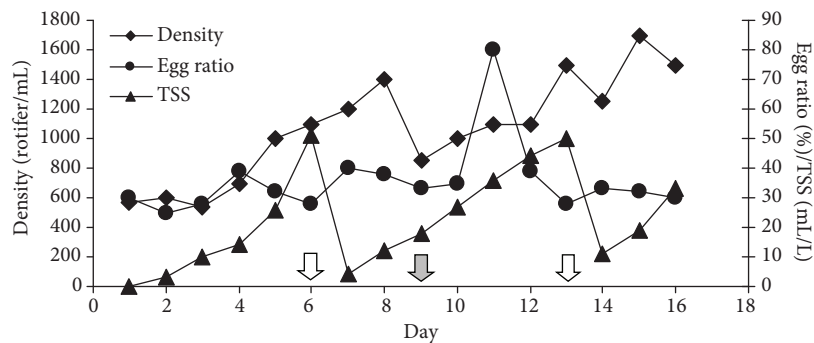


Figure 3. Daily changes in rotifer density and egg ratio during experiment 2. White arrows indicate system cleanup, gray arrow indicates removal of detritus filter.

### Experiment 3

The population increase was significant, with 1193, 1560, and 2400 individuals/mL on days 3, 5, and 8, respectively. Rotifer density reached its maximum of 2750 individuals/mL on day 10 (Figure 4a). The mean egg rate was 33% during the experimental period, reaching a maximum of 47% on day 2.

Between days 4 and 7 rotifer density increased along with the egg rate, but overall a significant relationship between population density and the egg rate was not observed. The maximum specific growth rate was 0.27 (mean: 0.15) during the experimental period.

The concentration of ammonia remover was kept constant (200 g/day). Nevertheless, the NH<sub>3</sub>-N

concentration increased steadily as the population density increased, reaching 3.2 mg/L on day 5, 4.2 mg/L on day 8, and 6.4 mg/L on day 10 (Figure 4b).

TSS accumulation was rapid and the rotifer tank was cleaned only 6 days after start up. Following the cleaning, however, the rate of TSS accumulation increased due to an increase in the feeding rate in order to maintain the increasing rotifer density. Temperature ranged between 25.0 and 26.5 °C, the O<sub>2</sub> concentration fluctuated between 4.0 and 21.6 mg/L, and pH values were 7.0-7.9 throughout the experimental period (Table 2).

#### Experiment 4

The rate of increase in rotifer density was faster than in the other experiments, reaching 1230, 3000, and a maximum of 3760 individuals/mL on day 3, 6, and 8, respectively. On day 2 the egg rate was <10%, but increased to 36% the next day. The maximum egg rate was 50% on day 5 (mean: 36% throughout the experimental period). There was a significant relationship observed between the population density and the egg rate on days 2-5. Rotifer density increased every day until day 9, despite harvesting and a

fluctuating egg rate (Figure 5). The rotifers were harvested on days 6, 8, 9, 12, and 13 once the population density exceeded 2500 individual/mL. Rotifer density recovered 1-2 days following harvest. The maximum SGR was 0.22 (mean: 0.09). The experiment was terminated on day 13.

Temperature ranged between 25.5 and 29.0 °C, with daily fluctuations of up to 2.5 °C (Table 2). The O<sub>2</sub> concentration ranged between 5.8 and 14 mg/L and pH values ranged between 7.2 and 7.9 (Table 2). The low fluctuation in pH may have been due to the high rate of water exchange.

#### Discussion

In the present study flow-through, continuous culturing of the rotifer *Brachionus plicatilis*, using concentrated algae and water exchange rates of up to 500%, was performed in a small-scale system that resulted in a maximum density of 3760 individuals/mL. In addition, during 57 days (total days for experiments 1-4) the rotifer population did not crash. In contrast, unpredictability and frequent crashes of rotifer populations fed baker's yeast or other

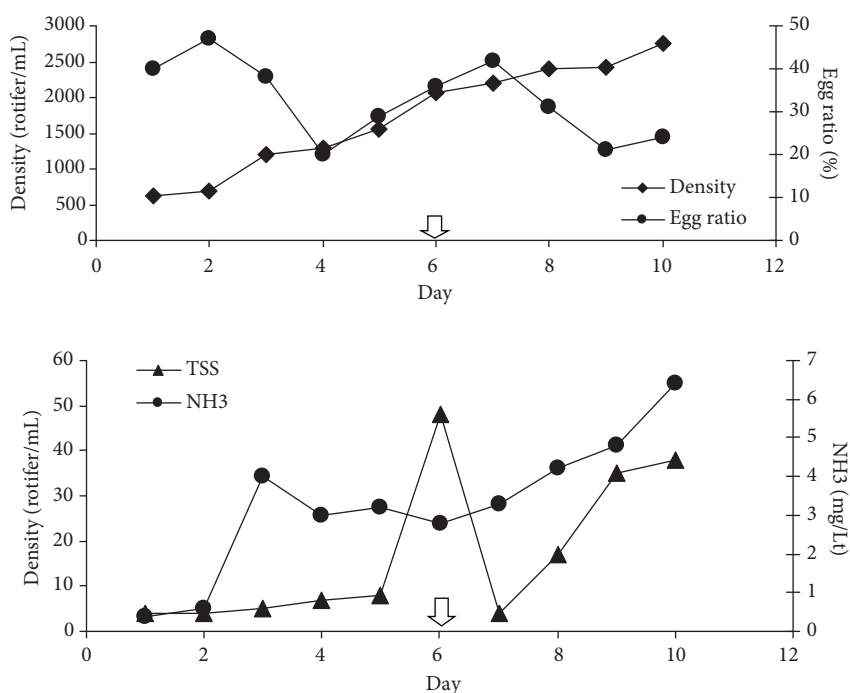


Figure 4. a. Daily changes in rotifer density and egg ratio during experiment 3. Arrow indicates system cleanup.  
 b. Daily changes in NH<sub>3</sub>-N and TSS concentrations during experiment 3. Arrow indicates system cleanup.

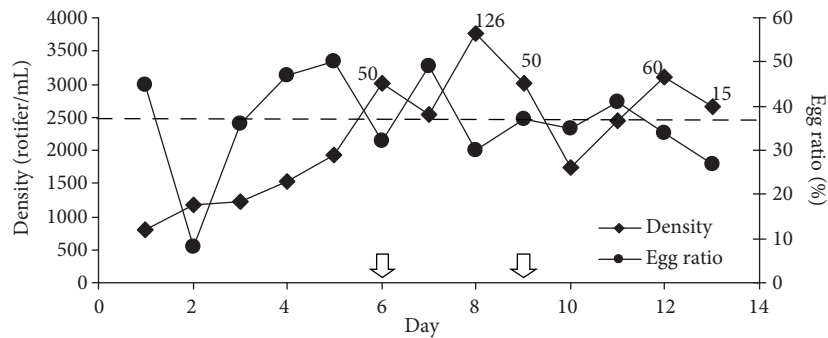


Figure 5. Daily changes in rotifer density and egg ratio during experiment 4. Numbers indicate amounts of rotifers ( $\times 10^6$ ) harvested. Arrows indicate system cleanup.

commercial yeast-based products are commonly reported (3,4). Using a continuous flow-through system in combination with an ammonia remover, pH stabilizers, and pure oxygen stabilized the physico-chemical parameters, and periodic harvesting of rotifers was possible. Our findings support those of others that reported high-density rotifer cultivation using concentrated algae may help solve the major problems that commonly occur in traditional batch-culture systems using yeast-based diets and other commercial products (3).

Periodic increases and decreases in rotifer density were, in part, associated with the daily egg rate. Although the maximum egg rate reached 80%, mean values were considerably lower and ranged from 33% to 36%. However, higher rates are desirable and our findings indicate that rotifer density always increased irrespective of the other parameters as long as the egg rate was  $\geq 40\%$ . The egg rate is, therefore, a good indication of the general health and status of rotifer populations, and may help to predict culture density and calculate feeding rates the following day.

In the present study concentrated *Nannochloropsis oculata* was used successfully and increased rotifer density in a very short time, especially during start up when rotifers were in poor condition, as indicated by egg rates  $< 10\%$  in experiments 1 and 4. This also indicates that initial culture conditions, such as the rotifer feeding rate and physico-chemical parameters, were optimal and suffered little or no mechanical damage via aeration. However, despite adequate feeding, as indicated by the presence of algae in the digestive system of the rotifers during microscopic

evaluation, we were unable to consistently maintain an egg rate  $\geq 40\%$  for successive days, which in turn resulted in lower specific growth rates and fluctuations in population density. Therefore, because other factors, including water quality, also affect rotifer density, predictions based on the egg rate alone may not be realistic and should be considered only when all the other parameters, such as  $\text{NH}_3\text{-N}$ , TSS, DO, and pH, are within acceptable ranges. Similarly, Bentley et al. (10) reported that declines in rotifer density in a recirculating, continuous system were not associated with changes in water quality parameters of an unknown cause.

In high-density rotifer cultures, the maintenance of physico-chemical conditions at optimum levels is difficult. In the present study, despite daily water exchange rates of up to 500% of the total culture volume, the accumulation of TSS was a major problem that limited rotifer growth and increased labor. TSS concentrations had to be reduced every 4-10 days after start up by increasing flow rates into the culture tank and rinsing the rotifers thoroughly. In order to prevent detritus build up, periodic cleaning and restocking were reported to be effective measures in a continuous rotifer culture system (11). Similarly, accumulation of organic material was reported to be a major factor that increases the  $\text{NH}_3\text{-N}$  concentration and interferes with rotifer feeding (12,13). Therefore, researchers have removed TSS using novel filters, though without providing the technical details (3,6,13). In the present study, in addition to periodic rinsing and cleaning, the potential of filtration mats to control the accumulation of TSS was also tested in

experiment 2. The use of filtration mats was previously described for high-density cultures and reported to be effective, removing up to 80% of solid substances and contaminating protozoans such as *Vorticella* (10,12). However, our findings indicate that these passive filters, although effective at removing organic material, also removed a significant amount of rotifers from the water column. Therefore, it is of considerable importance to develop simple, cost effective, and practical filtration methods that remove TSS with minimum effect on rotifer density.

Significant concentrations of  $\text{NH}_3\text{-N}$  have been reported in rotifer cultures once densities reach  $10^3$ - $10^4$  individuals (12). In the present study the use of sodium hydroxymethanesulfonate (ClorAm-X<sup>®</sup>) for controlling toxic ammonia levels resulted in lower concentrations. As the rotifer population increased,  $\text{NH}_3\text{-N}$  levels increased to a maximum of 8.8 mg/L, but increasing the amount of ammonia remover and the water exchange rate (up to 500%) prevented higher concentrations and possible negative effects on population growth. Sodium hydroxymethanesulfonate neutralizes  $\text{NH}_3\text{-N}$ , creating a non-toxic molecule; its potential has been evaluated for use in shipping fish, crustaceans, and mollusks, as well as in rotifer culturing (10,11). In batch-cultures  $\text{NH}_3\text{-N}$  concentrations were reported to be the major limiting factor when reaching 10 mg/L, which necessitated rinsing and restocking (2). The toxic  $\text{LC}_{50}$  concentration of unionized ammonia for marine *Brachionus* was reported to be 17.0 mg/L at 23 °C during a period of 24 h, which resulted in 50% lower growth and fecundity, as compared to treatments that had 7.8 and 13.2 mg/L of  $\text{NH}_3\text{-N}$  (14). In addition, up to  $22\text{-}34 \times 10^3$  rotifers/mL were cultured at total ammonia nitrogen (TAN) levels of 1000 mg/mL at pH 7.0 (12). However, in contrast to the  $\text{NH}_3\text{-N}$  concentrations reported in the present study, recently reported values for  $\text{NH}_3\text{-N}$  ranged between 0.06 and 0.1 mg/L in a high-density rotifer system in which sodium hydroxymethanesulfonate (ClorAm-X<sup>®</sup>) was used to control TAN concentrations (10). Despite higher concentrations of ammonia remover in the present study, the differences between the 2 studies are significant; therefore, further research is required to determine the optimal dose of ammonia remover and to determine its effects on  $\text{NH}_3\text{-N}$  concentrations.

Increased rates of water exchange had positive effects on water quality, with results similar to those reported in other studies. For example, a 1.5-fold increase in rotifer production was reported when the water exchange rate increased from 100% to 300% in a recirculating system (2,7). Rotifer densities up to 3000-6500 individuals/mL in recirculating systems with water exchange rates ranging between 28% and 70% of total culture volume per day have also been reported (7,10). The continuous replenishment of culture water with fresh seawater reduces the accumulation of toxic substances and stabilizes the culture medium (7). However, increased rates of water flow into rotifer culture tanks may also reduce the algae concentration due to dilution. For example, Bentley et al. (10) reported that feed loss occurred due to foam fractionation in a recirculating rotifer culture system. On the other hand, feed loss can be minimized by adjusting the feeding rate and water flow into the culture tank. Our observations indicate that when rotifer density reached 1500-2000 individuals/mL, the rotifers consumed concentrated algae within 10-15 min, as indicated by the color of the culture water. Hence, the rate of feed loss can be minimized by feeding rotifers multiple times a day, i.e. every hour, and by turning off the water flow into the rotifer tank during feeding. This may result in effective use of concentrated algae, as it was reported that feed cost was the most expensive component of a continuous system, which is 45% higher than that of a batch system (10). Therefore, there is a need to develop a cost-effective feeding protocol that will result in the economical production of rotifers in high-density systems.

It was reported that in finfish hatcheries 68%-89% of available space is designated for live feed production, such as rotifers and *Artemia*, and therefore, despite existing technology for the successful culture of marine finfish, further increases in production has been difficult due to limited space (12). Bentley et al. (10) reported that a 34% reduction in hatchery floor space is possible when a recirculating, continuous system is used, as compared to a batch-culture system. Our findings indicate that rotifer density can reach 3760 rotifers/mL and continuous harvesting of rotifers is possible at 1- or 2-day intervals. Although further research is required to control and fine-tune the changes in physico-



chemical parameters and scale-up of small-size experimental units to produce a consistent supply of rotifers, our results indicate that at a minimum 30% less space is required to culture the same amount of rotifers in comparison to conventional batch-culture systems, with rotifer density ranging from 500 to 1000 individuals/mL.

In conclusion, a high-density, continuous culture system for rotifers using concentrated algae is potentially an effective alternative to traditional, batch-culture systems. When used in combination with an ammonia remover, the continuous system cost-effectively provides a more stable and reliable environment, as compared to batch systems or recirculating systems that use biofilters or other expensive components, such as protein skimmers. In the present study, although an extensive cost analysis was not performed due to limited local availability of concentrated algae, the local production and supply of concentrated algae will, without doubt, further

decrease production costs. In addition, feeding rotifers with different species of concentrated algae presents another window of opportunity for further research and improvement, with the potential of eliminating the enrichment step using commercial enrichment diets. Such improvements in rotifer culture have the potential to increase the rate of success in marine finfish production.

### Acknowledgements

This research was funded by Çanakkale Onsekiz Mart University, Scientific Research Commission (BAP; grant no: 2005/72). We thank Reed Mariculture for supplying the concentrated microalgae. We also thank Güngör Muhtaroglu and his staff of Akuatek Su Ürünleri A.Ş. (Şakran, İzmir, Turkey), and İsmail Avcılar and Emel Dönmez Şentürk of İda Gıda Su Ürünleri A.Ş. (Lapseki, Çanakkale, Turkey) for their cooperation and technical assistance during the study.

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