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RAS based culture system for continuous production of Rotifers (*Brachionus calyciflorus*) in mass

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ABSTRACT

The present study was undertaken to find out preferred food type, minimum effective algal cell requirement, effective harvest rate of rotifers and to design and construct re-circulatory aquaculture system (RAS) based rotifer, *Brachionus calyciflorus* production system at Aquatic Rainbow Technology Park, Dr MGR Fisheries College and Research Institute, TNJFU, Madhavaram campus, Chennai during 2017–2019. In the first experiment, food types, viz. *Chlorella vulgaris, Spirulina major, Scenedismus obliquus* and Baker's yeast were extensively fed to rotifers with control (5000 L). The highest rotifer count was observed with *C. vulgaris* food type (365.33±2.18 rotifers/ml) followed by Baker's yeast (245.33±5.36 rotifers/ml) on 8th day which started declining gradually to 30th day because of lower water quality. Second experiment on algal consumption rate by rotifer postulated that 22,000–25,000 cells/day/rotifer was the minimum cell requirement. Third experiment on standardizing effective harvest rate (10%, 30%, 50% and 70%) suggested that daily harvest at 30% maintained the rotifer culture at its stationary phase (381±19.45 rotifers/ml). After the experiments, we have designed, built and operated RAS based live food production unit for 17 months where the harvested water was treated using series of filters (rapidsand, cartridge, UV filters) and reused for algal culture. The seasonal data showed that temperature plays a major role with the highest production during summer (404.43±24.33 rotifers/ml) and consequently lower in winter (301.21±14.33 rotifers/ml). The system opens a new perspective of commercial scale production of rotifers with standard culture and harvesting practices.

Keywords: Algal consumption, *Brachionus calyciflorus,* Harvest rate, RAS, Rotifers

In aquaculture, larvae culture is still a main bottleneck and needs live feed at the right time in adequate quantity (Felix 2013, Rojo-Cebreros *et al.* 2017). Rotifers, *Brachionus calyciflorus* (Phylum Rotifera) are small (≈200 µm) metazoan and have been widely used as an essential live food source in raising aquaculture due to its unique characteristics and apposite biochemical composition (Gilbert 2017). In addition, they have the habit of staying suspended in high density with high reproductive rate in the water column (Sananurak *et al.* 2009). Various mechanisms have been suggested to explain poor survival of rotifers viz. scarcity of favorite algal species in required quantity, energy loss, reduced swimming rate, low hatching rate, failure of osmoregulation due to biochemical changes in poor water quality, etc. (Oeie and Olsen 1993, Odo *et al.* 2015). Identification of appropriate food species and control of bio-chemical factors hinder the mass production of rotifers (Odo *et al.* 2015).

Studies on the effect of food type and water quality on rotifer production have been documented by various researchers (Arimoro 2006, Odo *et al.* 2015). More than 40 different species of micro-algae are cultured intensively for direct or indirect feeding of zooplankton (Ashraf *et al.* 2011). Most common cultured algal species are diatoms, *Skeletonema costatum*, *Thalassiosira psuedonanna, Chaetoceros gracilis, C. calcitrans*; the flagellates *Isochrysis galbana*, *Tetraselmis suecica*, *Monochrysis lutheri* and Chlorococcalean, *Chlorella*. Out of them, *Chlorella vulgaris* became an important source of food for rotifers due to their small spherical size (2–10 µm), asexual reproduction, nutritional value, non-motility and physical compatibility (Yamamoto *et al.* 2005).

In aquaculture, rotifers are commonly cultured using either baker's yeast or yeast-based products in batch systems where production rarely exceed 500–1,000 rotifers/ml which was subjected to sudden crashes due to poor water quality and thus needed backup systems (Suantika *et al.* 2000). Densities up to 2×10^4 – 1.6×10^5 rotifers/ml have been reported from high-density and ultra-high density systems where the optimum water quality was maintained by partial recirculation through the series of filters but unionized ammonia (NH_3^-N), foam formation and proliferation of pathogenic bacteria were the major problems in high-density cultures (Suantika *et al.* 2003). Successful culture of fish and shrimp in various parts of the world is depending on live food sources (Yuan *et al.* 2018) and accordingly demand is increasing progressively for rotifer, *B. calyciflorus,* in ornamental, food fish and shrimp hatcheries due to increased awareness among fish

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farmers. In general, not much attention has been given on its culture in freshwater and specifically in our local environment. Hence, we need this minuscule in abundance to make our fish hatcheries as a successful venture by developing reliable, sustainable and simple system to produce rotifers throughout the season in required quantum with zero water exchange.

MATERIALS AND METHODS

Experimental setup: Present study was conducted at Aquatic Rainbow Technology Park (ARTP), Dr. MGR FC and RI, TNJFU Madhavaram Campus, Chennai, India. Experiment was done following completely randomized design with three trials in 5000 L capacity tanks (4.5×1×1.2 m; l×b×h) attached to re-circulatory aquaculture system (RAS). Four feed types (*Chlorella vulgaris, Spirulina major, Scenedismus obliquus* and Baker's Yeast), algal consumption rate and daily harvest rates (at 10%, 30%, 50% and 70%) were observed for mass production and for maintaining culture at its stationary phase throughout the season, respectively.

Fresh water rotifer, *B. calyciflorus* (L-type) of 350–500 µ lorica length carrying eggs and algal species (*C. vulgaris, S. major, S. obliquus*) were procured from live feed unit, Advance Research Farm Facility (ARFF), TNJFU, Madhavaram campus. Culture of algal species was performed in 4,000 L capacity rectangular tanks (3.5×1×1.2 m; l×b×h) after fertilization with groundnut oil cake, urea and single super phosphate at 25%, 1%, 0.5% respectively, using an underwater light system (5,000 lux) and aeration facilities.

Rotifers were inoculated at a density of 10–12 rotifers/ml in tanks and extensively fed with algae using submersible pump. The water *p*H, temperature, hardness and total ammonia nitrogen level of culture were maintained at 7.2±*0*.8, 28±4°C, 250±40 mg/l and ≤0.5 mg/l, respectively.

Sampling of rotifers: Rotifer density in each tank was estimated by taking three random samples in the morning between 8:00 to 10:00 AM to minimize stress, on every 2nd day using micropipette (1.0 ml). Rotifers were observed in a Sedgewick Rafter Counting Chamber (S50, PYSER, SGI), after fixation with two drops of ethanol under a trinocular microscope (NLCD-120E Lawrence and Mayo). The rotifers with empty and transparent lorica were not counted.

Estimation of algal ingestion rate: The rotifers (200±1.12 rotifers) were housed in 500 ml fibreglass jars provided with varying quantities of algae in triplicates, while one was served as control without food for 24 h. Algal cell ingestion rate was estimated using Neubauer haemo cytometer (Bauer 1990). Ruled areas were centrally covered with cover slip and algal sample was placed in the "V" groove of the metal surface. Chambers were filled gradually for even distribution of algal cells without air bubbles. Counting was done from the top left square and cells were counted which lay within or on the boundary line of the square. All the cells counted in individual block were added up and cell density was calculated by the mathematical expression:

$$D \text{ (cells/ml)} = (\text{total count} \times 10^4/\text{blocks})$$

Ingestion rates on the basis of filtration rate of rotifer were calculated following Yufera and Pascual (1985) mathematical expression:

$$\text{Ingestion rate (IR)} = F \times \sqrt{C_0} \times C_t$$
$$\text{Filtration rate (F)} = (\text{Ln } C_0 - L_n C_t)/V \times t$$

where C_0 and C_t are initial and final cell densities; t, time; F, filtration rate and V, rotifer density.

Mass culture and harvesting protocol: The third experiment was conducted in 5,000 L capacity tanks to standardize harvesting rate for maintaining the rotifer culture at its stationary phase throughout the season. After rotifer inoculation (10 rotifers/ml), *C. vulgaris* was extensively used to feed rotifers at the rate of 2.3×10^5 cells/ml initially and was gradually increased to meet the food requirement. Before harvesting rotifer, count was observed at every 2nd day by taking three random samples from each tank using 1 ml micropipette in a Sedgewick Rafter Counting Chamber. Tanks were harvested daily at 10, 30, 50 and 70% of the total volume and checked quantum of harvest using mathematical expression:

$$\text{Total harvest} = \text{rotifers/ml} \times \text{quantity of water harvested (ml)}$$

Rotifer harvested tanks were refilled with same quantum of algal water while algal tanks were filled up by reutilization of rotifer harvested water after filtration. Daily observation for proper aeration, pathogenic contamination, sufficient light, water colour, smell, deposition of culture media and accumulation of algae on tank walls were monitored. Algal culture tanks were monitored periodically for water quality parameters, maximum cell count, dead algae and deficiency of required nutrients. To fulfill the nutrient requirements, algal tanks were fertilized regularly every two weeks for preventing death of algae.

RAS facility for continuous culture system: The RAS facility for continuous culture system consists of four components; algal culture, rotifer culture, filtrations facility and harvest units. Algal culture tanks were facilitated with under water aeration and light facilities. Commonly used aeration system was modified by making 1 mm holes in polyvinyl chloride (PVC) pipes for aeration purpose operated by 2 HP air blower (CLEANTEK) and under water light facility was developed by inserting green light emitting diodes (LED) of 5000 lux capacity in a waterproof transparent acrylic pipe of 1.27 cm diameter. PVC pipes for aeration were placed at the bottom of tanks while acrylic pipes with LED were placed horizontally in water column. Rotifer culture tanks were provided with under water aeration facility following same method with separate inlet and outlet valve without light. The filtration facility consists of one sand filter assembled with a layer of activated carbon and sand (500 µ), four wound type cartridge filters (10 µ) of 50.8 cm diameter arranged in series and one UV filter

with three lamps of 30 watts assembled in a stainless steel body (15.24 cm diameter) of 5 m^3/h capacity. Harvesting facility was provided with separate valves for each tank which opens in a harvesting pit. After collection of rotifers through plankton net (20 µ mesh), the water moves to 5 m^3 capacity sump constructed near culture tanks through underground PVC pipes by gravity. The water collected in sump was re-utilized for algal culture after filtration with the help of centrifugal pump (2 HP).

Statistical analysis: Initially experimental data was tested for normality of distribution using Shapiro-Wilk's W-statistic (PAST, version 16.0). One-way analysis of variance (ANOVA) was used to compare significant differences between treatments. After getting significant differences of triplicate mean (P<0.05), Duncan's multiple range test (Duncan D 1995) was used to rank the groups on SPSS software, version 20.0 (SPSS Inc., Michigan Avenue, Chicago, Illinois, USA). The results are presented as mean±SEM.

RESULTS AND DISCUSSION

The data obtained was kept for normality testing of food type showed normal distribution (P<0.05). Effect of food type on rotifer production, determination of algal (*C. vulgaris*) consumption rate of a rotifer and effect of daily harvest on rotifer population has been illustrated in Tables 1, 2 and 3.

Effect of food types on rotifer production: Overall effect of different food type on rotifer population was investigated and significant difference was observed (P<0.05). The highest population growth was observed in tanks fed with *C. vulgaris* (365.33±2.18 rotifers/ml) followed by Baker's

Table 1. Effect of food type on rotifer (*B. calyciflorus*) production (rotifers/ml)

Day	Control	*Spirulina major*	*Scenedismus obliquus*	*Chlorella vulgaris*	Baker's yeast
Day 0	10.00±2.1	10.00±1.5	10.00±1.4	10.00±2.1	10.00±1.1
Day 2	15.00±1.73^{a}	23.66±1.33^{a}	18.33±3.84^{a}	39.33±1.33^{b}	22.66±3.96^{a}
Day 4	27.00±8.88^{a}	54.66±7.96^{ab}	69.00±2.64^{ab}	131.33±6.35^{c}	90.66±5.66^{bc}
Day 6	12.33±2.72^{a}	75.33±3.13^{ab}	83.00±1.52^{b}	167.33±7.33^{c}	228.33±6.83^{c}
Day 8	4.33±0.88^{a}	86.00±10.21^{ab}	212.67±10.80^{c}	365.33±2.18^{d}	245.33±5.36^{c}
Day 10	1.00±0.57^{a}	92.33±4.09^{ab}	204.00±2.00^{c}	351.67±9.27^{d}	152.00±9.03^{bc}
Day 12	00^{a}	71.67±14.52^{b}	141.33±7.21^{bc}	346.67±2.84^{d}	104.00±2.00^{b}
Day 14	00^{a}	58.00±3.64^{a}	155.00±10.00^{bc}	362.67±8.48^{d}	141.33±7.21^{c}
Day 16	00^{a}	62.24±8.30^{a}	141.23±6.35^{bc}	241.61±9.31^{cd}	155.23±8.35^{bc}
Day 18	00^{a}	60.29±7.98^{a}	92.24±6.52^{b}	239.71±8.96^{cd}	141.32±9.68^{bc}
Day 20	00^{a}	55.33±6.93^{a}	104.11±6.48^{ab}	237.11±8.54^{cd}	92.24±5.36^{b}
Day 22	00^{a}	57.34±4.56^{a}	113.24±7.21^{ab}	228.61±7.96^{cd}	127.86±5.66^{c}
Day 24	00^{a}	82.74±6.33^{ab}	115.31±3.61^{ab}	218.94±7.33^{c}	131.74±7.71^{bc}
Day 26	00^{a}	90.56±9.20^{ab}	79.78±5.15^{b}	203.31±9.27^{c}	117.96±7.49^{bc}
Day 28	00^{a}	69.26±4.78^{a}	109.79±4.33^{b}	211.66±2.64^{c}	136.11±8.02^{bc}
Day 30	00^{a}	57.44±6.28^{a}	139.88±3.32^{ab}	229.74±6.32^{cd}	144.23±8.41^{bc}

Table 2. Estimation of algal (*C. vulgaris*) consumption rate by rotifers in jar (500 ml)

Treatment	Rotifers/ Jar	Algal cells/Jar (×1000)	Algal cells/ml (×1000)	Algal cells/ Rotifer (×1000)	Algal cells recovered after 24 h/Jar (×1000)	Algal cells consumed/ Rotifer (×1000)	Rotifers recovered/ Jar	Rotifers/ml
Control	200±5.4	0	0	0	0	0	7.33±17.38^{a}	0.015±0.04^{a}
T_1	200±4.7	60524±47.89	121.05±0.95	202.62±2.39	21036.70±88.22^{a}	25.1±9.41^{a}	544.63±44.03^{b}	1.08±0.09^{b}
T_2	200±4.2	39892±63.98	79.60±1.27	199.01±3.20	2416.70±65.01^{a}	22.08±0.33^{ab}	541.33±21.18^{b}	1.08±0.04^{b}
T_3	200±3.1	20803±14.33	41.61±2.87	104.01±7.17	981.10±75.97^{b}	14.91±0.38^{b}	240.61±11.60^{c}	0.48±0.02^{c}
T_4	200±3.2	10987±73.61	21.97±1.47	54.93±3.68	375.63±10.43^{c}	7.88±0.90^{c}	43.33±14.49^{a}	0.08±0.03^{a}

Table 3. Effect of daily harvest on rotifer population in RAS system

Treatment	Rotifer/ml	Harvested Rotifers × 1E+05	Rotifers/ml after 2 days	Harvested Rotifers × 1E+05	Rotifers/ml after 4 days	Harvested Rotifers × 1E+05	Rotifers/ml after 6 days	Harvested Rotifers × 1E+05	Rotifers/ml after 8 days
Control	234±12.30	0	263±14.78	0	198±9.65	0	162±29.00	0	98±18.55
10%	257±18.20	1286±15.87	284±18.79	1420±18.55	260±10.48	1300±11.20	182±16.25	4100±23.15	157±29.80
30%	248±18.24	3720±14.28	319±25.24	4745±23.69	356±28.13	5340±15.11	395±11.12	5925±35.14	381±19.45
50%	251±21.00	6275±29.80	296±16.56	7400±25.29	209±16.13	5220±29.41	82±24.42	2050±31.26	82±11.20
70%	260±19.45	9100±19.75	204±21.11	7140±13.84	64±9.37	2240±14.57	14±7.96	490±48.14	11±9.37

yeast (245.33±5.36 rotifers/ml) while the lowest growth was observed in tanks fed with *S. major* (86.00±10.21 rotifers/ml) and *S. obliquus* (212.67±10.80 rotifers/ml) on 8th day (Table 1).

Rotifers preferred primarily *C. vulgaris* and then Baker's yeast rather than *S. major* and *S. obliquus* might be due to difficulties in ingesting long, spiral thread like structures and irregular shape of alga, respectively (Ajah 2010). Similarly, Ashraf *et al.* (2010) reported that tanks fed with *C. vulgaris* had the maximum rotifer production (380 rotifers/ml) followed by baker's yeast and *Spirulina* sp. while Kennari *et al.* (2008) observed that *B. calyciflorus* had better production and fatty acid content when fed with *Chlorella* sp. compared to *Scenedesmus* sp. Odo *et al.* (2015) also reported that rotifers fed with *Chlorella sp.* (650 mg/ml) showed highest growth (213.81±9.94 rotifers/ml) rather than *Spirulina sp*. Though rotifers preferred Baker's yeast, its lower performance compared with *C. vulgaris* could be attributed to vitamin B_{12} deficiency (Treece and Davis 2000) and change in water quality (Kennari *et al.* 2008). This indicated that size, shape and nutrient composition of *C. vulgaris* made it more accessible to prey (Loka *et al.* 2016) and also water quality can be maintained by avoiding yeast based products (Suantika *et al.* 2003).

Estimation of algal consumption rate: After knowing preferred food type, trials to know ingestion rate of a rotifer by applying algal cells at different proportions to the same quantity of rotifers showed significant difference on consumption and multiplication rate ($P<0.05$). The highest quantity of rotifers was recovered from T_1 (544.63±44.03 rotifers/jar) followed by T_2 (541.33±21.18 rotifers/jar). Almost all algal cells were consumed in T_3 (104010 cells/rotifer) and T_4 (54930 cells/rotifer) with the mean of 0.48±0.02 and 0.08±0.03 rotifers/ml, respectively, within 24 h. While in T_1 (202620 cells/rotifer) and T_2 (199010 cells/rotifer), the quantum of rotifers recovered from jar was almost equal (1.08±0.09 and 1.08±0.04 rotifers/ml, respectively) with the consumption rate of 25,100 and 22,080 cells/rotifer/day, respectively (Table 2).

In a similar way, Loka *et al.* (2016) reported high filtration (12.2×10^{-5} cells/ml/rotifer) and ingestion rate (5.4×10^{-3} cells/ml/rotifer/min) in *B. plicatilis* when fed with *I. galbana* and *Nannochloropsis oculata* while Ashraf (2010) reported daily algal cells requirement of a rotifer, *B. calyciflorus,* was approximately 33,000–35,000 cells/day. On the contrary, Bentley *et al.* (2008) reported that much higher number of algal cells not only affects environmental conditions but also affects algal requirements of an individual rotifer. These observations suggest that higher production of rotifer, *B. calyciflorus*, can be achieved by application of *C. vulgaris* at 22,000–25,000 cells/rotifer/day without affecting rotifer culture environment.

Effect of daily harvest rate on rotifer population: Daily harvest rate showed significant role on rotifer production ($P<0.05$). Initial rotifer quantity was maintained at an average of 250±18.20 rotifers/ml. The highest quantity of harvested rotifers was observed at 70% ($9100\pm19.75\times10^5$ rotifers/tank) followed by 50% harvest rate ($6275\pm29.80\times10^5$ rotifers/tank) on 2nd day but was decreased gradually to $490\pm48.14\times10^5$ and $2050\pm31.26\times10^5$ rotifers/tank, respectively, on 8th day. Daily harvest at 30% showed stagnant quantity of rotifers (360.52±25.41 rotifers/ml) throughout the experiment with $5425\pm78.24\times10^5$ rotifers/day/ tank without affecting water quality and rotifer quantity (Table 3).

Similarly, Sananurak *et al.* (2009) reported a closed-recirculating continuous culture system for micro algae (*T. suecica*) and rotifers (*B. plicatilis*) with 0.4×10^4 cells/ml and 35 rotifers/ml productions, respectively, for the culture period of only 28 days. According to Lawrence *et al.* (2012), rotifer production in continuous culture system was most productive with regular feeding and frequent harvesting. Suantika *et al.* (2000) reported that daily water exchange at 30–50% help to maintain rotifer production at optimum level. A high-density continuous recirculating system for rotifers (*B. rotundiformis*) fed with non-viable *N. oculata* and using sodium hydroxymethane sulfonate to neutralize ammonia was also developed by Bentley *et al.* (2008) with 3000 rotifers/ml production for 30 days. Another high density rotifer production was reported in a closed recirculation system in 1.0 m^3 tank with a daily rotifer harvest of 2.1×10^9 rotifers (Suantika *et al.* 2003).

Effect of RAS system on rotifer production: RAS system has played a significant role on rotifer production by maintaining water quality throughout the culture period. The highest rotifer production was observed during summer (394.12±15.41 rotifers/ml) but was started declining gradually during monsoon (349.57±25.44 rotifers/ml) followed by winter (301.21±14.33 rotifers/ml) and again reached to its normal stage during summer (404.43±24.33 rotifers/ml). Water quality parameters during the culture period remained within the acceptable limits. The ranges of different water quality parameters during seasons are given in Table 4.

The highest rotifer count during summer was due to the increased temperature. Similarly, Park *et al.* (2001) reported higher rotifer growth in increased temperature although dissolved oxygen is one of the limiting factors for rotifer growth, no difference in rotifer quantity was observed as it was maintained always above 3.0 mg/l. Sananurak *et al.* (2009) also suggested to maintain DO level always above 3.0 mg/l. On the contrary, Rojo-Cebreros *et al.* (2017) had maintained DO level 8.6–8.7 mg/l using respironics to supply pure oxygen in high density culture. Total hardness and alkalinity has not showed any effect on rotifer production while pH was maintained between 7.4–8.8 as suggested by Suantika *et al.* (2000, 2003) and Rojo-Cebreros *et al.* (2017) to acquire higher growth rate. Ammonia was the major threat in high density rotifer production, as it causes cessation in reproduction at 3–5 mg/l concentration. In the present study, ammonia concentration above 1.16 mg/l was not observed throughout the culture period. Similarly, in Suantika *et al.* (2000) report, daily harvest at 30–50% maintained ammonia level between

Table 4. Water quality parameters and rotifer numbers (range) observed

Parameter	Summer	Monsoon	Winter	Summer	Monsoon
Temperature (°C)	27.6–31.4	24.0–31.4	24.2–30.1	27.0–32.9	24.0–31.1
D.O. (mg/l)	3.8–6.8	4.0–5.4	4.0–5.4	4.0–5.8	3.8–5.8
*p*H	7.4–8.4	7.6–8.2	7.4–8.4	7.4–8.4	7.6–8.4
Ammonia (mg/l)	0.1–1.1	0.1–1.0	0.2–1.0	0.3–1.1	0.1–0.8
Alkalinity (mg/l)	260–330	260–224	260–330	280–320	260–320
Hardness (mg/l)	280–650	280–650	420–520	300–650	300–650
Rotifer quantity (rotifers/ml)	384–406	301–396	254–403	398–415	370–400

2–5 mg/l. Liang *et al.* (2018) also reported that more ammonia concentration has negative effect on reproductive performance, algal grazing rate, swimming speed and defensive phenotypes of *B. calyciflorus*. The minimum level of ammonia throughout the culture may be accomplished by the incorporation of activated charcoal in sand filter. In similar way, Zeng *et al.* (2017) reported that ammonia can be removed from the aquaculture water using activated carbon.

Most of the rotifer and algal culture systems were used in batch or in semi-continuous way (Bentley *et al.* 2008, Odo *et al.* 2015). If we compare our RAS based culture system with batch or semi-continuous culture practices, the system has many advantages which includes substantially shorter start-up time, less complicated, big tanks, more stable culture, simplified food source for the rotifers, less contamination, control over the culture process, much reduced need of water, more consistent harvest densities, simple and continuous harvest procedures with daily production of around $5{,}425\pm78.24\times10^5$ rotifers/tank.

Production of rotifers throughout the season is critical for making hatcheries as a successful venture. Based on these experiments, RAS based continuous culture system could produce efficient quantity of rotifers throughout the season without crash and contamination. This RAS based system have significant advantages over traditional culture methods in the commercial world and could be adopted my emerging fish hatchery operators to reduce huge losses during earlier fish developmental stages.

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