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Development of a closed-recirculating, continuous culture system for microalga (*Tetraselmis suecica*) and rotifer (*Brachionus plicatilis*) production

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ABSTRACT: We have designed, built and operated a closed-recirculating, continuous culture system to produce microalgae and rotifers in seawater (25% salinity) for larval fish culture. The system opens a new perspective in terms of automated production of rotifers without labour cost. Rotifers can be easily harvested daily by a conical harvest net and there is no routine maintenance work. This new, automated system has three components: a microalga culture, a rotifer culture and storage with harvest, and a water treatment and re-use component. After treatment using mechanical filtration, biofiltration, UV sterilization, ozonation, and protein skimmers, water is re-used, thus nearly eliminating the need for seawater imports to the system. Our trial demonstrated that this culture system is capable of producing sustained and acceptable levels of rotifer production for at least 28 days. Microalga densities and yields were quite stable, while rotifer yield varied more during our 28 day culture period. Nitrogenous waste compounds were stable and well within acceptable levels, although nitrate and phosphate concentrations increased throughout the culture period, but did not negatively impact on rotifer production.

KEYWORDS: aquaculture

INTRODUCTION

Live rotifers, *Brachionus plicatilis*, are one of the most important and widely used feed for larviculture of fish and other aquatic animals that require live food of small size with high nutritional value. Three economically important, cultured fish species in Thailand requiring live rotifers during their larviculture are seabass (*Lates calcarifer*), grouper (*Epinephelus coioides*), and mullet (*Liza subviridis*)[1]. Rotifers are cultured using a wide variety of culture systems, including batch, semi-continuous, and continuous culture[2]. In Thai hatcheries, rotifers are most often cultured using open, batch culture systems in tanks or ponds with microalgae and/or yeast as food sources[3]. Although these batch culture systems are relatively simple, rotifer production is often unpredictable and requires considerable labour to operate and maintain. Rotifer production is therefore often insufficient to meet the hatchery needs during critical larviculture stages. Continuous, mass culture systems for microalgae and rotifers are generally much smaller than batch culture systems, but require more intensive management. Chemostats are the most advanced type of continuous culture used in aquaculture for microalga production[4,5]. Alga production is the critical first step in the production of rotifers and other live, larval fish feed. Considerable effort has gone into development of substitutes for live microalgae, but as yet these substitutes have not proven satisfactory[6–9]. Microalga substitutes often lack adequate nutritional value, are difficult to maintain, or have other functional problems, especially in tropical places like Thailand. Consequently, live culture of *Chlorella* spp., *Nannochloropsis* spp., and *Tetraselmis* spp. microalgae still provide the basis for rotifer culture in Asia[3]. Batch culture of microalgae typically involves a multi-step process using backups for each step, where each step is used to inoculate the next step leading to rotifer food production. Although this process is conceptually simple, it does require considerable labour, equipment, materials, and space resources. Furthermore, it is also susceptible to unpredictable 'crashes' caused by protozoans, alga contamination, and/or other causes[10]. In addition, it is often difficult to operate batch culture systems for marine species at inland locations, or at locations without a reliable source of high quality seawater. Inland hatcheries in Thailand typically must import brine (150–200% salinity) at considerable expense from salt farms located in coastal areas. Effective reuse of this seawater is therefore desirable[8,11]. These

needs generated considerable interest in development of closed-recirculation systems for larviculture food production as an alternative to open, batch culture systems[5, 12–14]. To date, however, most of these closed systems are too costly, technically complex, and difficult to operate in most commercial hatchery settings.

We know of no continuous, closed-recirculation system for algae and rotifers that is appropriate for widespread use in Thai hatcheries. Our motivation, therefore, was to develop such a system using microalga *T. suecica* and rotifer *B. plicatilis* that would overcome the problems identified above. Our aim was to develop an efficient but simple culture system for rotifers that could produce sufficient quantities of rotifers on demand, while at the same time reducing labour, risks, and resource costs. The culture system we describe herein met our objectives for both small and large-scale rotifer production needs.

MATERIALS AND METHODS

Microalga and rotifer Culture

Microalga (*T. suecica*) and rotifers (*B. plicatilis*) were obtained from the Department of Marine Science, Chulalongkorn University and Bangsaen Institute of Marine Science, Burapha University, respectively. Stock cultures of these organisms were maintained using batch culture techniques at Angsila Marine Station, Chulalongkorn University. *T. suecica* were maintained using 25% Conway medium[15] at 28 ± 2 °C, 24:0 h dark/light cycle, with white fluorescent light at an intensity of 40µmol photon m^{-2} s^{-1} under continuous aeration. Batch cultures were scaled up to 20 to 30 l and cultured until reaching exponential growth before transfer to the microalga culture tank of the continuous culture system. Batch cultures of *B. plicatilis* were maintained in 25% seawater at 28 ± 2 °C with continuous aeration and fed *T. suecica* daily. Batch cultures were scaled-up to 200 l at a density of 150-200 rotifers per ml before transfer to the rotifer culture tank of the continuous culture system.

EXPERIMENTAL SETUP

Description of continuous culture system

Our continuous culture system consisted of three components; microalga culture, rotifers culture and harvest, and water treatment and re-use component (Fig. 1). The microalga culture component included a nutrient source, culture medium tank, and microalga culture tank. Flow of concentrated nutrient solution from the nutrient source reservoir (Fig. 1; 4) to the culture medium tank was controlled by an electronic on-off switch (1) that regulated a small water pump (2). A magnetic stirrer (3) kept nutrients well mixed, starting one minute before pumping to the culture medium tank. While nutrients were pumped, seawater was also pumped from the water re-use tanks into the culture medium tank. The culture medium tank has a capacity of 150 l when full. Water flow from the recycled/new water tanks was regulated by a liquid level control (LLC) switch (8) that sensed water levels inside the culture medium tank. When water volume in the culture medium tank was reduced to 20 l (LLC switch turned on), re-used water was automatically and synchronously pumped into the culture medium tank along with concentrated nutrients at a ratio of 750:1. When the water volume reached 10 l in the culture medium tank, pumps were turned off. All LLC switches were from Shin Tung Electronics Industry Co., Ltd., Taiwan, model ST-65AB.

Refill water passed through a 20 µm filter before entering the culture medium tank, and was then recycled through an ultraviolet (UV) sterilizer (Fig. 1; 6) below the tank by a separate pump (7). The culture medium tank had two LLC switches. One LLC switch (8) controlled refill water and nutrient inflows into the culture medium tank as described above, while the second LLC switch (5) controlled ozonation (27) of water in the water re-use tanks. The second LLC switch (5) in the culture medium tank turned off ozonation in the water re-use tanks when water volume fell to 40 l (nearly empty) in the culture medium tank. When the water volume increased to 150 l, ozonation resumed in the water re-use tanks. Discontinuous ozonation was necessary to prevent ozone from entering the microalga culture tank where it could kill algae. The ozonator was a Hailea model HLO-810, 10 watt capacity that operated using airflow of 3.5 l/min and air stone injection. This model produced 200 mg O_3 h^{-1}. All tanks in the system were made of polyethylene, while hard pipes were of blue PVC plastic, and flexible pipes and tubes were of soft PVC.

The microalga culture tank was 72 cm in diameter and 1 m in height with a volume of 260 l. The light energy source for alga growth consisted of three 36 W white florescent lamps (Fig. 1; 12) inside three layers of concentric, clear acrylic tubes (11). Water temperature in the microalga culture tank was maintained using a cooling system that consisted of water exchange between the first space created by the concentric acrylic tubes (11) and a cooling system tank next to the microalga culture tank. Mechanical cooling was not provided in the cooling water tank,

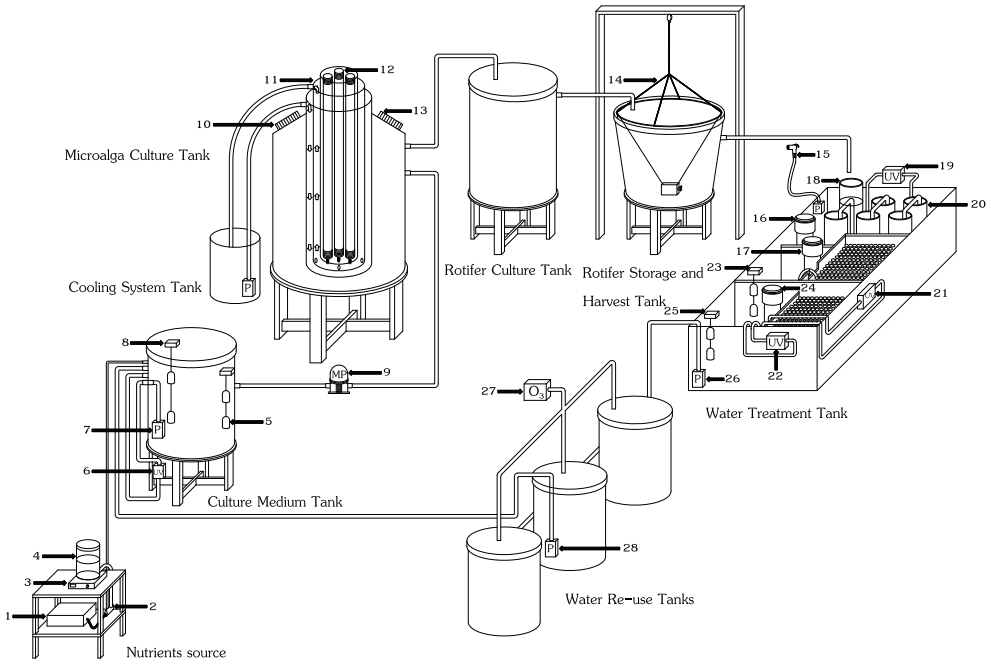

Fig. 1 Diagram of a closed-recirculating, continuous culture system for microalga (*T. suecica*) and rotifers (*B. plicatilis*). See text for description of numbered labels on system components.

but water was circulated between the cooling water tank and the centre of the microalga tank in order to remove heat generated by the light source. The water temperature in the cooling water tank was 30 °C and 27–28 °C in the microalga culture water. Room temperature was maintained at 28 ± 2 °C using air conditioning. In addition, two small ventilation 30 W fans (10, 13) circulated air through the top of the culture tank. One fan pumped outside air into the culture tank, while the other fan pumped air out. Culture medium water was pumped at a constant rate from the culture medium tank into the microalga culture tank using a metering pump, while continuous aeration was provided at 4 to 5 l/min.

The rotifer culture tank was 55 cm in diameter, 88 cm height and had a volume of 200 l (Fig. 1). Water flows into the rotifer culture tank were by gravity from the microalga culture tank, which in turn matched the constant rate of water inflows from the culture medium tank. The amount of microalga food thus supplied to rotifers was related to water flow rates (l/min) and alga densities (cells/l) in the microalga culture tank. Rotifers were harvested in the 200 l rotifer harvest tank using a conical harvest net (14; 0.6 m diameter, 0.6 m height, 58 µm mesh size). Water flowed by gravity from the rotifer culture tank into the harvest net. During rotifer harvest, the net was raised using a rope and pulley arrangement and rotifers were concentrated in a small plastic container at the cod-end of the net. Rotifers adhering to the net were washed into this container using a hand-held sprayer (15) that operated on a separate water pump that drew water from the first compartment of the water treatment tank. Effluent from the rotifer harvest tank then flowed into the water treatment tank.

The water treatment tank (Fig. 1) measured 1.2 m $\times$ 0.6 m $\times$ 0.65 m (volume 300 l). It contained several sub-components designed to restore water quality by removing both solid and dissolved wastes and contaminants. These sub-components were contained within five compartments of the water treatment tank. Water flows into compartment 1 of the water treatment tank were gravity flows from the rotifer harvest tank into a PVC pipe (18) containing a 38 µm mesh net that removed most rotifers and other debris. After passing through this net, water was pumped through an UV sterilizer (19) into five additional PVC pipes (20), each measuring 10.2 cm in diameter and 55 cm in height. All six PVC pipes contained three layers of charcoal (150 g) on the bottom, oyster-shells (1.5 kg) in the middle layer, and spun-fibreglass filter material on top. Water flow was nearly equal between the five pipes with total flow of 23 l/min. The UV sterilizer (19) operated 3 h on and 1 h off, while the protein skimmer (16) operated continuously with of 33 l/min water flow. The protein skimmer (16; Queen

Turbo-Skimmers model TS-2000) operated independent of the pump providing water to the six pipes and to the UV sterilizer (19) in compartment 1 of the water treatment tank. Intermittent operation of all UV sterilizers (Atman 11 W) was necessary to prevent damage to their lamps. Wastes from protein skimmers were discharged to the drain.

Water from compartment 1 of the water treatment tank flowed by gravity into compartment 2. Compartment 2 contained a protein skimmer (Fig. 1; 17) for removing suspended solids and remaining dissolved proteins. Compartment 2 also had an LLC switch (23) that actuated when water levels reached a pre-set level, pumping water into compartment 3. The LLC switch turned off the pump when water levels dropped to the lower pre-set level. Compartments 3 and 4 of the water treatment tank contained biofilter media for nitrification. Water flowed from compartment 3 into section 4 by gravity flow. When compartment 4 was full, water flowed by gravity into compartment 5. Compartment 5 contained two UV sterilizers (21, 22) for water entering the re-circulation pumps and a protein skimmer (24) that also recirculated water between the UV sterilizers and the compartment. The protein skimmer removed bacterial flock from the biofilters. The UV sterilizers were on a timer switch (3 h on, 1 h off). Water from compartment 5 of the water treatment tank was then pumped (26) into 3 water re-use tanks (total volume 400 l). Water flows were controlled by a LLC switch (25). A control board contained protection and electrical control for the continuous culture system. This board included a ground-fault control for all circuits to protect system operators, timers for the UV sterilizers, and circuit breakers for pumps and other electrical components.

Operation and performance of continuous culture system

We assessed performance of the continuous culture system during a 28 day trial at a water flow-through rate of 121 l/d and dilution rates [Dilution rate (D) = Medium Flow Rate (F)/Tank Volume (V)][16], in the microalga culture tank and rotifer culture tank of 0.47 l/d and 0.61 l/d, respectively. We then observed the effects of these dilution rates on microalga and rotifer densities and production, and on water quality. Microalga yields from the microalga culture tank were calculated as the water flow rate from the microalga culture tank times average microalga density in this tank on that date. Rotifer yields from the rotifer culture tank (stock) were calculated as the water flow rate from the rotifer culture tank times average rotifer density in this tank on that date, while rotifer yields for the rotifer storage and harvest tank were actual numbers of rotifers harvested from the net in that tank on that date. The system operated at 28 ± 2 °C with continuous aeration, as described above.

Water used in our continuous culture system was from seawater diluted to 25% and treated using ozone injection for 1 day, then aerated for 4 h to remove any residual ozone. Treated seawater was filtered through 20 µm nylon netting before transfer to the culture medium tank, microalga culture tank, rotifer culture tank, and the water re-use tanks. Several months before our trials began, we preconditioned nitrifying bacteria in the continuous culture system using continuous aeration and NH_4Cl at 2 mg/l with trace elements added. The nutrient solution for *T. suecica* in the continuous culture system consisted of four types of cost-effective modified agricultural-grade fertilizers[17]. We initially stocked the microalga culture tank with 26 l of *T. suecica* inoculum to achieve a density of $1–2 \times 10^5$ cells/ml, while the rotifer culture tank was initially stocked with 150–200 rotifers/ml. After stocking, the metering pump from the culture medium tank was turned on and continuous flow-through began at 121 l/d and remained constant during the 28 days trial.

Sampling and counting

Microalga and rotifer densities were measured daily using a haemacytometer and Sedgewick-Rafter slide under a light microscope with triplicate samples preserved using Lugol's iodine solution. Microalgae were counted in both the microalga culture tank and rotifer culture tank, while rotifers were counted in the rotifer culture tank only.

Physico-chemical parameters

Inorganic nitrogen (ammonia-N, nitrite-N, nitrate-N) and total phosphorus (P) were measured every five days at five locations in the continuous culture system[18,19]. Temperature and salinity were measured daily, while freshwater and/or seawater was added to maintain salinity at 25% and to compensate for evaporation and other water losses.

RESULTS

Daily microalga densities in the microalga culture tank ranged from $10.1 \pm 0.5–16.8 \pm 1.1 \times 10^4$ cells/ml, while the average density for the culture period was 13.4×10^4 cells/ml (Table 1). Estimated average microalga yield from the microalga culture tank was $1.63 \pm 0.1 \times 10^{10}$ cells/day ($6.28 \pm 0.38 \times 10^7$ cells/l/day). These alga yields provided food for rotifers in the rotifer culture tank and, to a lesser extent, for rotifers in the rotifer storage and harvest

Table 1 Resultant densities of microalga (*T. suecica*) and rotifers (*B. plicatilis*) in a closed-recirculating, continuous culture system during a 28 days culture trial.

	Microalga culture tank	Rotifer culture tank	Rotifer storage and harvest tank
microalga daily densities ($\times 10^4$ cells ml^{-1})	10.1 ± 0.5–16.8 ± 1.1	0.2 ± 0.1–0.8 ± 0.2	-
rotifer daily densities (rotifers ml^{-1})	-	16.7 ± 2.1–64 ± 5	29 ± 11–343 ± 18
mean microalga 28 day density ($\times 10^4$ cells ml^{-1})	13.4 ± 0.8	0.4 ± 0.1	-
mean rotifer 28 day density (rotifers ml^{-1})	-	35 ± 5	119 ± 10

tank. Microalga densities in the rotifer culture tank averaged 0.4×10^4 cells/ml, or about 3% of alga densities in the microalga culture tank. This reduction in alga density was the result of grazing by rotifers on algae and reduced light intensities in the rotifer culture tank. It is noteworthy that daily fluctuations in alga densities in the microalga culture tank were relatively small. Standard deviation for daily alga densities was 6% of mean densities, indicating rather stable alga production under the water flow rates, nutrient concentrations, and light intensities used.

Rotifer densities in the rotifer culture tank during the 28 days culture period were more variable than alga densities, ranging from 17 to 64 rotifers/ml, with peak values between days 9 and 13, and with lowest values on days 19 and 26–28. Average rotifer densities in the rotifer culture tank were 35 rotifers/ml and in rotifer storage and harvest tank were 119 rotifers/ml for the culture period (Table 1). There were no clear responses in rotifer densities in the rotifer culture tank related to alga densities in influent waters. Alga densities in influent waters and in the rotifer culture tank were relatively stable, as noted above. Estimated rotifer yields from the rotifer culture tank ranged from 2.02–7.77×10^6 rotifers/day (1–4×10^4 rotifers/l/day), with a mean estimated daily yield of $4.24 \pm 0.55 \times 10^6$ rotifers/day ($2.12 \pm 0.27 \times 10^4$ rotifers/l/day) (Table 1). These estimated yields based on rotifer densities in the rotifer culture tank and water flow rates agree well with mean observed rotifer harvests of $4.66 \pm 0.40 \times 10^6$ rotifers/day ($11.9 \pm 1.0 \times 10^4$ rotifers/l/day) in the rotifer storage and harvest net. Average estimated and observed values differed by only 10%.

Ammonia at five locations in the continuous culture system during 28 days culture ranged from 0.0 to 0.86 mg/l, with maximum values in the rotifer storage and harvest tank on day 9 (Fig. 2). Nitrite ranged from 0.03 to 3.09 mg/l, with maximum values in the rotifer culture tank. Nitrate ranged from 1.1 to 60 mg/l in all tanks during 28 days. Phosphate ranged from 0.0 to 3.5 mg/l, with continuous increases at all locations during the culture period.

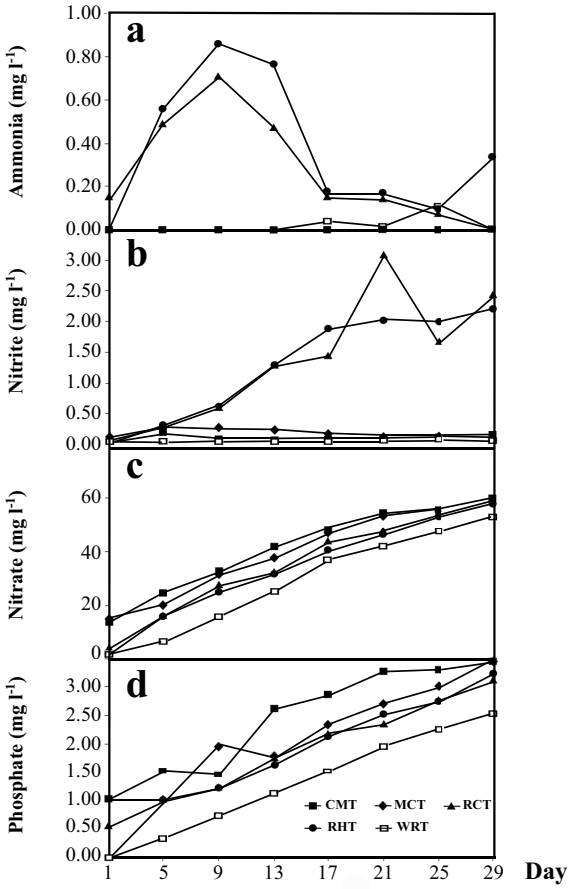

Fig. 2 Concentrations of N and P in the continuous culture system during a 28 days culture trial. CMT = culture medium tank, MCT = microalga culture tank, RCT = rotifer culture tank, RHT = rotifer storage and harvest tank, WRT = water re-use tanks.

DISCUSSION

Our trials with the closed-recirculating, continuous culture system demonstrated that this culture system is capable of sustained and acceptable levels of rotifer production for at least 28 days. Microalga densities and yields were quite stable, while rotifer yield varied more during our 28 day culture period. Rotifer variability seemed unrelated to microalga densities and yields, but rotifer production was still entirely acceptable. We did not observe serious problems with

either microalga or rotifer contamination with this system. We did, however, find that microalgae became attached to the acrylic plastic around the light source in the microalga culture tank by day 10. This required daily scrubbing to remove the microalgae.

Water quality was within an acceptable range for all parameters measured. Ammonia-N is known to cause cessation of rotifer reproduction when concentrations approach 3–5 mg/l, and rotifer mortality within two days at >5 mg/l[20]. However, we did not observe more than 0.86 mg/l ammonia-N at any time or location with our continuous culture system (Fig. 2). Nitrite-N toxicity for rotifers is said to begin in the range of 90–140 mg/l[2]. Again, we did not observe nitrite-N concentrations much greater than 2.2 mg/l at any time. Nitrate-N and phosphate did increase during our 28 days culture trial, but neither of these appeared to cause rotifer mortality or problems with rotifer production. Both nitrates and phosphates continued to increase throughout the culture period.

In some marine and freshwater organisms, lethal and sub-lethal effects of high concentrations of nitrates have been documented. An amount of 50 mg/l is a generally accepted safe limit for nitrate nitrogen in fish culture, but this concentration varies widely for different species and developmental stages[21]. The effects of excess nitrates can mean slower growth, susceptibility to diseases, retardation in development, low fertility and poor survival[22]. In marine species the ranges are 2.2–5050 mg/l of nitrate with larvae and broodstock as the most sensitive stages. In addition, a nitrate concentration of 75 mg/l reduced the fertilization rate, delayed hatching time, reduced the hatching rate of the eggs and decreased the growth rate of juveniles[23]. These observations suggest that at the production rates we achieved, nitrate concentrations would not negatively impact the system operation under most anticipated scenarios.

Most alga culture systems used in batch and other culture systems typically use a light source suspended 0.5 to 1.5 m above the alga culture tank[24], or next to polyethylene culture bags[25]. These light sources are outside the alga culture vessel[5], and much of the light is therefore not directly available for alga growth. With our continuous culture system, the light source was inside the alga culture tank. We therefore expect much greater photosynthetic efficiencies per unit of light energy. We did not, however, measure this efficiency during our initial trials.

If we compare our closed-recirculating, continuous culture system with batch or semi-continuous culture practices in Thailand, we see that our system has many advantages. These include a shorter start-up time, less complicated start-up procedures, greater cell densities, lower light power requirements, no labour, fewer problems that could lead to alga culture 'crashes', and much reduced need for seawater imports[3,26]. Likewise, our system has similar advantages over batch or semi-continuous culture practices for rotifers. These advantages for rotifer production included substantially shorter start-up time, less complicated and shorter start-up procedures, smaller tanks, more stable culture temperatures, simplified food sources for the rotifers, more consistent harvest densities, simple and continuous harvest procedures, greater production per unit labour and tank space, less contamination, greater control over the culture process, and much reduced need for seawater imports[3,26]. This culture trial demonstrated the benefits and advantages of this culture system compared with more commonly used batch or semi-continuous culture system for microalgae and rotifers. A chemostat culture system[27] for rotifer production achieved a daily output of 186×10^6 rotifers in a 1 m^3 tank. An automatic continuous culture system[6] has been developed with a rotifer production yield of 1.7×10^8 rotifers/l/day. Another high density rotifer production has been achieved in a closed recirculation system[11] that test on a commercial scale in 1 m^3 tank with a daily rotifer harvest of 2.1×10^9 rotifers.

For the rotifer production of 1.2×10^5 rotifers/l/day in our system, the total investment cost is about 160 000 THB, of which 37% is spent for investment on fixed assets and 63% is consumed by electricity, chemicals, fertilizers, seawater, and tap water. The investment cost and operational costs in our system were also lower than the Suantika[11] system that needed 2 700 000 THB to produce 2×10^6 rotifers/l/day. In addition, our system needed around 1560 l less water than the Suantika[11] system.

Our system clearly demonstrated successful and continuous culture of microalgae and rotifers. It is easy to operate our systems for marine species at inland locations or at locations without a reliable source of high quality seawater. Our continuous culture system met the design criteria of system simplicity, minimal contamination of culture organisms, low cost, and system stability and reliability. We anticipate that as culturists gain experience using this system under different operating conditions, it will become even more applicable to culture needs. We anticipate changes in system size and capacity, use of additional culture species, and perhaps microprocessor control of the continuous culture system in order to achieve even greater control over production results.

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