Chapter 3
Mass Culture and Preservation of *Brachionus plicatilis* sp. Complex

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Abstract The quality and quantity of rotifer production in a hatchery primarily determine the yield of larval production; therefore, considerable studies geared toward mass culture and preservation of rotifers. Rotifer mass production, attaining ultrahigh density and greater culture stability, was attained when condensed microalgal pastes (*Chlorella* and *Nannochloropsis*) were introduced and used in a closed recirculation system. The rotifer culture health status can be determined by measuring water quality, rotifer physiological conditions such as egg ratio, swimming speed, ingestion rate, and in vivo enzyme that takes 1–2 days to finish. Addition of chemicals and hormones such as γ-aminobutyric acid (GABA), porcine growth hormone, serotonin, and human chorionic gonadotropin is known to improve the health status of cultured rotifers. Mass-produced rotifers can be preserved at low temperature; these rotifers can either be directly used as feed for the larvae or as starters of rotifer culture. For long-term storage, collection and storage of resting eggs are recommended. For species which do not produce resting eggs, cryopreservation of amictic eggs is recommended.

3.1 Introduction

The rotifer *Brachionus plicatilis* species complex is being used worldwide as a live food for the initial stages of larval rearing of fish and shellfish because of their suitable size and shape. Based on morphometric characteristics, rotiferologists have classified *B. plicatilis* into three morphotypes (Fu et al. 1991): L-type (130–340 μm), S-type (100–210 μm), and SS-type (90–150 μm; see review Hagiwara et al. 1995, 2001). This classification is supported by biochemical and ecological differences

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(Snell 1998), including mating behavior (based on 29-kDa glycoprotein sex pheromone; see Snell et al. 1995), hence, assigned a name to distinguish these different species as *B. plicatilis* for L-type, *B. ibericus* for S-type, and *B. rotundiformis* for SS-type (Kotani et al. 2005). However, this physiology-based classification is considered a difficult approach with weak boundary species (Kotani et al. 1997); thus, molecular phylogeny was adapted to classify rotifer species complex. Several cryptic species were found within *B. plicatilis* sp. complex using ITS1and COI sequences (Gómez et al. 2002), and to date, 15 different species are classified (Mills et al. 2016). Among rotifers, this taxon is well studied because of its ease in culture and high reproductive performance and thus commonly used in fish and crustacean hatcheries (Hagiwara et al. 2007). The quality and quantity of rotifer production in a hatchery primarily determine the yield of larval production; therefore, considerable attention and researches are being done in the mass culture and preservation of this species complex (Hagiwara 1994; Lubzens and Zmora 2003).

Mass production of *B. plicatilis* sp. complex was initiated in the early 1960s with the use of *Nannochloropsis oculata* as diet (Hirata 1964). Thereafter, rotifer mass culture trials were conducted using various diets such as baker's yeast, marine yeast, bacteria, omega yeast, and *Tetraselmis tetrathele* (reviewed by Nagata and Hirata 1986). Rotifer mass production has been systematized with the combination of improved diet formulation (Yoshimatsu and Hossain 2014), and rotifer densities reached to 2.3×10^4 ind/mL (Dhert et al. 2001). However, in spite of these advancements, fresh phytoplankton and/or baker's yeast is still generally used for the mass culture of rotifers worldwide. The use of baker's yeast is beneficial for aquaculturists since it lowers the cost for rotifer production. However, rotifer cultures fed yeast are less stable owing to the rapid decline of water quality. Moreover, rotifers fed yeast need further nutritional enrichment before being fed to fish larvae; therefore, phytoplankton remains the ideal food for rotifer cultures (Lubzens et al. 1995a). However, culturing of phytoplankton requires considerable space in a hatchery. Condensed microalgal paste was found to be the ideal food for ultrahigh production of rotifers. During the late 1980s, a phytoplankton industry has developed new products such as condensed microalgal paste (*Chlorella* and *Nannochloropsis*), which enabled culturists to mass culture rotifers at a high density as much as 1.6×10^5 ind/mL (Lubzens et al. 1995a; Yoshimura et al. 1996, 2003; Hagiwara and Kuwada 2004) with higher stability of culture. Furthermore, it was the most convenient, since it could be stored at low temperature (refrigerator or freezer) for at least 2 months or even longer without significant loss of its essential fatty acid content and maintaining its nutrient composition in cultured rotifers (Lubzens et al. 1995a; Welladsen et al. 2014). However, condensed microalgal pastes are not commercially available in developing countries because of higher price. Recently, detritus from macroalga, *Ulva pertusa*, was shown to sustain high population growth and enhance the nutritional value of *B. plicatilis* (Yin et al. 2013). Detrital macroalgae can also be stored and collected from the wild; thus, it is considered economical in large-scale production of rotifers.

In this chapter, various culture methods commonly used for mass culture of clonal rotifer *B. plicatilis* sp. complex are discussed. Since the major problem

encountered by aquaculturists is the unpredictable culture collapse, some of the techniques used to diagnose and treat rotifer culture are presented. To address the inadequate supply of larval food, some preservation methods that have been proposed by many researchers are also discussed.

3.2 Culture Methods

3.2.1 Batch Culture

Batch culture is a common method for rotifer mass production in marine fish hatcheries (Fig. 3.1a). The culture maintains a constant volume with an increasing rotifer density or a constant rotifer density by increasing the culture volume (Dhert et al. 2001). When the desired density is achieved, the culture is entirely harvested at once by draining the culture medium and collecting the rotifers in a net. The harvested rotifers are used to feed the larvae, and the remaining is used as the inoculum for the next culture (Lubzens 1987; Hino 1993; Dhert et al. 2001). The size of culture vessels is flexible: 500–1000 L for plastic tanks or up to 10 ton for concrete tanks. In case of S-type rotifers, the obtainable densities at harvest time are over 1000 ind/mL when fed with *Nannochloropsis oculata* and/or backer's yeast (Hino 1993) and 600 ind/mL when fed with artificial diet (Culture Selco®) (Suantika et al. 2000). This method is used in many countries; however, it has many disadvantages, including low efficiency in terms of labor and utilization of infrastructure, low production yield, unstable and unpredictable culture, and high costs of operation (Dhert et al. 2001).

3.2.2 Semicontinuous Culture

The semicontinuous culture method is also known as "thinning out" culture, because the rotifer density is maintained constant by periodic harvesting (Hino 1993). The size of the culture tank is usually larger (usually between 20 and 480 tons) than that used in batch culture. The initial density of rotifers inoculated into the system varies between 50 and 200 ind/mL and might reach 300 to over 1000 ind/mL in 3–7 days when fed fresh microalgae and/or baker's yeast (Dhert et al. 2001). Unlike in the batch culture method, in this system, a fixed fraction of culture water (which contains rotifers and residual food) is harvested at regular intervals and replaced by an equal quantity of fresh culture medium (Fig.3.1b). The dilution rate determines the rotifer population dynamics. Monod kinetics and related mathematical concepts are used to model the quasi-steady state of periodically diluted cultures used in this system (Schlüter et al. 1987). Navarro and Yufera (1998) used the quasi-steady state method and freeze-dried microalgae and used dilution rates of 0.3/day and 0.2/day,

which resulted in the best production (mg rotifer/day) and food conversion efficiency (mg rotifer developed/mg microalgae consumed) for L- and S-type rotifers, respectively.

3.2.3 High-Density Culture

An intensive mass culture system for rotifers was developed by Japanese scientists in the late 1990s. In the culture trial with the use of concentrated freshwater *Chlorella* diet, an ultrahigh S-type rotifer density ranging from 10,000 to 30,000 ind/mL was obtained (Yoshimura et al. 1996). Under this batch, culture method, culture instability due to ammonia accumulation, presence of bacteria/protozoa, and food shortage or oxygen decline, however, are often encountered (Hagiwara and Kuwada 2004). To solve these problems, Yoshimura et al. (2003) employed several remedies including oxygen gas supplementation, regulation of pH (adjusted to 7), and a filtering equipment which prevents particulate organic matter, debris, and bacteria from clogging the collection net during harvest. In addition, Fu et al. (1997) introduced an automatic *continuous culture system* as the solution to maintain culture stability, as well as to reduce labor and create more space in the hatchery (Fig. 3.1c). This system consists of a filtration unit, culture unit, and harvest unit. Filtered water and food are continuously supplied into the rotifer culture tank at a predetermined rate, and the same amount of culture water is transferred into the harvest tank to obtain a significant rotifer biomass. By using this system, Fu et al. (1997) successfully mass produced about 2.1 billion rotifers/day for S-type in a 1-m^3 tank with densities ranging from 3000 to 6000 ind/mL and about 0.17 billion rotifers/day for L-type in a 500-L tank with densities ranging from 1100 to 2200 ind/mL. The longest duration of their culture was over 110 days. Hagiwara and Kuwada (2004) described continuous rotifer culture system using a large tank (20 ton), which uses facilities in old hatcheries. Commercially available concentrated freshwater *Chlorella vulgaris* was used as feed for both species. In order to achieve high-density rotifers, a *closed recirculation culture system* (Fig. 3.1d) was lastly performed. This system comprised of protein skimmers, novel filters, ozone, and an addition of sodium hydroxymethanesulfonate to neutralize ammonia in the tank. With these modifications, ultrahigh rotifer densities (over 5000 ind/mL) were maintained for longer culture period (up to 30 days) without compromising the water quality (Suantika et al. 2000, 2001; Bentley et al. 2008). The development of a high-density rotifer culture system significantly reduced the space needed at the hatchery for live food production, allowing the production of sufficient amount of rotifers even in small tanks, improved the water quality, and decreased the harmful bacterial load of the culture; thus, this system was significantly economical (Suantika et al. 2003).

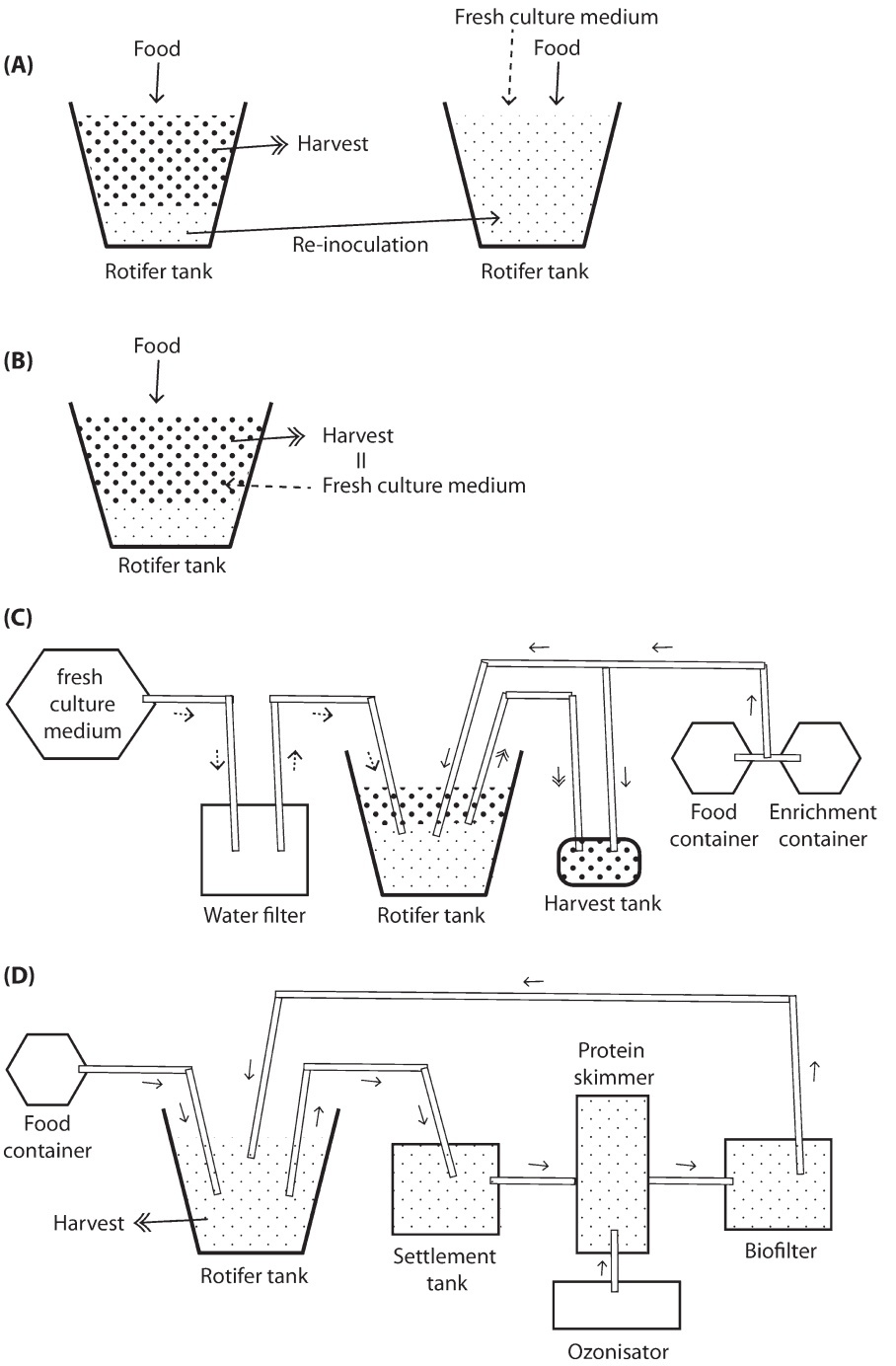

Fig. 3.1 Various mass culture systems of rotifer *Brachionus plicatilis* sp. complex. (**a**) Batch culture, (**b**) semicontinuous culture, and high-density culture including (**c**) automatic continuous and (**d**) closed recirculation systems. Normal, *dot*, and *double head arrows* indicate major inflows of materials, fresh medium, and rotifer harvest line, respectively

Table 3.1 Effects of chemicals on reproduction (i.e., population growth and mixis induction) and body size of the rotifer *B. plicatilis* sensu stricto

Chemicals (concentrations affect reproduction)	Population growth	Mixis induction	Body size
γ-Aminobutyric acid (50 mg/L)	++	++	+
Porcine growth hormone (0.025 I.U./mL)	++	++	n.e.
Serotonin (0.05 and 5 mg/L)	+	++	n.e.
Human chorionic gonadotropin (2.5 I.U./mL)	+	n.e.	−
Juvenile hormone (0.05 and 0.5 mg/L)	n.e.	++	++
Estradiol-17β (50 mg/L)	n.e.	+	−
20-hydroxyecdysone (0.05 mg/L)	n.e.	+	−

Adopted from Hagiwara et al. (2001) and Hagiwara and Kuwada (2004)
++, increased phenomenon ($P < 0.01$); +, increased phenomenon ($P < 0.05$); −, decreased phenomenon ($P < 0.05$); n.e., no effect ($P > 0.05$).

3.3 Culture Diagnosis and Treatment

Rotifers have been successfully mass produced; however, maintenance of the culture for a long time remains a critical unresolved problem. Several factors, including decreased feeding activity, protozoan contamination, and poor water quality, caused rotifer cultures to collapse (Yu and Hirayama 1986; Jung et al. 1997). The water quality in the rotifer culture can be assessed by measuring pH, ammonia level, and viscosity (Snell et al. 1987; Hagiwara et al. 1998). The reported critical pH and unionize ammonia concentrations are at 7 (Yoshimura et al. 1996) and at less than 2.1 ppm (Yu and Hirayama 1986), respectively. The viscosity of rotifer culture medium increases with the accumulation of dissolve organic substances, and the higher viscosity causes a decline in the rotifer population (Araujo et al. 2001). It is therefore necessary to continuously monitor rotifer cultures in order to determine their health status. Several methods are recommended to assess the physiological status of cultured rotifers, as well as to predict culture collapse (for review see Hagiwara and Kuwada 2004). Egg ratio (the number of parthenogenetic eggs per female) was used by Snell et al. (1987); however, this method required 1–2 days for the assessment and was not a sensitive end point for culture diagnosis. Swimming speed (Snell et al. 1987; Janssen et al. 1994) and ingestion rate (Ferrando et al. 1993; Juchelka and Snell 1994) were also found to be sensitive indicators to detect stress in rotifer culture. These two characteristics can be detected easily and rapidly (require around 1–2 h). Furthermore, in vivo enzyme activity test, which is considered to be the most sensitive method (Araujo et al. 2001), is another way to assess the health of rotifer cultures; however, it requires instruments such as a computer, fluorometer, and an image analyzer, which increases the cost for operation.

Several treatment methods have been suggested to improve rotifer culture and prevent collapse (Table 3.1). Araujo and Hagiwara (2005) found that the addition of γ-aminobutyric acid (GABA) can improve the health condition of rotifers when they are exposed to stressful environment (e.g., increase of unionized ammonia and

protozoa contamination). Addition of GABA during nutritional enrichment culture is also shown to improve survival and swimming activity of rotifers (Gallardo et al. 2001). In addition, the supplementation of porcine growth hormone significantly enhances the rotifer population growth when environmental stressors are low, such as under optimal food conditions with a low concentration of free ammonia (Gallardo et al. 1999). Serotonin and human chorionic gonadotropin are also effective to increase the rotifer population growth. For the improvement of rotifer sexual reproduction, in addition to GABA, serotonin can also be applied under low food conditions. On the other hand, juvenile hormone increases mixis production under optimal and suboptimal food conditions. To apply the mentioned chemicals into rotifer mass cultures, the following issues have to be dealt with: (1) several chemicals also affect body size of rotifers (Gallardo et al. 1997) and unknown influences remain, (2) chemical-treated rotifers would influence fish larvae, and (3) effluents containing these hormones would have effects on indigenous species in receiving waters (Hagiwara et al. 2001).

3.4 Preservation

Despite the progress of successful establishment of mass culture of rotifers, diagnosis of culture status, treatment of culture for recovery, and methods to preserve rotifers for either feeding fish stocks or starting a new culture are necessary. Mass preservation is essential because of the usual problem of culture collapse. Rotifers can be preserved for a short time (few days to 4 weeks) at low temperatures (between −2 and 8 °C) and for long term (years) by producing and collecting resting eggs.

Rotifers preserved at low temperature can either be directly used as feed for the larvae or as starters of rotifer culture (Assavaaree et al. 2001; reviewed by Hagiwara et al. (2001)). However, *B. plicatilis* sp. complex respond differently to various conditions during preservation. Assavaaree et al. (2001) found that *B. plicatilis* s. s. were more resistant to low-temperature (4 °C) preservation than *B. rotundiformis* (Fig. 3.2). They also found that exchange of culture media during the incubation at low temperature is necessary to maintain the viability of the rotifers. The rotifer survival at low temperature is also significantly related to the rotifer culture conditions, e.g., food and salinity before exposure to low temperature (Lubzens et al. 1990), where syntheses of specific proteins, including 94 kD and HSP 60, are being synthesized (Lubzens et al. 1995b). The recovery rate of preserved *B. plicatilis* for 14 days at density of 20,000 ind/mL was about 50%. The S- and SS-type *B. rotundiformis* strains were comparatively less resistant to low-temperature preservation but can be improved by GABA treatment (Assavaaree and Hagiwara 2011).

For long-term storage, resting eggs of rotifers can be produced and hatched when needed. The resting egg is a notable feature of rotifer life cycle and is the end product of sexual reproduction which is resistant to harsh environment. The advantages of rotifer resting eggs are highlighted for preservation (reviewed by Hagiwara and Hirayama 1993; Hagiwara et al. 1997). These eggs can be stored for more than

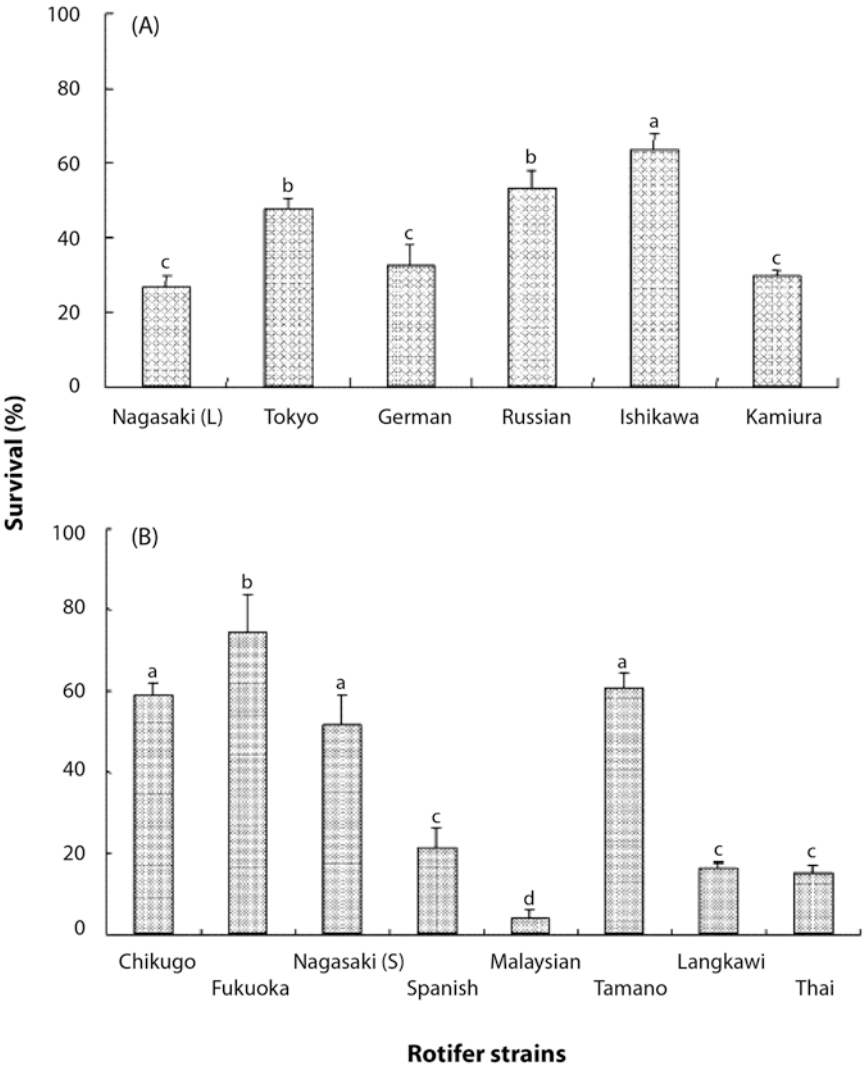

Fig. 3.2 Percent survival after preservation at 4 °C. (**a**) Survival of 6 L-type strains on day 30 and (**b**) survival of 8 S- and SS-type strains on day 5. Columns and vertical bars indicate mean rotifer survival of three replicates and standard deviation, respectively. Results of Tukey test were presented (a>b>c, $P < 0.05$) (Adopted from Hagiwara et al. 2001)

20 years in sterilized seawater under complete darkness at 5 °C. The hatching rates of dried resting egg gradually decrease, but those preserved by canning at an atmospheric pressure of 48–61 kPa after lyophilization (at −30 °C) can be maintained for up to 6 months (Hagiwara et al. 1997). The resting egg production, however, is an obstacle for mass production of rotifers because of the appearance of mictic females

which cannot contribute to population growth. Resting eggs, however, could be a by-product during hatchery off-season operation and then can be hatched with the same manner as *Artemia* cysts when needed. However, even with these merits, not all species of *B. plicatilis* sp. complex produce resting eggs, and hatchlings from resting eggs would have different reproductive characteristics from their parent because of gene recombination during resting egg formation. As solution to these issues, other methods such as cryopreservation of amictic eggs were tested (Toledo and Kurokura 1990; Toledo et al. 1991; Lubzens et al. 2001). With this method, rotifers showed around 50% of survival rate. This method is recommended and is useful for the maintenance of a certain strain with desired morphological and genetic characteristics. Nevertheless, there are several drawbacks to this technique such as practical only in small-scale and low population growth after thawing (Toledo et al. 1991).

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