# Study on the tank shape and aeration for small-scale larviculture of marine fishes

小型飼育水槽の形状と流場が海産仔魚の飼育成績に与える影響

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# Study on the tank shape and aeration for small-scale larviculture of marine fishes

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## ABSTRACT

Many larviculture trials of marine fishes have been conducted in large-scale ($m^3$), and little attention has been paid to the small-scale (<50-l) larviculture. Large rearing tanks are known to be preferable for marine fish larvae due to the stable flow field and stabilized water quality, and a lesser chance of damage from the contacting the tank walls for larvae. Small tanks are easily manipulated, however, survival rates are usually low comparing to the large tanks due to the difficulty of maintaining stable flow field. Tank shapes and sizes can differ the flow field in larval rearing tanks. Therefore, to seek for optimal flow field in small-scale larviculture, I conducted rearing experiments using 50-l small tanks in different shapes, cylindrical (CT) and rectangular tank (RT), with different aerators and compared rearing results and flow field.

First, I chose red seabream *Pagrus major* as experimental fish and compared survival, growth and swim-bladder inflation between CT ($1.7\times10^3$ $cm^2$ water surface area, 30 cm depth) and RT ($1.8\times10^3$ $cm^2$ water surface area, 28 cm depth, Chapter 2). This species is an important cultured marine fish in Japan and aquaculture technique has been established. However, little attention has been paid to the small-scale larviculture experiments for this species. One air stone with 100 ml/min aeration rate was set at the bottom center of CT and RT (RT1AS), and two air stones with 50 ml/min aeration rate were set at the bottom center of half of RT (RT2AS). Then fish were reared until 14 days

post hatch (dph). Two-phase bubbly flow simulations were performed in the experimental tanks using a dispersed flow model. Survival rate in CT (54.7±11.0%) and RT1AS (55.3±6.0%) at 14 dph were significantly higher than that in RT2AS (29.6±9.3%; n=3, $p<0.05$). Growth of larvae was not significantly different between tank shapes and aerators. Swimbladder inflation rates were not significantly different between tank shapes and aerations, however, CT (58.9±28.3%) showed lower trends in swimbladder inflation than RTs (80-100%). The low-flow areas in tanks were defined as the area where the water velocity is slower than the swimming speed of red seabream larvae (4.6 mm/s) at 14 dph (4.8±0.2 mm in total length). At the water surface in tanks, the low flow areas were observed along the sidewalls in CT and RT1AS, and along the sidewalls and in the middle in RT2AS. These areas at tank bottom varied by tank shape, occurring at the edge of the tank wall on the bottom in the CT, from the air stone to the tank wall in RT1AS, and at the center (between air stones) and from the air stones to the tank wall in RT2AS. Further, high rotifer distribution at tank bottom coincided with the stagnate areas where the water velocity is lower than the swimming velocity of rotifers (1.3 mm/s). Hence, more complicated flow field was observed in the RT2AS. Velocity of water surface in CT (24.6 mm/s) was higher than those in RTs (19.3-22.1 mm/s). High water velocity at water surface was assumed to prevent the larvae from accessing water surface for swim bladder inflation. These results revealed that small-scale (50-l) experiments can be conducted for red

seabream larviculture using either a CT or RT1AS with present aeration system.

Second, larviculture of Pacific bluefin tuna (PBT) *Thunnus orientalis* larvae was performed using CT and RT (Chapter 3). One air stone with 100 ml/min aeration rate was set at the bottom center of three CT and RT. Since high mortality of PBT larvae due to sinking syndrome during the first 10 days is known to be obstacle, continuous illumination at 2000 lx was set to reduce the sinking syndrome. Survival of larvae in the CT (52.7±5.1%) at 8 dph was about 60-folds higher than that in the RT1AS (0.8±0.7%, $p<0.01$). Larval growth was not significantly different between tank shapes either in body length (CT: 4.23±0.26 mm, RT1AS: 4.09±0.20 mm) or dry weights (CT: 95.1±17.6 μg, RT1AS: 67.7±10.9 μg). The swimbladder inflation rates were not different significantly between tank shapes but similar trend as red seabream larvae was observed (CT: 16.5±14.5%, RT1AS: 56.9±3.5%). Low flow areas in RT, where the water velocity is lower than the swimming speed of PBT larvae (8.8 mm/s) at 8 dph (4.2±0.1 mm in total length), were larger than those in CT. High rotifer distribution was observed in the areas where the water velocity is slower than the swimming speed of rotifers (1.3 mm/s). Therefore, these low flow areas in RT1AS may be a cause of sinking syndrome of PBT larvae. These results indicated that small-scale (50-l) PBT larviculture experiments can be conducted using a CT with the present aeration system, but RT requires improvement in aeration.

To improve the aeration system in RTs for PBT larviculture, I tested several

different aerators (Chapter 4). One spherical air stone (5 cm in diameter) was set at the bottom center of RT as control. Then, long bar-shaped aerators were set at three patterns; two long aerators (30 cm) with 50 ml/min were set at the both narrow sides of bottom, one long aerator (60 cm) was set at the long side of bottom adjusting 400-500 ml/min aeration rates, and 4 baculiform aerators (15 cm) were set at the edge of the bottom walls with 100 ml/min aeration rates. Survival, growth and swimbladder inflation of PBT larvae were similar or lower in these 3 different aeration systems than control.

The flow field in different larval rearing tank shapes affected the survival of red seabream and PBT larvae. Sinking syndrome occurred in PBT larvae but not in red seabream. The CT and RT1AS with a single-pair vortex system were more suitable for survival of red seabream larvae than RT2AS with two single-pair vortex systems. Only the CT was feasible for survival of PBT larvae due to the stagnate areas at the bottom of the tank, which may cause the sinking syndrome. On the other hand, larger stagnate areas at the water surface of RTs may support fish larvae to access the air through the water surface for the swimbladder inflation. However, RTs are generally easier to handle and clean than CTs. Therefore, the improvement of aeration beyond a single air stone or different aerator types were required to get the optimal flow velocity in RTs. Measuring the rotifer density at the various points of a rearing tank is proposed for estimating the flow field in the tank, especially for the stagnate areas with higher rotifer densities than the average density.

# Chapter 1

# General introduction

A successful marine fish larviculture depends on the numerous factors such as optimal environmental conditions, nutritional composition and biochemical composition of larvae (Planas and Cunha, 1999). All of these factors can be controlled effectively under artificial conditions and this study emphasizes the physical environments in larval rearing tanks for marine fish larviculture.

Physical environments of tanks play important roles for marine fish larviculture (Sakakura et al., 2014, 2019). Many studies have been done to enhance marine fish larviculture by manipulating physical environmental factors, such as water temperature and salinity (Akatsu et al., 1983), aeration rate and water flow (Sakakura et al., 2007; Nakagawa et al., 2011), tank volumes (Başaran et al., 2004), tank proportion/shape (Ruttanapornvareesakul et al., 2007; Moore and Prange, 1994), water quality (Guillén et al., 1993), and illuminations (Cerqueira and Brügger, 2001; Kurata et al., 2017). Furthermore, the combination of tank shapes and flow field controlled by aeration rate was examined to improve the marine fish larviculture (Sakakura et al., 2019). However, this combination that will affect the flow field still needs to be investigated for marine fish larviculture to obtain the optimal flow field for larvae in rearing tanks.

It is assumed that flow fields in rearing tank have great impact on marine fish larvae

and provide a basis for tank design for larviculture (Harboe et al., 1998; Kolkovski et al., 2004). Flow field generated by aerators in larviculture tanks is important to prevent stratification, to insure oxygenation and to disperse live and artificial food (Backhurst and Harker, 1988). Both flow field and performance of fish larvae were influenced by tank proportions in larviculture and tank with smaller water surface area is suitable to reduce the mass mortality by surface tension related death of marine fish larvae (Ruttanapornvareesakul et al., 2007), in which mucus secreted on the body surface of larvae functions as a glue when larvae are attracted or carried to the water surface causing high mortality (Yamaoka et al., 2000).

The tank geometry and the water inlet and outlet are the main factors to define the flow patterns and velocities in tank (Klapsis and Burley, 1984; Timmons et al., 1998). Moore and Prange (1994) reported that the tank shapes and sizes can differ the flow field structure in rearing tanks. Cylindrical tank provides more stable flow patterns, more homogenous distribution of dissolved oxygen and better self-cleaning features (Oca and Masalo, 2013) and a more uniform water quality (Timmons et al., 1998). In the case of rectangular tank, it provides plug-flow patterns, an irregular and unpredictable flow patterns, and generates poor water mixing conditions which can influence the welfare of fish in several ways (Oca et al., 2004; Duarte et al., 2011).

The optimum tank size varies with fish species, and the large tanks are preferable to

conduct the larval rearing and the larvae may grow fast without showing any signs of starvation (Theilacker, 1980). Larger rearing tanks are better for the early larvae because of (1) the wide space to swim, (2) a lesser chance to be damaged from contacting the tank walls and (3) the getting preferable water quality, although it is hard to maintain the required greater qualities of food (Estudillo et al., 1998). On the other hand, small tanks (40-l) are advantageous for larval rearing because these tanks are (1) easily manipulated and (2) cost-effective (Estudillo et al., 1998).

Two marine fishes, red seabream *Pagrus major* and Pacific bluefin tuna *Thunnus orientalis*, were chosen as experimental animals in this study. Red seabream is an important species for commercial coastal fisheries in Japan and larval rearing techniques are well established on commercial scale (Foscarini, 1988). Fertilized eggs are easily obtained from the mature culture fish without depending on the unstable catch of fry in the ocean, and the larvae fast growth and tolerance to a relatively wide temperature range (Foscarini, 1988). However, little is known about the small-scale (<50-l) larviculture for this species.

Aquaculture of bluefin tunas has been developed in some Mediterranean countries, Japan, Australia and Mexico as an economically important industry (Benetti et al. 2016). Although the life cycle of Pacific bluefin tuna (PBT) under aquaculture conditions had completed, heavy mortality in the early life stages still has been observed in mass-culture,

including cannibalism during the late larval and juvenile stages and collision with tank or net walls during the juvenile stage (Sawada et al., 2005). Sinking syndrome occurs in PBT larvae during the dark period because larvae with higher specific gravity than the seawater sink to the bottom of rearing tank due to low swimming activity at dark (Takashi et al., 2006; Tanaka et al., 2009; Nakagawa et al., 2011). This cause of high mortality during early larval stages must be solved for mass production of this species to gain a stable large-scale supply of seedlings (Sawada et al., 2005; Nakagawa et al., 2011; Woolley et al., 2013). Therefore, I tried to prevent the early larval mortality of PBT larvae, using the different small tank shapes.

The hypothesis of my study is that tank shapes and aerations may affect the survival, growth and swimbladder inflation of marine fish larvae. The larviculture experiments were conducted in small 50-l tanks of different shapes, cylindrical (CT) with axisymmetrical flow field patterns (Sumida et al., 2013) and rectangular tank (RT) with three-dimensional complicated flow field patterns (Takakuwa et al., 2018), to examine the hypothesis. I started investigating the effects of tank shapes and aerators on survival, growth and swimbladder inflation of red seabream larvae (Chapter 2) and the effect of tank shape on survival, growth and swimbladder inflation of PBT larvae to improve the early mortality due to sinking syndrome (Chapter 3). Final experiments investigated the effects of aerations on survival, growth and swimbladder inflation of PBT larvae in RT to improve

the aeration system, using different aerator types (Chapter 4). All findings were generally discussed in Chapter 5.

# Chapter 2

# Effects of tank shapes and aerations on survival, growth and swim bladder inflation of red seabream *Pagrus major* larvae

## 2.1 Introduction

Physical conditions in larval rearing tanks are important factors for survival and growth of marine fish larviculture (Nour et al., 2004; Tanaka et al., 2018). In these environmental factors, many studies have focused on water temperature (e.g., Seikai et al., 1986; Fukuhara, 1990; Guillén et al., 2014; Kim et al., 2015; Tanaka et al., 2018; Honryo et al., 2018), salinity (e.g., Hart et al., 1996; Shi et al., 2008; Kim et al., 2015) and illuminations (e.g., Hart et al., 1996; Partridge et. al., 2011; Villamizar et al., 2011; Stuart and Drawbridge, 2012; Kurata et al. 2017; Honryo et al., 2018) for enhancing larval survival and growth. However, little attention has been paid to the flow field structures generated by aerators in larval rearing tanks as a factor of physical environments (Klapsis and Burley, 1984), which is an important aspect for tank designs in larviculture (Harboe et al., 1998; Kolkovski et al., 2004). Previous studies revealed that the flow field in rearing tanks influenced survival and growth of some marine fish larvae (Shiotani et al., 2003; Ruttanapornvareesakul et al. 2007; Sakakura et al. 2007, 2014, 2019). Flow field can prevent the stratification, insure oxygenation and disperse live and artificial food to obtain

the high survival and growth of larvae (Backhurst and Harker, 1988). Moreover, the fast flow can produce the fast rate air bubbles which will directly damage to larvae, and can have caused a less chance to encounter the prey organisms and the reducing direct supply of oxygen for larvae (Shiotani et al., 2003). However, interactive factors of flow field and larval fish behavior in rearing tanks remain to be investigated (Aung Naing Win et al. 2020).

Red seabream *Pagrus major* is important cultured marine fish in Japan (Foscarini, 1988). Rearing techniques of this species had been established at the commercial scale and many studies regarding larviculture have been done with various aspects, such as water quality (Guillén et al., 1993), water temperature and light intensity (Honryo et al., 2018), nutritional requirements (Matsunari et al, 2008; Linn et al., 2014; Kim et al., 2014; Dossou et al., 2018; Hossain et al., 2018) and larval development (Fukuhara, 1985; Khoa et al., 2019a, b). However, little attention has been paid to the small-scale larviculture experiments for this species. In this study, I hypothesized that tank shapes and aerations may affect the survival and growth of red seabream larvae. To test this hypothesis, larviculture experiments were conducted in small 50-l tanks of different shapes with different aeration systems. I chose a cylindrical tank (CT) with axisymmetrical flow field patterns (Sumida et al., 2013), and a rectangular tank (RT) with three-dimensional complicated flow field patterns (Takakuwa et al., 2018), to investigate the effect of tank

shapes and aerations on survival, growth and swimbladder inflation of red seabream larvae. I also examined the rotifer distribution to estimate the flow field in tanks, and visualized the flow fields by simulation.

## 2.2 Materials and methods

### 2.2.1 Experiment 1

Three blue plastic CT (46 cm in diameter) and blue acrylic RT (60 cm × 30 cm × 28 cm depth) with a 50-l working volume were used for experiment 1. The aspect ratio (liquid depth/internal radius of tank) of CT is 1.3. I assumed that the aspect ratio of RT is also 1.3 because the water surface area and depth of RT ($1.8 \times 10^3$ $cm^2$ × 28 cm) are almost equal to those of the CT ($1.7 \times 10^3$ $cm^2$ × 30 cm). Artificial seawater (Marine Art Hi, Tomita Pharmaceutical, Japan) of 32 parts per thousand (ppt) was filled into each experimental tank in a 22ºC temperature-controlled room of the Aquaculture Biology Laboratory, Nagasaki University, Japan. A spherical aerator (5 cm in diameter) was set at the center on the bottom of each experimental tank (three CT and RT) with 50 ml/min to generate the water flow in tanks. The low velocity areas were defined as the area where the water velocity is slower than the swimming speed of red seabream larvae (4.6 mm/s) at 14 dph (4.8±0.2 mm in total length). Swimming speed of larvae was estimated by following the method of Fukuhara (1985), in which swimming velocity of red seabream larvae was less

than 1 standard length/s during larval phase. Maximum light intensity was 1000 lx at the water surface with natural photoperiod.

Fertilized eggs of red seabream were purchased from a private hatchery (Ogata Suisan Co., Ltd., Kumamoto Prefecture) and were transported to the Aquaculture Biology Laboratory, Nagasaki University on 6 April 2018. Eggs were firstly transferred into a 100-l polycarbonate tank and larvae were kept until 3 day post hatch (dph) in the same tank at 22ºC and 32 ppt. On 3 dph, larvae were distributed into each experimental tank and reared until 14 dph with static conditions.

Super Chlorella V12 (Chlorella Industry Co., Fukuoka, Japan) was added into each experimental tank as green water and the density of Chlorella V12 was adjusted at $5 \times 10^5$ cells/ml once daily. Super Chlorella V12 was also used to enrich the rotifers *Brachionus plicatilis* which were fed to larvae at 10 individuals/ml when the mouth opened (3 dph). I changed 1/3 water of experimental tanks on 9 dph. Water samples to assess rotifer density were collected from 9 stations (3 ml for each) in a vertical cross-section of CT (Figure. 2.1 (a)) and 27 stations in the quarter segments of RT (Figure 2.1 (b)), using pipet. I defined the stagnate areas in tanks as high rotifer density areas. To estimate the stagnate areas in tank, the swimming speed of rotifer was referred from Yúfera et al. (2005), in which swimming speed of rotifers was 1.3 mm/s. Since the rotifer numbers in the tanks increased during the experimental period, I standardized the rotifer distribution in

the tanks each sampling day using the following equations:

$$deviation\ value\ at\ station\ x\ on\ day\ i = \frac{rotifer\ density\ at\ station\ x - mean\ rotifer\ density\ on\ day\ i}{standard\ deviation\ of\ rotifer\ density\ on\ day\ i}$$

On the last day of experiment (14 dph), the survival rate was calculated after counting all surviving larvae in the tested tanks. Then, 30 fish in each tank were anaesthetized with 200 ppm of MS222 (Tricaine; Sigma-Aldrich) and were fixed with 5% formalin solution. Formalin-preserved fish were individually measured for morphometric characters by a digital microscope (VH-6300; Keyence, Osaka, Japan).

An air-exposure test was conducted by following the method of Hagiwara et al. (2016) to compare the viability of the fish larvae in different tank shapes on the last day of larviculture. I caught larvae by using a net (130×145 mm, Super Net M; SANY, Kanagawa Japan) for each tank (n=3) and exposed them to air for 1 min. After this, the fish larvae were immediately returned to seawater and their survival was observed every 4 hour for 24 hours.

Physical environmental parameters during the experiment were as follows: water temperature 19.0ºC, salinity 32.0-32.1 ppt, dissolved oxygen 7.0 mg/l, pH 7.93-7.96 and total ammonia ($NH_3$+$NH_4^+$) 0.35-0.36 mg/l.

### 2.2.2 Experiment 2

Three blue plastic CT (46 cm in diameter) and six blue acrylic RT (60 cm × 30 cm × 28 cm depth) containing a 50-l working volume were used in this study. The CT has aspect ratio of 1.3 (liquid depth/internal radius of the tank). I assumed that the aspect ratio of RT is also 1.3 because the water surface area and depth of RT ($1.8 \times 10^3$ $cm^2 \times 28$ cm) are almost equal to those of the CT ($1.7\times 10^3$ $cm^2 \times 30$ cm). Artificial seawater (Marine Art Hi, Tomita Pharmaceutical, Japan) of 32 parts per thousand (ppt) was filled into each experimental tank in a 22ºC temperature-controlled room of the Aquaculture Biology Laboratory, Nagasaki University, Japan. Water flow was generated by a spherical aerator (5 cm in diameter) setting at the center on the bottom of three CTs and RTs (RT1AS) with 100 ml/min aeration rate, and two spherical aerators were placed at the bottom center of half of three RTs (RT2AS) with 50 ml/min aeration rate. The low velocity areas were defined as the place where the water velocity is lower than the swimming velocity of red seabream larvae (4.6 mm/s) at 14 dph (4.8±0.2 mm in total length). Swimming velocity of larvae was estimated to define the low velocity areas in larval rearing tanks by following the method of Fukuhara (1985), in which swimming velocity of red seabream larvae was less than 1 standard length/s during larval phase. Maximum light intensity at the water surface was 1000 lx with natural photoperiod.

On 20 February 2020, fertilized eggs of red seabream were purchased from a

private hatchery (Ogata Suisan Co., Ltd., Kumamoto Prefecture) and were transported to the Aquaculture Biology Laboratory, Nagasaki University. Five hundred eggs were directly distributed into each experimental tank. Larvae were reared until 14 days post hatch (dph) with static conditions.

Super Chlorella V12 (Chlorella Industry Co., Fukuoka, Japan) was added to the experimental tanks as green water, adjusting the density at $5\times10^5$ cells/ml once daily. Rotifers *Brachionus plicatilis* enriched with Super Chlorella V12 were fed to larvae at 10 individuals/ml when the mouth opened (3 dph). I changed 1/3 water of experimental tanks on 9 dph. Water samples to assess rotifer density were collected from 9 stations (3 ml for each) in a vertical cross-section of CTs (Figure 2.1. (a)) and 27 stations in the quarter segments of RTs (Figure 2.1. (b), (c)), using a pipet. The swimming speed of rotifer (1.3 mm/s) was referred from Yúfera et al. (2005) for estimating the stagnate areas in rearing tanks. Since the rotifer numbers in the experimental tanks increased during the experimental period, I standardized the rotifer distribution in the tanks each sampling day using the following equations:

$$deviation\ value\ at\ station\ x\ on\ day\ i = \frac{rotifer\ density\ at\ station\ x - mean\ rotifer\ density\ on\ day\ i}{standard\ deviation\ of\ rotifer\ density\ on\ day\ i}$$

On 14 dph, all surviving larvae in the experimental tanks were counted to calculate the survival rate. Then, 30 fish in each tank were anaesthetized with 200 ppm of

MS222 (Tricaine; Sigma-Aldrich) and observed under a dissecting microscope with transmitted light to see whether the swimbladder was inflated by checking air bubbles in the bladder. Then, larvae were fixed with 5% formalin solution. Formalin-preserved fish were individually measured for morphometric characters by a digital microscope (VH-6300; Keyence, Osaka, Japan), and then dried at 60°C for 24 h for measurement of the dry body weight by an ultra-micro balance (UMX2; Mettler Toledo, Columbus, OH, USA).

Two-phase bubbly flow simulations were performed in the experimental tanks using a dispersed flow model that was developed by Takakuwa et al. (2018). Its governing equations are composed of the conservation laws of mass and momentum of liquid (water) and gas (air bubble) phases, in which the effects of pressure gradient, drag and lift forces acting on bubbles, gravitational acceleration and flow viscosity are taken into account. A simplified marker and cell (SMAC) method was used to solve the governing equations. For the liquid phase, the free surface was assumed to be flat, and a no-slip boundary condition was used. On the other hand, an outflow condition was given for the gas phase at the free surface. As boundary conditions on the wall surface of tanks, a no-slip condition was given for the liquid phase, while a slip condition was given for the gas phase. An air inlet (square with a side length of 22 mm; aeration rate, 50 or 100 ml/min) was set at the center of the bottom surface. The diameter of a bubble was set to 2.0 mm. Flow simulations were

performed for 450 s, and averaged flow fields for the last 150 s are discussed in this research.

Physical environmental parameters during the experiments were as follows: water temperature 21.1-21.9ºC, salinity 32.4-32.7 ppt, dissolved oxygen 7.0-7.4 mg/l, pH 7.9, and total ammonia ($NH_3$+$NH_4^+$) 0.30-0.31 mg/l.

### 2.2.3 Statistical analysis

The differences of survival, growth and viability of larvae between different tank shapes were determined by either Student's t-test for parametric test which the variance of the two groups are equal after a random variable of two groups is normally distributed by Shapiro-Wilk normality test or Mann-Whitney *U* test for non-parametric test which a random variable of single or two groups is not normally distributed by Shapiro-Wilk normality test in the experiment 1. Differences in the survival, growth and swimbladder inflation rates of larvae among tanks with different aeration systems of the experiment 2 were determined by one-way ANOVA followed by Tukey HSD test. The rotifer distribution in tanks for both experiment 1 and 2 was standardized by the deviation value on day *i*, and were compared by two-way ANOVA followed by Tukey HSD test. All analyses were done by R 3.4.1 software, and a 5% level of confidence was considered as a significant difference.

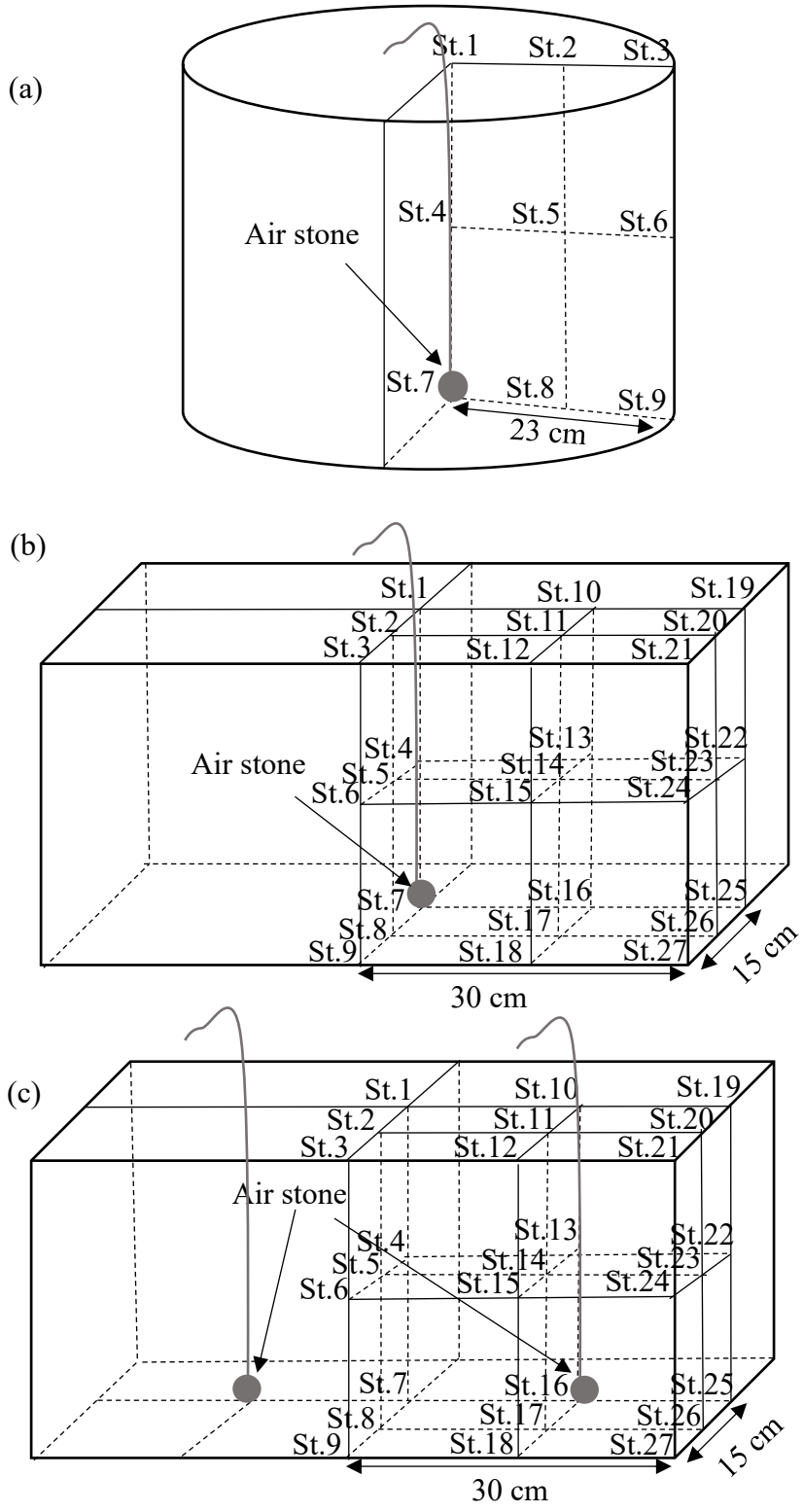



**Figure 2.1.** Sites for rotifer distribution in (a) cylindrical tank with one air stone at the center of tank bottom, (b) rectangular tank with one air stone at the center of tank bottom and (c) rectangular tank with two air stone at the each center of half of tank bottom.

## 2.3 Results

### 2.3.1 Survival, growth and viability of larvae of experiment 1

For the experiment 1, the hatching of larvae was 95.0±1.7%. Survival rates of larvae at 14 dph were not significantly difference between CT (67.6±13.7%) and RT1AS (73.6±14.2%, Student's t-test, n=3, *df*=4, *t*=0.0560, *p*=0.9580; Table 2.1). For growth of larvae at 14 dph (Table 2.2), although standard length of larvae was significantly different between different tank shapes (Student's t-test, n=3, *df*=4, *t*=3.5980, *p*=0.0228), other morphological parameters of larvae were not significant different between different tank shapes (Mann-Whitney *U* test, n=3, *W*=9, *p*=1.0000 for total length; Student's t-test, n=3, *df*=4, *t*=0.3971, *p*=0.7115 for body depth/standard length; Mann-Whitney *U* test, n=3, *W*=3, *p*=0.6428 for head length/standard length; Student's t-test, n=3, *df*=4, *t*=-2.2942, *p*=0.0834 for eye length/standard length). The viability of larvae at 14 dph in CT (73.3±15.3%) and RT1AS (73.3±5.8%) was not significantly different between different tank shapes (Mann-Whitney *U* test, n=3, *W*=4, *p*=1.0000, Table 2.1).

### 2.3.2 Survival, growth and swimbladder inflation rates of larvae of experiment 2

The hatching rate of eggs was 98.3±2.9%. The survival rates at 14 dph in CT (54.7±11.0%) and RT1AS (55.3±6.0%) were significantly higher than RT2AS (29.6±9.3%, one-way ANOVA, n=3, *df*=2, *F*=7.5200, *p*=0.0232, Tukey HSD test, $p<0.05$; Table 2.3). The body

length (one-way ANOVA, n=3, *df*=2, *F*=2.1380, *p*=0.1990) and dry weight (one-way ANOVA, n=3, *df*=2, *F*=0.0830, *p*=0.9210) of larvae at 14 dph were not significantly different among tanks (Table 2.3). Swimbladder inflation rates were also not significantly different between tank shapes and aerators (one-way ANOVA, n=3, *df*=2, *F*=3.7650, *p*=0.0872, Table 2.3), but CT (58.9±28.3%) showed lower trend than RTs (80-100%). Morphological parameters were not significantly different between tank shapes and aerations, either (one-way ANOVA, n=3, *df*=2, *F*=2.2370, *p*=0.1880 for standard length; *df*=2, *F*=1.1720, *p*=0.3720 for body depth/standard length; *df*=2, *F*=1.0890, *p*=0.3950 for head length/standard length; *df*=2, *F*=0.1770, *p*=0.8420 for eye diameter/standard length; Table 2.4).

### 2.3.3 Flow field in rearing tanks

Three-dimension visualization of streamlines in the experimental tanks are shown in Figure 2.2. In the case of the CT, upward flows by the effect of air bubbles generated from the air stone radiated outward in the vicinity of the water surface, and then downward flows were observed along the sidewalls of the tank. In the RT1AS, the central upward flows radiated outward in the same manner as the CT, but a more complicated flow field was generated due to the effect of rectangular corners and the non-axisymmetric shape of the tank. In the RT2AS, two single-pair vortex systems forming two cubes were observed

by two air stones. Cumulative areas of flow velocity magnitudes in the water column obtained from the flow simulation results were shown in Figure 2.3. The average velocity of total water volume is around about 1.2 times higher in CT than RTs (Figure 2.3. (a)). Same trend is found at the surface area where CT has higher velocity areas than RTs (Figure 2.3. (b)), and the velocity in surface area of CT and RTs is about three times faster than that in the whole water column (Figure 2.3). Visualization of streamlines at the surface areas are shown in Figure 2.4, with color of the flow velocity magnitude. In the CT and the RT1AS, most streamlines eventually moved toward the sidewalls of tanks and streamed as downward flows. Low velocity regions in these tanks were observed along the sidewalls of tanks. In the RT2AS, streamlines moved toward the walls and at the center of tank (between two aerators). And then, they streamed as downward flows where low velocity regions were observed. Figure 2.5 shows visualization of streamlines colored by the flow velocity magnitude at the bottom areas (where water depth is more than 25 cm) in tanks. Streamlines at the bottom regions in the CT moved from the low-velocity areas along the sidewalls toward the air stone, where they reverted to the upward flows. Streamlines from both short sides of the rectangle created a vortex in RT1AS, colliding in the vicinity of the center of the long sides. Low velocity regions were observed along the sidewalls in the RT1AS and the RT2AS but they were also found from the aerator to tank walls and between two aerators in the RT2AS. Streamlines toward the air stones in the

RT2AS did not form a vortex like in RT1AS. Flow structures were more complicated in both the RT1AS and RT2AS than in the CT, and low velocity magnitude at the bottom regions were larger contrary to the surface area.

With the respect of the tank bottom, the low velocity areas were observed along the edge of the tank walls in CT. It was coincided with the end of downward flow areas where flows continue toward the aerator to be reverted to upward flow. In the RT1AS, low velocity areas and eddy at the bottom of tank were observed from the aerator to the tanks walls. Likewise, high rotifer densities were observed from aerator to the tanks walls in RT1AS. At the bottom of RT2AS, low velocity areas were found from the aerators to the tank walls and between two aerators. Similarly, high rotifer density areas in RT2AS were observed at the same areas with stagnate areas. For the whole water volume, velocity was higher in CT than in RTs. The velocity at the water surface areas was almost 1.2 times higher in CT than in RTs.

### 2.3.4 Rotifer distribution

For experiment 1, rotifer density increased throughout the experiment, reaching 24.5 individuals/ml in CT and 38.7 individuals/ml in RT1AS at 14 dph, respectively (n=6). In CT, the rotifer distribution was different among sampling stations (two-way ANOVA, $df$=8, $F$=19.2280, $p$<0.0001; Tukey HSD test $p$<0.05; Figure 2.6 (a)) but was not affected by

sampling days (two-way ANOVA, *df*=5, *F*=0.0010, *p*=1.0000). There were interactions between stations and days on rotifer density in CT (two-way ANOVA, *df*=40, *F*=2.6790, *p*<0.0001). With the respect of RT1AS, rotifer distribution was also affected by stations (two-way ANOVA, *df*=26, *F*=14.2210, *p*<0.0001; Tukey HSD test, *p*<0.05; Figure 2.6 (b)), although it was not associated with days (two-way ANOVA, *df*=5, *F*=0.0010, *p*=1.0000). Interactions between stations and days were detected (two-way ANOVA, *df*=130, *F*=1.5860, *p*=0.0008).

For the experiment 2, rotifer density also increased throughout the experimental period, reaching 49.9 individuals/ml in CT, 60.5 individuals/ml in RT1AS and 82.2 individuals/ml in RT2AS at 14 dph, respectively (n=6). The rotifer distribution in CT was affected by stations (two-way ANOVA, *df*=8, *F*=26.1420, *p*<0.0001; Tukey HSD test, *p*<0.05; Figure 2.7 (a)) but was not associated with sampling days (two-way ANOVA, *df*=5, *F*=0.0020, *p*=1.0000). There were no interactions between stations and days in CT (two-way ANOVA, *df*=40, *F*=0.7560, *p*=0.8370). In RT1AS, the rotifer distribution was associated with stations (two-way ANOVA, *df*=26, *F*=24.2660, *p*<0.0001; Tukey HSD test, *p*<0.05; Figure 2.7 (b)) but not with days (two-way ANOVA, *df*=5, *F*=0.0070, *p*=1.0000). There were interactions between stations and days in RT1AS (two-way ANOVA, *df*=130, *F*=2.1510, *p*<0.0001). For RT2AS, the rotifer distribution was associated with stations (two-way ANOVA, *df*=26, *F*=6.5440, *p*<0.0001; Tukey HSD test, *p*<0.05; Figure 2.7 (c))

but not with days (two-way ANOVA, $df$=5, $F$=0.0060, $p$=1.0000). There were no interactions between stations and days (two-way ANOVA, $df$=130, $F$=1.0710, $p$=0.3188).

**Table 2.1.** Survival rate and Viability of *Pagrus major* larvae at 14 dph in different tank shapes of the experiment 1.

| Tank | Survival rate (%) | Viability (%) |
|---|---|---|
| Cylindrical | 67.6±13.7 | 73.3±15.3 |
| Rectangular | 73.6±14.2 | 73.3±5.8 |

Results are mean values ± SD (n=3).

**Table 2.2.** Morphological parameters of *P. major* larvae at 14 dph in different tank shapes of the experiment 1.

| Tank | TL (mm) | SL (mm)* | BD/SL (mm) | HL/SL (mm) | ED/SL (mm) |
|---|---|---|---|---|---|
| Cylindrical | 3.93±0.08 | 3.73±0.08 | 0.223±0.015 | 0.272±0.005 | 0.106±0.003 |
| Rectangular | 3.70±0.04 | 3.53±0.05 | 0.219±0.005 | 0.266±0.008 | 0.113±0.004 |

Results are mean values ± SD (n=3). The asterisk indicates significant differences by Student's t-test ($p$<0.05). Abbreviation: TL=Total length, SL=Standard length, BD Body depth, HL=Head length, ED=Eye diameter.

**Table 2.3.** Survival, growth and swimbladder inflation rates of *P. major* larvae at 14 dph in different tank shapes and aerations of the experiment 2.

| Tank | Survival (%) | Total length (mm) | Dry weight ($\mu$g) | Swimbladder inflation (%) |
|---|---|---|---|---|
| Cylindrical | 54.7±11.0[a] | 4.71±0.23 | 165.2±41.8 | 58.9±28.3 |
| Rectangular with one air stone | 55.3±6.0[a] | 4.94±0.13 | 152.6±38.1 | 94.4±6.9 |
| Rectangular with two air stones | 29.6±9.3[b] | 4.65±0.17 | 155.6±38.1 | 92.2±10.7 |

Results are mean values ± SD (n=3). Alphabets in superscripts in the column represent significant differences (a>b, Tukey HSD test, $p<0.05$).

**Table 2.4.** Morphological parameters of *P. major* larvae at 14 dph in different tank shapes and aerations of the experiment 2.

| Tank | SL (mm) | BD/SL (mm) | HL/SL (mm) | ED/SL (mm) |
|---|---|---|---|---|
| Cylindrical | 4.51±0.25 | 0.25±0.02 | 0.27±0.02 | 0.10±0.01 |
| Rectangular with one air stone | 4.76±0.12 | 0.23±0.02 | 0.26±0.02 | 0.10±0.01 |
| Rectangular with two air stones | 4.45±0.18 | 0.24±0.02 | 0.26±0.02 | 0.10±0.01 |

Results are mean values ± SD (n=3). Abbreviation: SL = Standard length, BD = Body depth, HL = Head length, ED = Eye diameter.

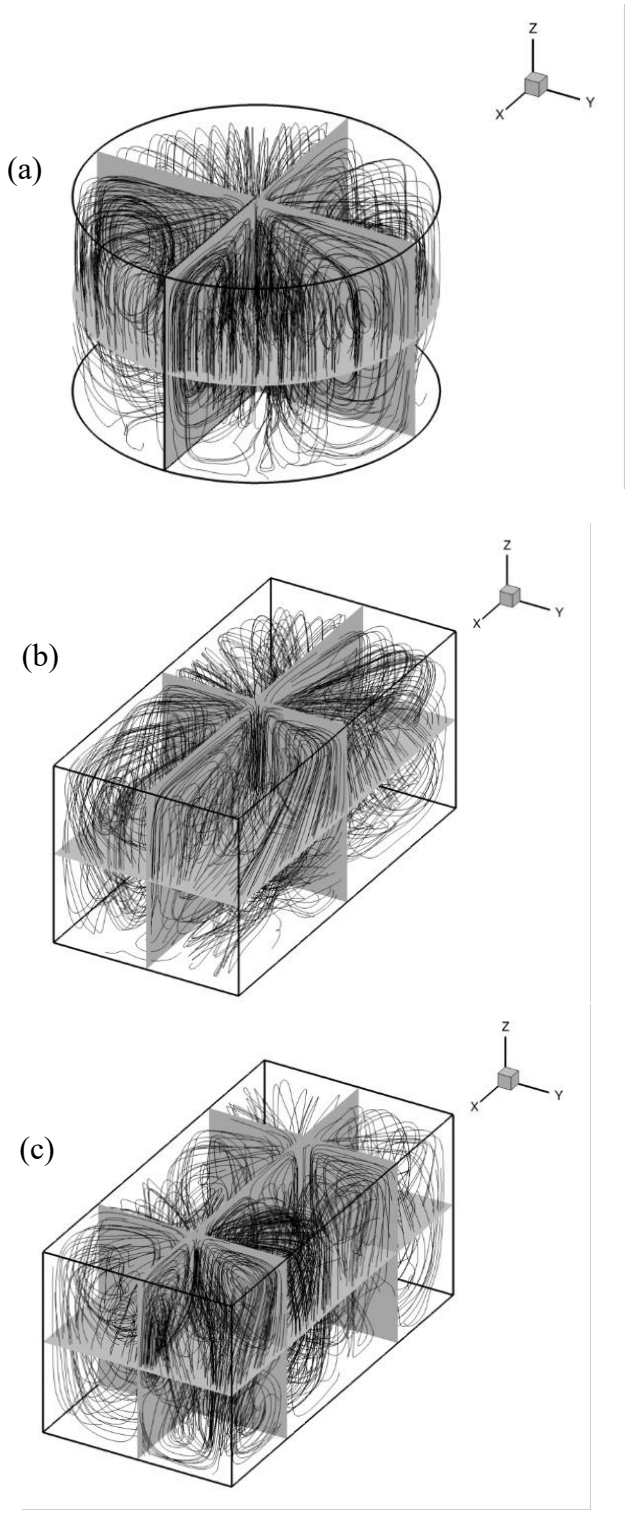
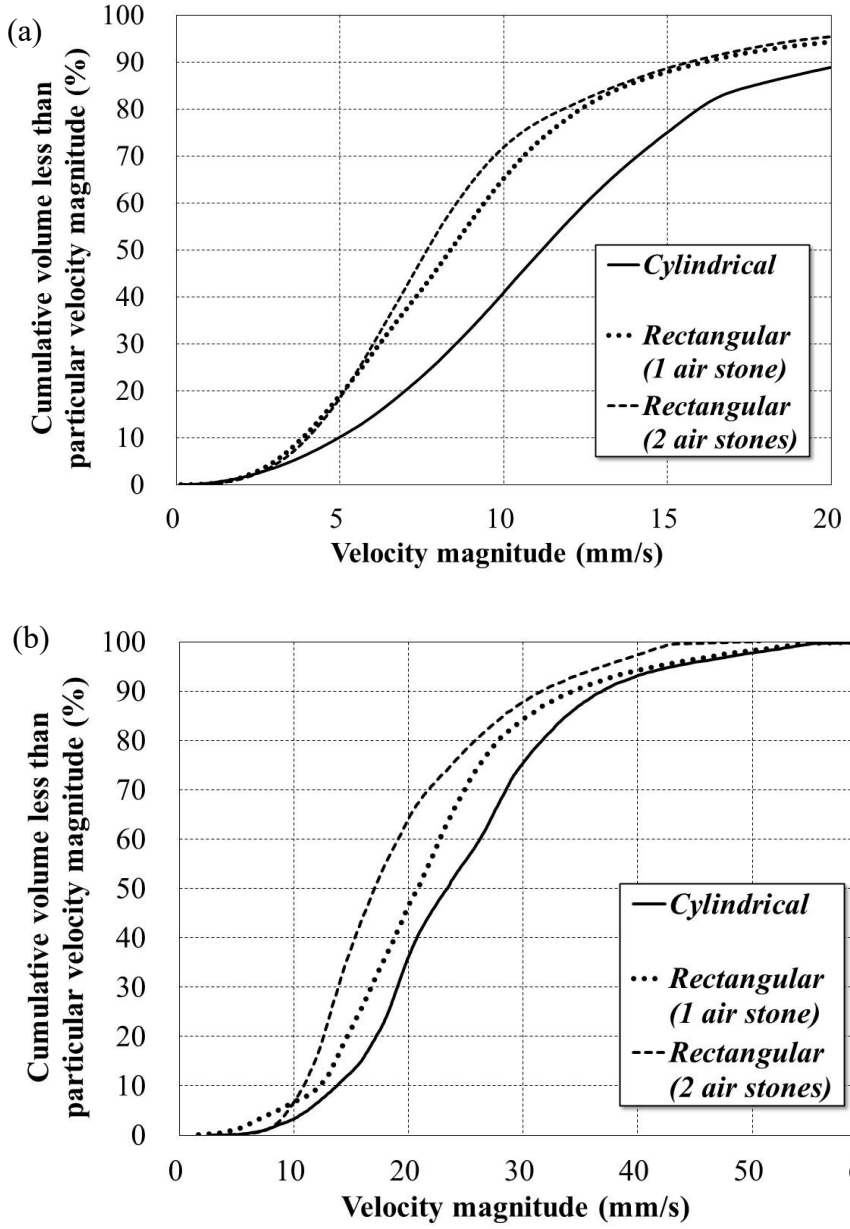



**Figure 2.2.** Three-dimensional streamlines predicted in (a) a cylindrical tank (ø 46 cm × 29 cm depth) with one aerator at the bottom center with 100 ml/min aeration rate in this study, (b) a rectangular tank (60×30×28 cm depth) with one aerator at the bottom center with 100 ml/min aeration rate and (c) a rectangular tank (60×30×28 cm depth) with two aerators at the bottom center of half of tank with 50 ml/min aeration rate for each aerator.




(a) Average velocity
Cylindrical : 12.5mm/s
Rectangular (1) : 9.76mm/s
Rectangular (2) : 9.32mm/s

(b) Average velocity
Cylindrical : 24.6mm/s
Rectangular (1) : 22.1mm/s
Rectangular (2) : 19.3mm/s

**Figure 2.3.** Cumulative area (%) of the velocity magnitude (< x mm/sec) (a) in total water volume and (b) at water surface between the tanks applied in this study. For instance, (a) the water mass having a velocity between 0 and 10 mm/s occupied 40%, 65% and 72% of total water volume in the CT, RT1AS and RT2AS and (b) the water mass having a velocity between 0 and 20 mm/s occupied 36%, 48% and 64% at the water surface areas in CT, RT1AS and RT2AS, respectively.

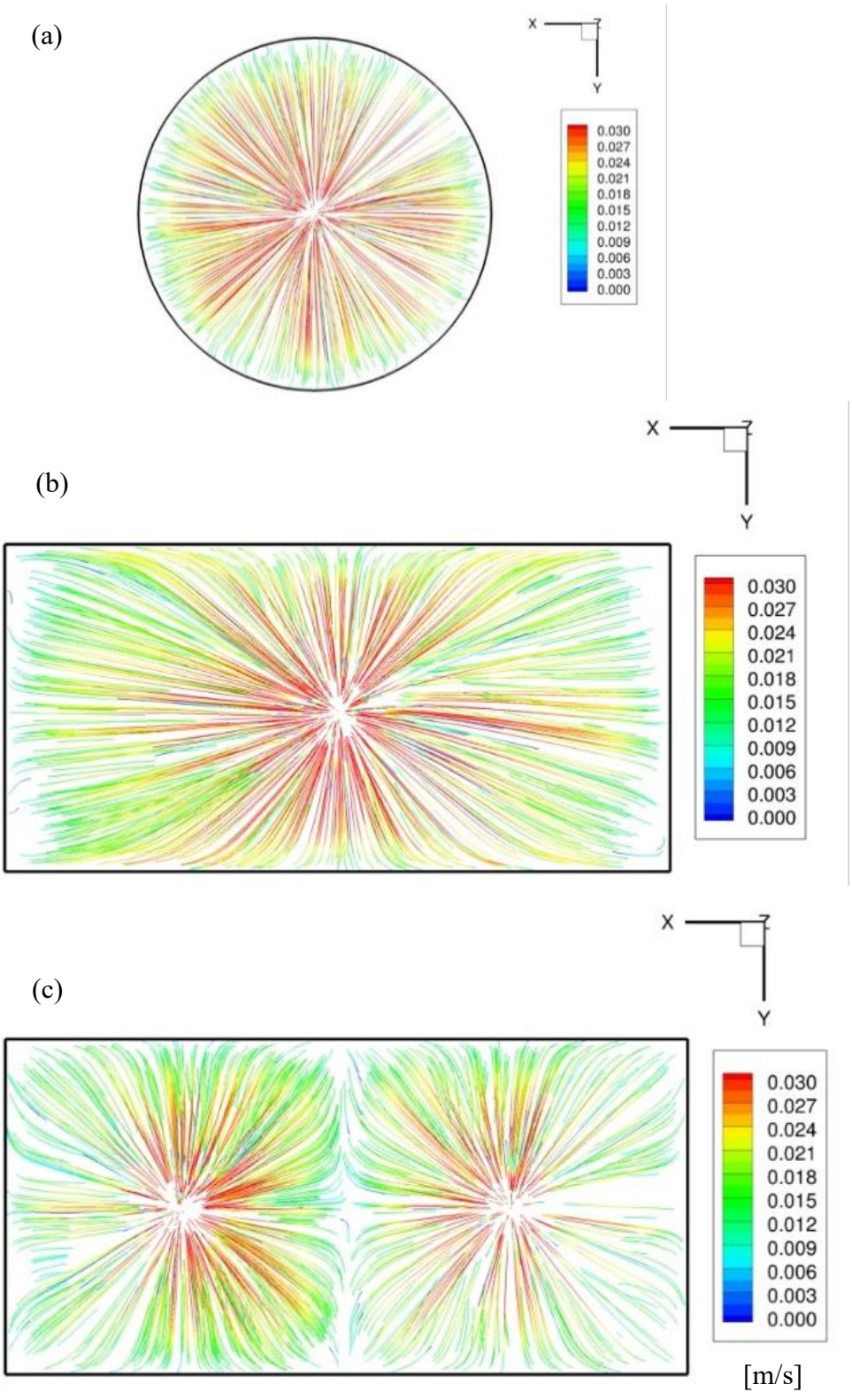



**Figure 2.4.** Visualization of streamlines at the surface areas of (a) a cylindrical tank (ø 46 cm) with one air stone at the center of the tank, (b) rectangular tank (60 × 30 cm) with one air stone at the center of the tank, and (c) rectangular tank (60 × 30 cm) with two air stones at the center of each half of the tank.

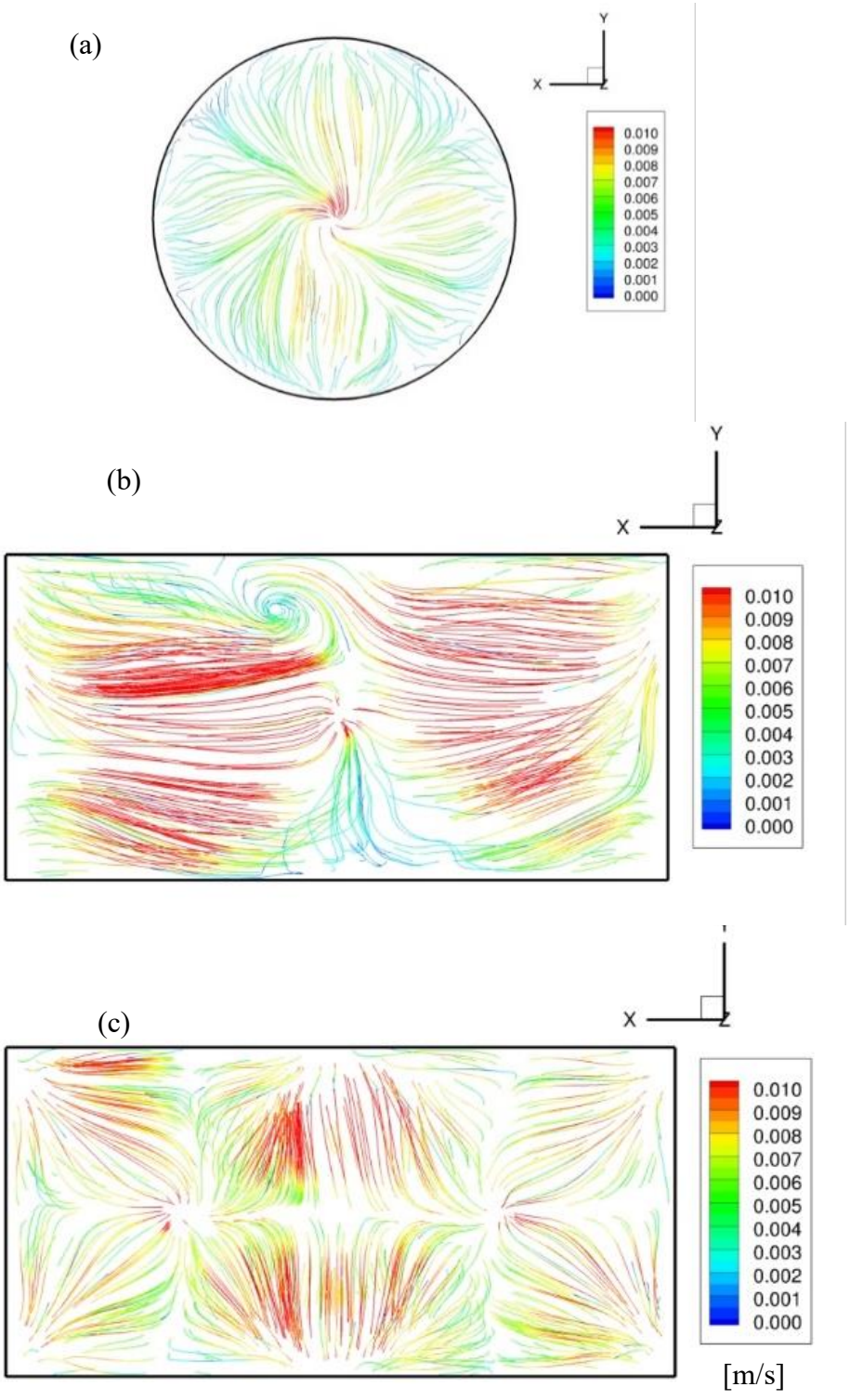



**Figure 2.5.** Visualization of streamlines at the bottom areas of (a) a cylindrical tank (ø 46 cm) with one air stone at the center of the tank, (b) rectangular tank (60 × 30 cm) with one air stone at the center of the tank and (c) rectangular tank (60 × 30 cm) with two air stones at the center of each half of the tank.

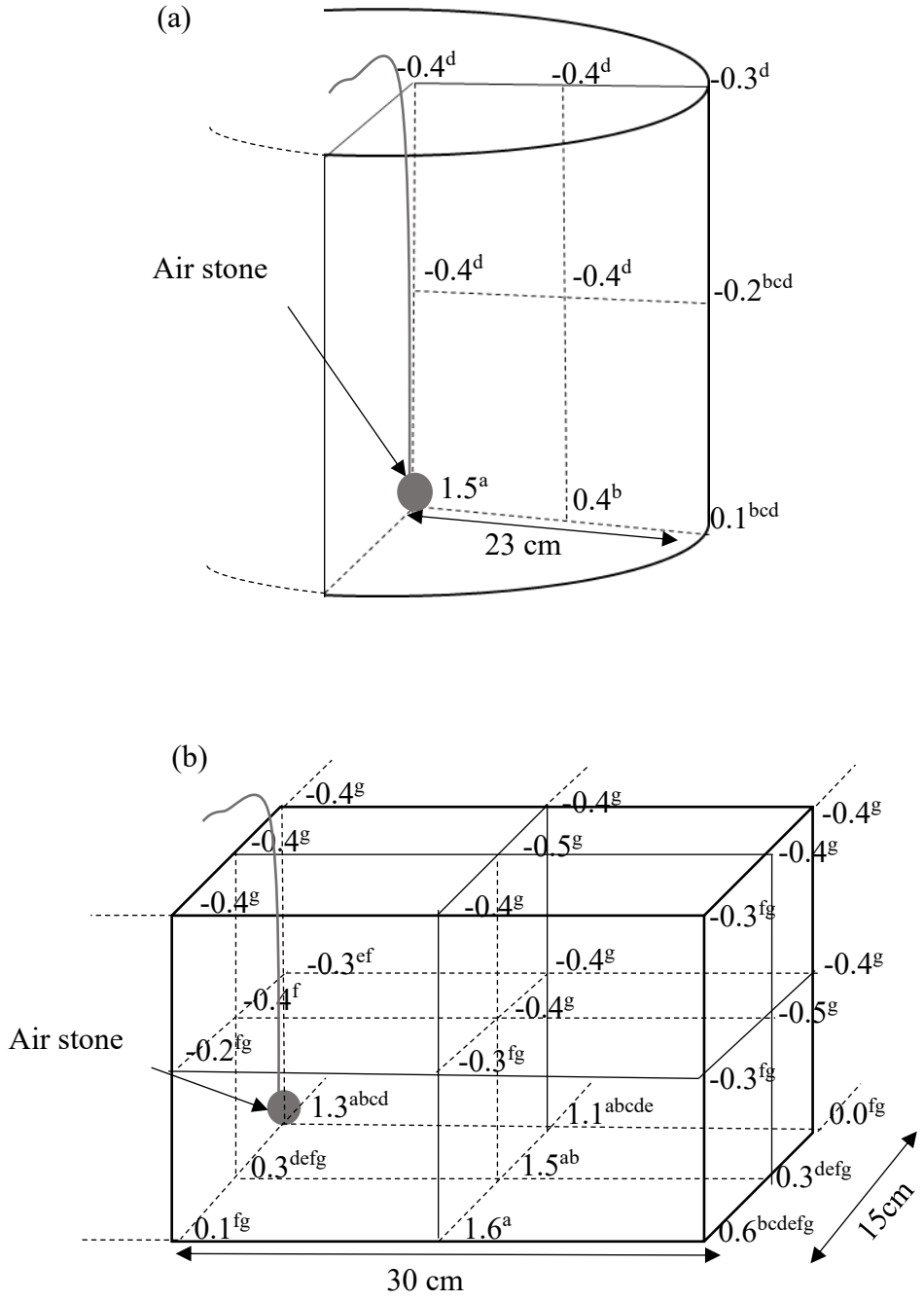



**Figure 2.6.** Rotifer distribution (a) at half cross section in cylindrical tank, (b) in the quadrisection in rectangular tank of the experiment 1. Values are average of deviation values during culture period and alphabets in superscripts represent the significant differences between the stations that is one of the factors of two-way ANOVA (n=6, a>b>c>d>e>f>g, Tukey HSD, $p<0.05$).

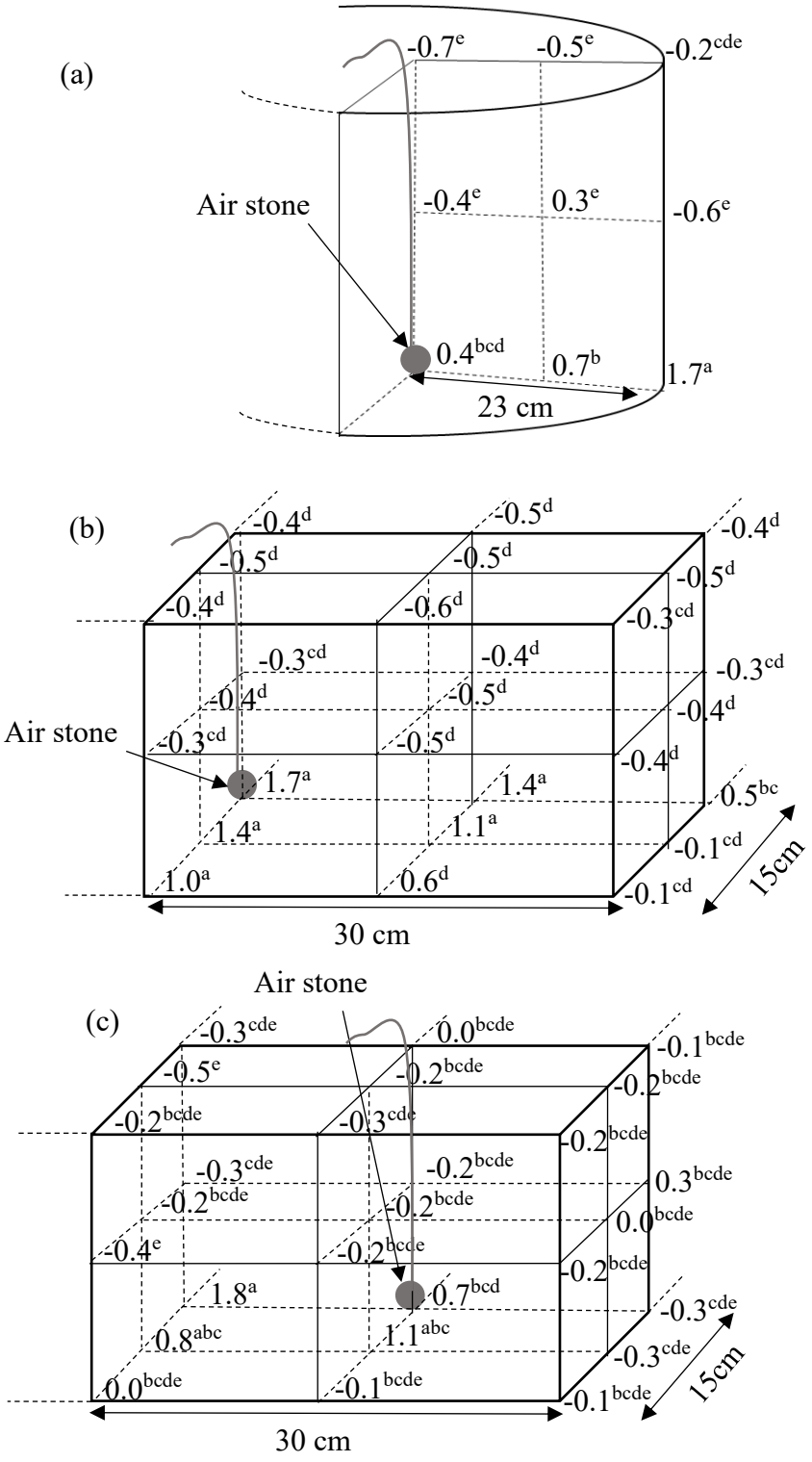



**Figure 2.7.** Rotifer distribution (a) at half cross section in cylindrical tank, (b) and (c) in the quadrisection in rectangular tank of the experiment 2. Values are average of deviation values during culture period and alphabets in superscripts represent the significant differences between the stations that is one of the factors of two-way ANOVA (n=6, a>b>c>d>e, Tukey HSD, $p<0.05$).

## 2.4 Discussion

### 2.4.1 Survival, growth and viability of larvae in the experiment 1

For the experiment 1, the survival rate of red seabream larvae at 14 dph was not significantly different between tank shapes. However, the survival rates of larvae at 14 dph in the experiment 1 was higher than those (29.6-55.3%) in the experiment 2 at 14 dph with 100 ml/min aeration rate. The differences of survival rates between the experiment 1 and 2 may be due to the different aeration rates because the fast flow can produce the fast rate air bubbles which will directly damage larvae (Shiotani et al., 2003). Standard length of larvae at 14 dph was larger in CT than that in RT but other morphological parameters were not significantly different between tank shapes. However, growth of larvae in experiment 1 (e.g., total length: 3.93±0.08 mm for CT and 3.70±0.04 mm for RT1AS) at 14 dph was smaller than that in the experiment 2 (total length: 4.71±0.23 mm for CT and 4.94±0.13 mm for RT1AS) at 14 dph. The different growth of larvae between the experiment 1 and 2 may be due to the higher density of larvae in the experiment 1 by the higher survival than that in experiment 2.

Viability of larvae was not significantly different between tank shapes in the experiment 1 although swimbladder inflation of larvae in the experiment 2 showed different trend between tank shapes with the same rearing system as the experiment 1.

Flow velocity in larval rearing tank may not affect the viability of red seabream larvae at 14 dph in this study although larval diet affected the viability of larvae at 28 dph in larviculture with a 100-l polycarbonate tank (Hagiwara et al., 2016). On the other hand, the viability of larvae (73.3±15.3 in CT and 73.3±5.8 in RT1AS) at 14 dph in 50-l different tank shapes in the present study were higher than that (60.0±32.2 with *Diaphanosoma celebensis* diet; 60.0±27.8 with *Tigriopus japonicus* diet; 44.4±12.3 with *Artemia franciscana* diet) at 28 dph in 100-l polycarbonate tank (Hagiwara et al., 2016). Further studies are needed to observe the effect of flow field in different tank sizes and/or shapes on viability and swim bladder inflation of red seabream larvae.

### 2.4.2 Survival, growth and swimbladder inflation of larvae in the experiment 2

Survival rate of red seabream larvae in CT and RT1AS at 14 dph was almost two-folds higher than that in RT2AS. The survival rate of red seabream in CT was comparable to that of Pacific bluefin tuna (PBT) *Thunnus orientalis* larvae at 8 dph in the same CT (52.7%; Aung Naing Win et al., 2020), whereas survival in the RT1AS was different between red seabream (55.3%) and PBT (0.8%). The different survival rates between red seabream and PBT in RT1AS may be due to the occurrence of sinking syndrome in PBT, where PBT larvae with higher specific gravity sink to the bottom of the tank during dark periods due to low swimming activity and cause mortality at the bottom of the rearing tank (Takashi et al.,

2006; Tanaka et al., 2009). Sinking PBT larvae were assumed to be trapped into low velocity areas at the bottom of RT1AS (Aung Naing Win et al., 2020). On the contrary, sinking syndrome is not observed in larval red seabream, and thus, similar survivals were observed between CT and RT1AS.

Survival rate of red seabream larvae in RT2AS (29.6%) was low in this study. In the CT and RT1AS, a single-pair vortex system could be observed at the central sections. Low velocity regions in these tanks were observed along the sidewalls of the tanks and bottom areas. The stagnate areas, where the water velocity is lower than the swimming speed of rotifer (1.3 mm/s), were coincided with higher density of rotifer distribution at the bottom (Figure 2.6. (a), (b)), but these areas are smaller in the CT than in the RT1AS (Aung Naing Win et al., 2020). In the RT2AS, two-vortex systems were formed between two aerators. The bubbles from two air stones may stun fish larvae more frequently and had larvae contacting the tank walls and bubbles than 1 air stone. Thus, I presume that the survival of red seabream larvae in RT2AS was lower than that in RT1AS and CT.

Growth and swimbladder inflation of red seabream larvae at 14 dph were not significantly different between different rearing tank shapes with different aeration systems. It is similar to the previous findings, in which tank shapes affected larval survival but not growth of seven-band grouper *Epinephelus septemfasciatus*, devil stinger *Inimicus japonicus* larvae (Ruttanapornvareesakul et al, 2007) and PBT (Aung Naing Win et al.,

2020). However, swimbladder inflation rate in the CT was slightly lower and varied rather than the RTs. Swimbladder inflation of fish larvae is initiated by gaping air from the water surface and introducing it into swimbladder through the pneumatic duct after mouth opened (Kitajima et al., 1981). With the respect of water surface in tanks of my study, streamlines radiated from air stone were straight forward to the side walls in CT and RT1AS, and straight forward to the side walls and to the middle of tanks in RT2AS (Figure 2.4). Flow velocity in CT was around 1.2 times higher than these in RT1AS and RT2AS (Figure 2.3). High water velocity at surface area in CT may prevent the larvae not only from attaching to water surface (Sakakura et al., 2019) but also gaping air through the water surface that will lead to failure of swimbladder inflation.

Flow fields in rearing tanks affected the survival of red seabream larvae. Flow field created by a single air stone in small-scale 50-l tanks was a preferable for red seabream larvae, but swimbladder inflation may be different by tank shapes. Further detailed studies are required to elucidate the influence of flow field on swimbladder inflation of marine fish larvae.

# Chapter 3

# Effect of tank shape on survival, growth and swim bladder inflation of Pacific bluefin tuna *Thunnus orientalis* larvae

## 3.1 Introduction

Pacific bluefin tuna (PBT) *Thunnus orientalis* is a commercially important fish in Japan, Korea, Taiwan and the United States (Craig et al., 2017). Aquaculture for PBT utilizes wild-caught juveniles as seedlings (Ottolenghi, 2008), and overfishing of PBT juveniles has led to a decline of the PBT population in the wild (Craig et al., 2017). Recently, the full life cycle of PBT was successfully completed under aquaculture conditions for the increasing demands of PBT seedlings (Sawada et al., 2005). However, high mortality occurred during the first 10 days post hatching (dph), followed by cannibalism during the late larval and juvenile stages, and high mortality occurred by collision with tank or net walls during the juvenile stage (Sawada et al., 2005). The high mortality during the first 10 days is an obstacle that must be solved for mass production of *Thunnus* species to gain a stable large-scale supply of seedlings (Woolley et al., 2013; Nakagawa et al., 2011; Sawada et al., 2005).

The sinking syndrome is a main cause of high mortality during the early larval stages in PBT larviculture (Masuma et al., 2011). This occurs when larvae sink to the

bottom during dark periods because larval swimming activity is low at night, and their specific gravity is higher than that of seawater (Nakagawa et al., 2011; Tanaka et al., 2009; Takashi et al., 2006). Sinking syndrome occurs in many marine fish larvae, including the striped trumpeter *Latris lineata* (Trotter et al., 2005), greater amberjack *Seriola dumerili* (Teruya et al., 2009), yellowtail kingfish *S. lalandi* (Woolley and Qin, 2013), leopard coral grouper *Plectropomus leopardus* (Takebe et al., 2011), kelp grouper *Epinephelus bruneus* (Ching et al., 2014) and tiger grouper *E. fuscoguttatus* (Ching et al., 2016). Sinking syndrome can be reduced by increasing the aeration rate at night (Tanaka et al., 2018; Nakagawa et al., 2011) and/or creating conditions in which the larvae are suspended within the water column of the rearing tank (Kurata et al., 2017; Ching et al., 2014, 2016; Takebe et al., 2011; Tanaka et al., 2009) and by continuous illumination (Kumon et al., 2018; Kurata et al., 2017).

In PBT larviculture, water temperature (Tanaka et al., 2018), aeration rates (Tanaka et al., 2018; Kurata et al., 2017; Nakagawa et al., 2011) and light conditions (Kurata et al., 2017) have been studied as physical environmental factors that affect sinking syndrome in PBT larvae. However, information on the effects of tank shape, which affect the early survival of marine fish larvae (Ruttanapornvareesakul et al., 2007) are lacking. In the present study, I hypothesized that tank shape may affect the survival and growth of marine fish larvae. To examine this possibility, I conducted larviculture experiments in small 50-l

tanks of different shapes. I chose a cylindrical tank (CT) with axisymmetrical flow field patterns (Sumida et al., 2013), and a rectangular tank (RT) with three-dimensional (3-D) complicated flow field patterns (Takakuwa et al., 2018), to investigate the effect of tank shape on survival and growth of PBT larvae. I also examined the distribution of rotifers to estimate the flow field in tanks, and visualized the flow fields by simulation.

### 3.2 Materials and methods

Three blue plastic CT (46 cm in diameter) and blue acrylic RT (60 cm×30 cm×35 cm depth) with a 50-l working volume were used in this study. The aspect ratio (liquid depth/internal radius of the tank) of the CT was 1.3. I assumed that the aspect ratio of the RT was also 1.3, since the water surface area and depth of the RT ($1.8 \times 10^3$ $cm^2 \times 28$ cm) were almost equal to those of the CT ($1.7 \times 10^3$ $cm^2 \times 30$ cm). Tanks were filled with 32 parts per thousand (ppt) artificial seawater (Marine Art Hi, Tomita Pharmaceutical, Japan), and placed in a 25°C temperature-controlled room of the Aquaculture Biology Laboratory, Nagasaki University, Japan. A spherical aerator (5 cm in diameter; 100 ml/min aeration rate) was placed at the bottom center of each tank to generate water flow. The low velocity areas were defined as the place where the water velocity is lower than the swimming velocity of PBT larvae (8.8 mm/s) at 8 dph (4.2±0.1 mm in total length). Swimming speed of PBT larvae was estimated from the equation proposed by Sabate et al. (2010), in which

swimming speed of PBT was 2.2 standard length/s during larval phase. Light intensity at the water surface was 2000 lx with a photoperiod of 24L:0D to decrease the sinking syndrome that occurs in PBT larvae during dark periods (Nakagawa et al., 2011; Tanaka et al., 2009; Takashi et al., 2006).

I followed the PBT larval rearing procedure described by Tanaka et al. (2018), who successfully reared PBT larvae for 7 dph in 200-l tanks under static conditions (no water exchange). Heavy mortality has been observed within 10 dph in the PBT mass culture process due to the sinking syndrome, and the percentage of sinking PBT larvae on the tank bottom has been shown to peak at around 5 dph (Tanaka et al., 2009). I thus decided on a rearing period of 8 dph. Fertilized eggs of PBT were obtained from Seikai National Fisheries Research Institute, and were transported to the Aquaculture Biology Laboratory, Nagasaki University, Japan, on 30 June 2018. Eggs were first transferred into a 100-l polycarbonate tank and larvae were kept until 2 dph in the same tank at 25°C and 32 ppt. Larvae were distributed into each experimental tank at 10 individuals/l on 2 dph, and reared until 8 dph under static conditions.

Super Chlorella V12 (Chlorella Industry Co., Fukuoka, Japan) was added to the experimental tanks as green water, and the density was adjusted to $5\times10^5$ cells/ml once daily. Rotifers *Brachionus plicatilis* enriched with Super Chlorella V12 were fed to larvae at 10 individuals/ml when the mouth opened (2 dph). Water samples to assess rotifer

density were collected from 9 stations (3 ml for each) in a vertical cross-section of CTs (Figure 3.1. (a)) and 27 stations in the quarter segments of RTs (Figure 3.1. (b)) using a pipet. The swimming speed of rotifers was referred to estimate the stagnate areas in rearing tanks and was set to 1.3 mm/s according to Yúfera et al. (2005). Since the rotifer numbers in the experimental tanks increased during the experimental period, I standardized the rotifer distribution in the tanks each sampling day using the following equations:

$$deviation\ value\ at\ station\ x\ on\ day\ i = \frac{rotifer\ density\ at\ station\ x - mean\ rotifer\ density\ on\ day\ i}{standard\ deviation\ of\ rotifer\ density\ on\ day\ i}.$$

On 8 dph, all surviving larvae in the experimental tanks were counted to calculate the survival rate. Then, about 30 fish in each tank were anaesthetized with 200 ppm of MS222 (Tricaine; Sigma-Aldrich) and observed under a dissecting microscope with transmitted light to see whether the swimbladder was inflated by checking air bubbles in the bladder. Larvae were then fixed with 5% formalin solution. Formalin-preserved fish were individually measured for morphometric characters by a digital microscope (VH-6300; Keyence, Osaka, Japan), and then dried at 60°C for 24 h for measurement of the dry body weight by an ultra-micro balance (UMX2; Mettler Toledo, Columbus, OH, USA).

Two-phase bubbly flow simulations were performed in the experimental tanks using a dispersed flow model that was developed by Takakuwa et al. (2018). Its governing

equations are composed of the conservation laws of mass and momentum of liquid (water) and gas (air bubble) phases, in which the effects of pressure gradient, drag and lift forces acting on bubbles, gravitational acceleration and flow viscosity are taken into account. A simplified marker and cell (SMAC) method was used to solve the governing equations. For the liquid phase, the free surface was assumed to be flat, and a no-slip boundary condition was used. On the other hand, an outflow condition was given for the gas phase at the free surface. As boundary conditions on the wall surface of tanks, a no-slip condition was given for the liquid phase, while a slip condition was given for the gas phase. An air inlet (square with a side length of 22 mm; aeration rate, 100 ml/min) was set at the center of the bottom surface. The diameter of a bubble was set to 2.0 mm. Flow simulations were performed for 450 s, and averaged flow fields for the last 150 s are discussed in this research.

Physical environmental parameters during the experiments were as follows: water temperature 24.3–24.8°C; salinity 32.1–32.2 ppt; dissolved oxygen 6.2 mg/l; pH 7.94–7.96; and total ammonia ($NH_3$-$NH_4^+$) 0.18–0.19 mg/l.

### 3.2.1 Statistical analysis

Differences in the survival, growth and swimbladder inflation rates of larvae between tanks were determined using either Student's t-test for parametric test which the variance of the two groups are equal after a random variable of the two groups is normally distributed by

Shapiro-Wilk normality test or Welch's t-test for parametric test which the variance of the two groups are unequal after a random variable of two groups is normally distributed by Shapiro-Wilk normality test. Mann-Whitney *U* test was also used to know those differences for non-parametric test which a random variable of single or two groups is not normally distributed by Shapiro-Wilk normality test. The rotifer distribution in tanks was standardized by the deviation value on day *i*, and determined by two-way ANOVA followed by Tukey HSD test. All analyses used R 3.4.1 software, and a 5% level of confidence was considered a significant difference.

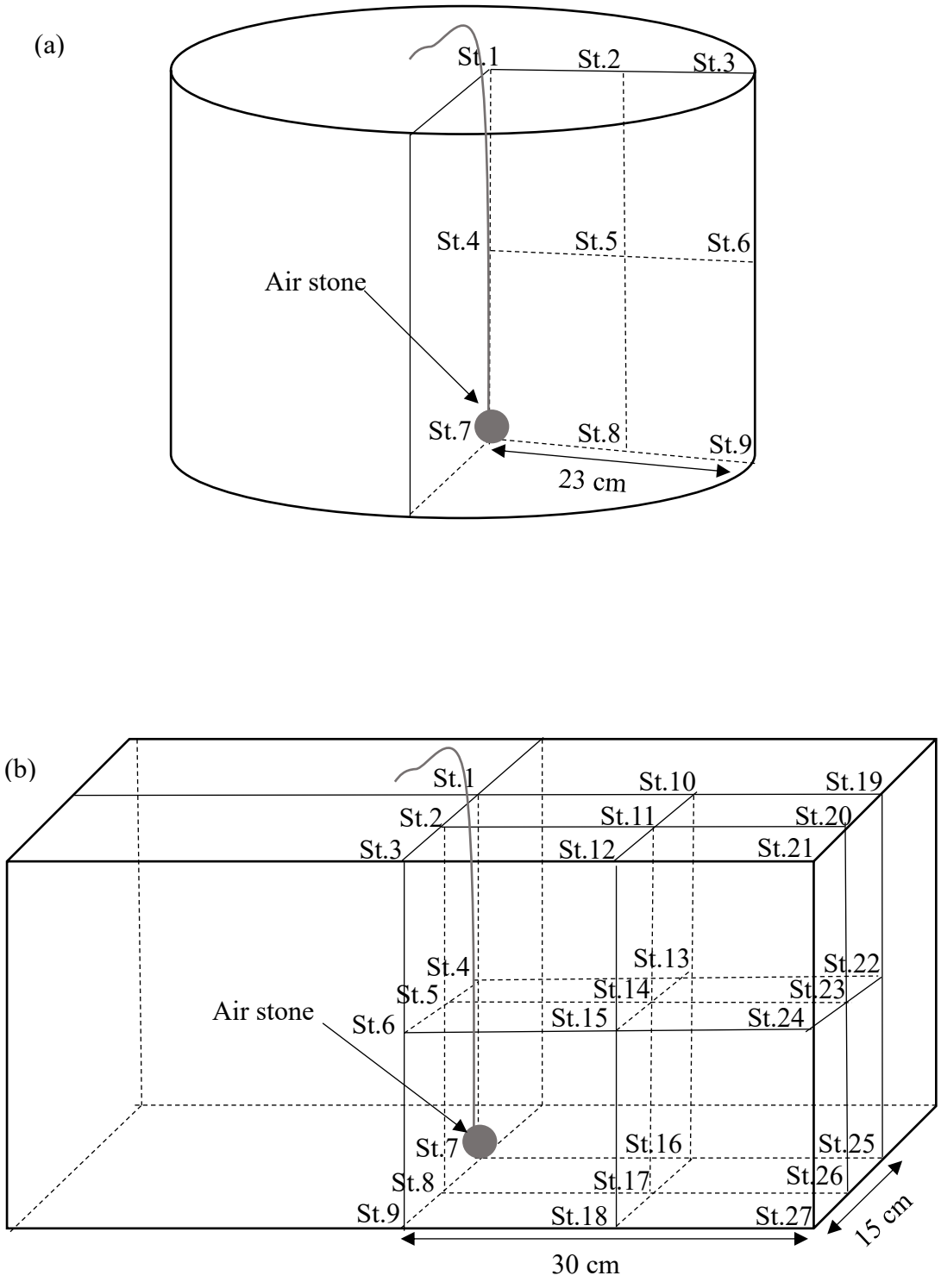



**Figure 3.1.** Sites for rotifer distribution in (a) cylindrical tank and (b) rectangular tank. One air stone was set at the center of tank bottom.

## 3.3 Results

### 3.3.1 Survival, growth and swimbladder inflation of larvae

The hatching rate of fish eggs was 100%. The survival rate of Pacific bluefin tuna (PBT) larvae at 8 dph in CTs (52.7±5.1%) was significantly higher than in RTs (0.8±0.7%, Welch's t-test, n=3, $df$=2.0566, $t$=15.5580, $p$=0.0036; Table 3.1). Neither standard length (Student's t-test, n=3, $df$=4, $t$=0.2524, $p$=0.8132) nor dry weight (Student's t-test, n=3, $df$=4, $t$=2.2896, $p$=0.0839) of larvae was significantly different between tank shapes (Table 3.1). Other morphological parameters of larvae at 8 dph were also not significantly different: total length (CT, 4.23±0.26 mm; RT, 4.09±0.20 mm; Student's t-test, n=3, $df$=4, $t$=0.1189, $p$=0.9111), body depth/standard length (0.18±0.02 mm; 0.17±0.02 mm; Student t-test, n=3, $df$=4, $t$=-0.1981, $p$=0.8526), head length/standard length (0.23±0.01 mm; 0.24±0.02 mm; Mann-Whitney $U$ test, n=3, $W$=9, $p$=0.0765) and eye diameter/standard length (0.11±0.01 mm; 0.11±0.01 mm; Mann-Whitney $U$ test, n=3, $W$=8, $p$=0.1642). The swimbladder inflation rate of larvae was not significantly different between CT and RT (Mann-Whitney $U$ test, n=3, $W$=9, $p$=0.0765; Table 3.1).

### 3.3.2 Flow field in the experimental tank

3-D visualizations of streamlines in the rearing tanks are shown in Figure 3.2. In the case of the CT, upward flows by the effect of air bubbles generated from the air stone radiated

outward in the vicinity of the water surface, and then downward flows were observed along the sidewalls of the tank. A single-pair vortex system could be observed at arbitrary central sections. In the case of the RT, the central upward flows radiated outward in the same manner as the CT. Due to the effect of rectangular corners and the non-axisymmetric shape of the tank, however, a more complicated flow field was generated. Figure 3.3., compares cumulative area distributions of the tanks with respect to the flow velocity magnitude obtained from the flow simulation results. Low velocity regions in the RT were larger than in the CT regardless of the increasing flow velocity. In Figure 3.4., streamlines at the bottom regions (where water depth is more than 25 cm) are visualized, colored by the flow velocity magnitude. In the CT, although low velocity regions were observed along the outside edge, most streamlines eventually moved toward the center of the tank, which reverted to the upward flow from the air stone. In the RT, the flow velocity magnitude at most bottom regions was larger than the CT, and the flow structures were more complicated. Streamlines from both short sides of the rectangle collided in the vicinity of the center of the long sides, creating a vortex at the upper side of Figure 3.4. (b), and a large low velocity area at the lower side of the figure.

### 3.3.3 Rotifer distribution

Rotifer density increased during the experimental period, reaching 54.7 individuals/ml in

CT and 52.2 individuals/ml in RT on 8 dph (n=6). Rotifer distribution in the CT was different among stations (two-way ANOVA, *df*=8, *F*=4.2610, *p*=0.0002, Tukey HSD test, $p<0.05$; Figure 3.5 (a)) but was not associated with days (two-way ANOVA, *df*=5, *F*=0.0020, *p*=1.0000). There were no interactions between stations and days (two-way ANOVA, *df*=40, *F*=1.4660, *p*=0.0690). In the RT, rotifer distribution was also different among stations (two-way ANOVA, *df*=26, *F*=4.0780, $p<0.0001$; Tukey HSD test, $p<0.05$; Figure 3.5 (b)) but not among days (two-way ANOVA, *df*=5, *F*=0.0090, *p*=0.9999). Interactions between stations and days were not detected in RT (two-way ANOVA, *df*=130, *F*=1.1530, *p*=0.1673).

**Table 3.1.** Survival, growth and swimbladder inflation rates of *T. orientalis* larvae at 8 dph in different tank shapes

| Tank | n | Survival (%)* | Standard Length (mm) | Dry weight (μg) | Swimbladder inflation (%) |
|---|---|---|---|---|---|
| Cylindrical | 3 | 52.7±5.1 | 4.06±0.25 | 95.1±17.6 | 16.5±14.5 |
| Rectangular | 3 | 0.8±0.7 | 3.91±0.20 | 67.7±10.9 | 56.9±37.4 |

Results are mean values ± SD. The asterisk indicates significant differences by Welch's t-test, ($p<0.01$).

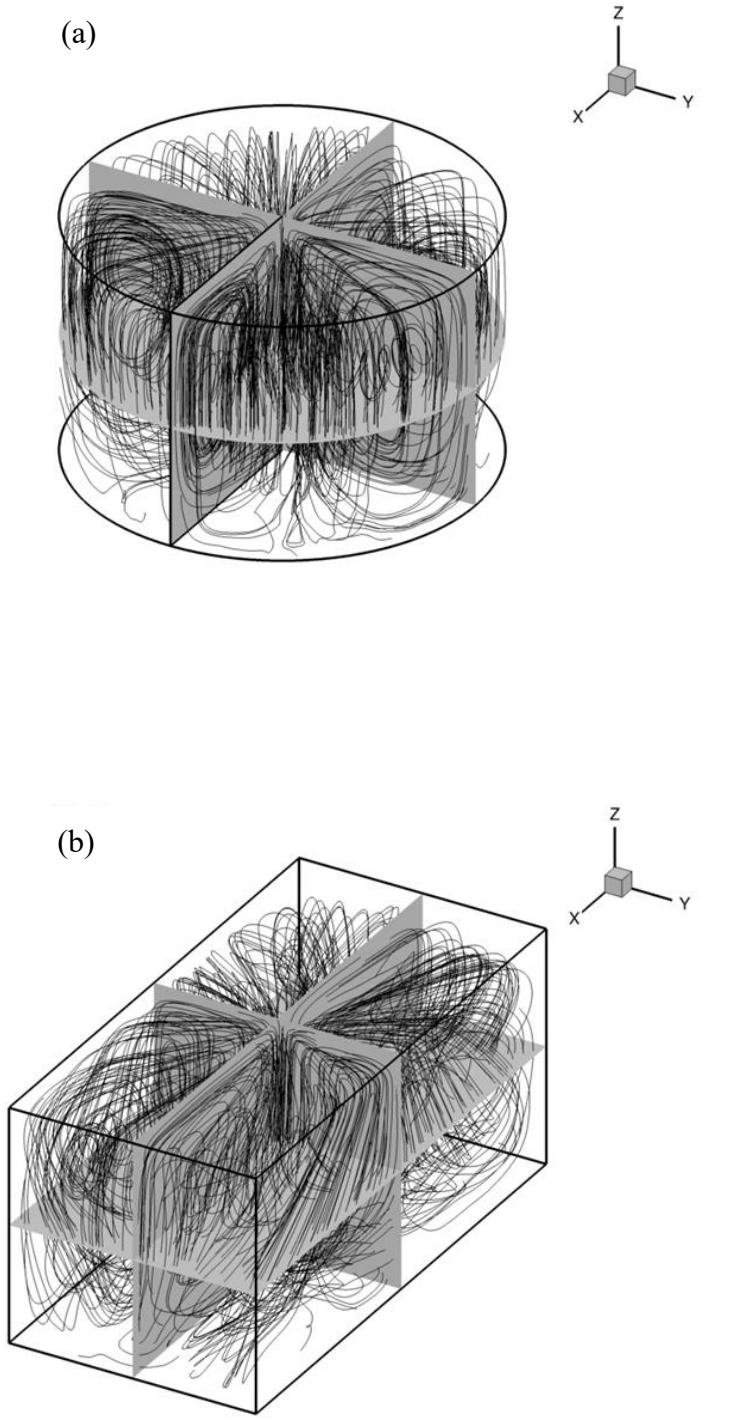



**Figure 3.2.** Three dimensional streamlines predicted in this study in (a) cylindrical tank and (b) rectangular tank with an aerator at the center of the bottom with 100 ml/min aeration.

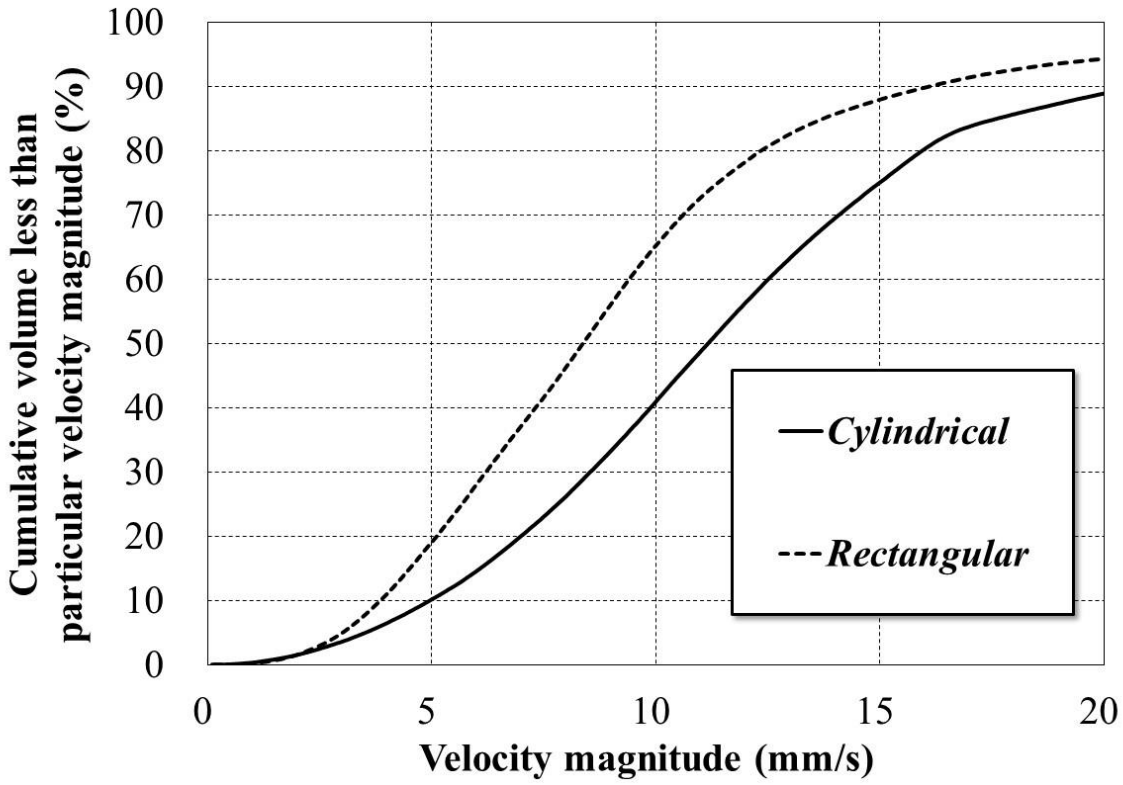



**Figure 3.3.** Cumulative area (%) of the velocity magnitude (< x mm/sec) between the tanks applied in this study. For instance, the water mass having a velocity between 0 and 10 mm/s occupied 40% and 65% of total water volume in the CT and RT in this study, respectively.

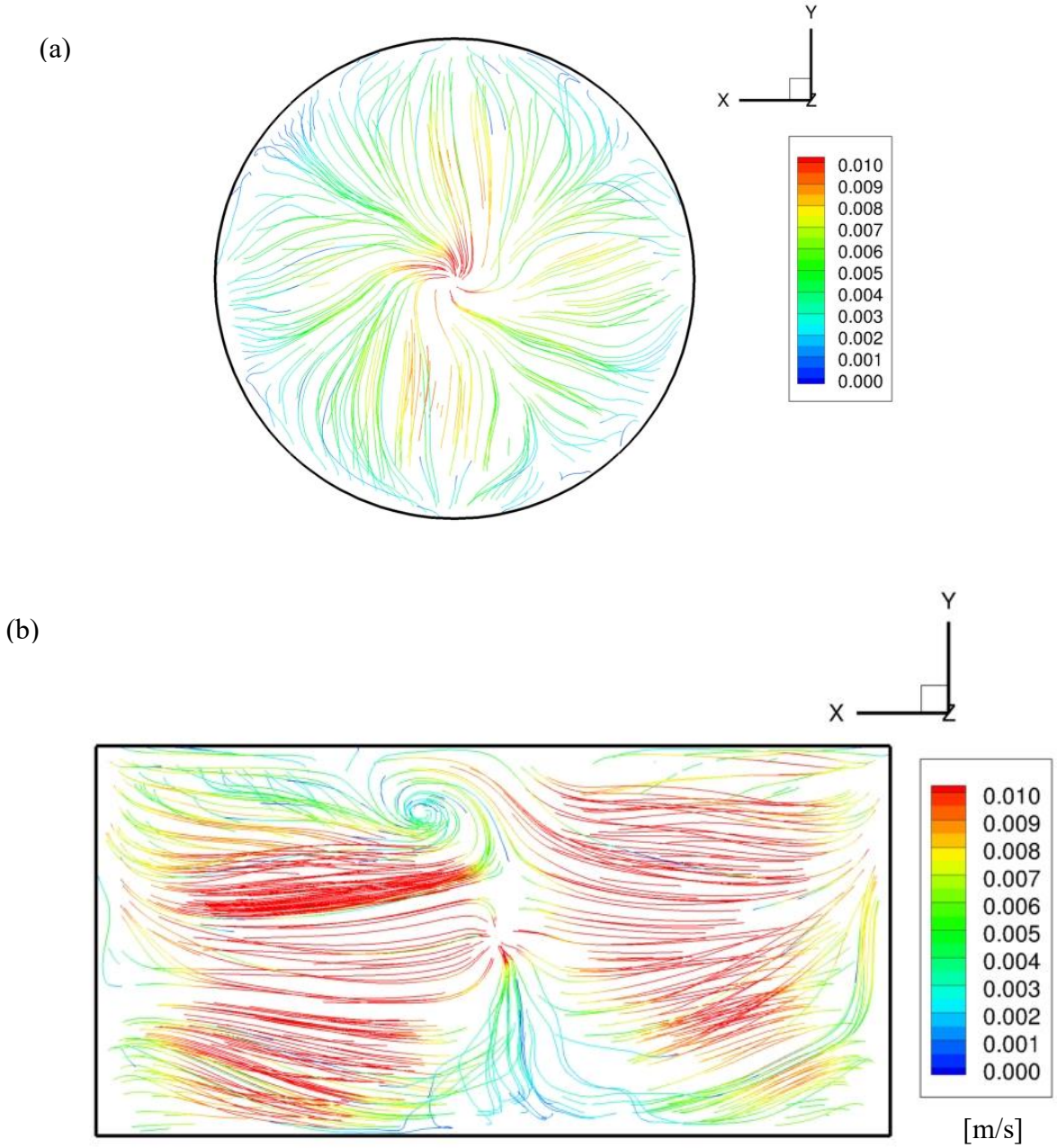



**Figure 3.4.** Visualization of streamlines at bottom regions of (a) a cylindrical tank (ø 46 cm) and (b) a rectangular tank (60 × 30 cm) in this study. An air stone was located at the center of each tank.

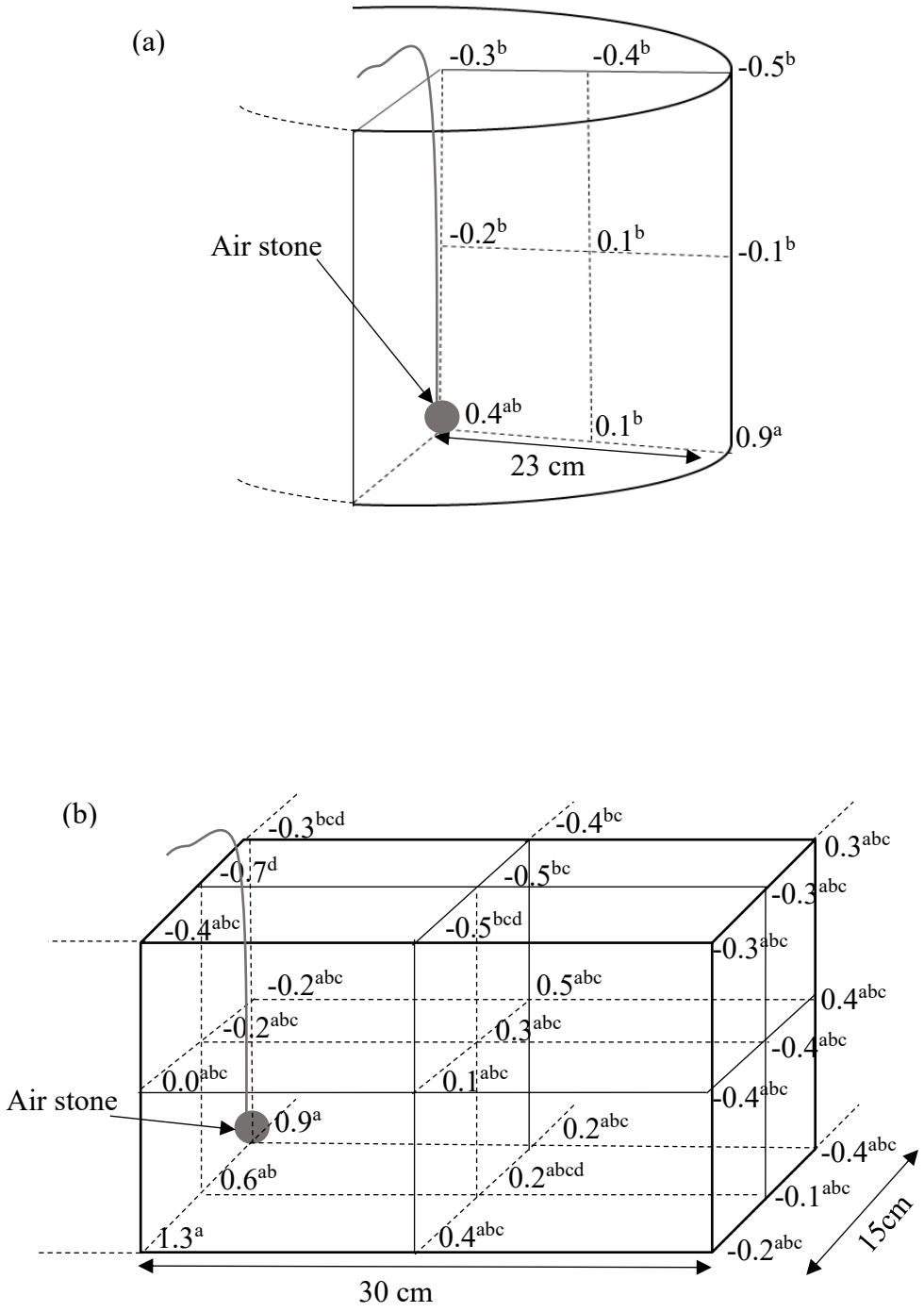



**Figure 3.5.** Rotifer distribution (a) at half cross section in cylindrical tank and (b) in quadrisection in a rectangular tank. Values are average of deviation values during culture period and alphabets in superscripts represent the significant differences between the stations that is one of the factors of two-way ANOVA (n=6, a>b>c>d, Tukey HSD, $p<0.05$).

## 3.4 Discussion

### 3.4.1 Survival, growth and swimbladder inflation of Pacific bluefin tuna (PBT) larvae

The present study examined whether the shape of small-scale larval rearing tanks affects the survival and growth of PBT larvae, together with the rotifer distribution in tanks. The survival rate of PBT larvae in CTs at 8 dph was about 60-folds higher than that in RTs. Usually, better survival and growth of larvae occurs in large rearing tanks rather than in smaller systems (Houde, 1972). However, the survival rate in this study (52.7%) in the 50-l CT under a 24-h photoperiod was higher than that in the 500-l CT of a previous study at 7 dph under natural photoperiod with strong aeration during darkness (about 35%; Tanaka et al., 2018), whose protocol with static conditions was followed here. Survival rates in mass-scale tanks at similar ages were also lower than this study: 19.3% in a 50 $m^3$ octagonal tank with a water pump system under a natural photoperiod at 8 dph (Tanaka et al., 2009), and 20.3% in 30 $m^3$ circular tanks with 1.7 l/min aeration rates in daytime and stronger aeration rates (3.0 l/min) during the dark period at 8 dph under a natural photoperiod (Kurata et al., 2014).

Moreover, the survival rate in the present study was comparable to that at 10 dph in 1 $m^3$ cylindrical fiberglass tanks (22.2–42.3%) with 130 ml/min daytime aeration rates, and 1.2 l/min dark-period aeration rates under natural and artificial fluorescent lighting (Kurata

et al., 2012), as well as those at 10 dph in 500-l cylindrical polycarbonate tanks (43.2–48.6%) with 300 ml/min daytime aeration rates and 900 ml/min dark-period aeration rates at 12L:12D (Nakagawa et al., 2011). Therefore, it proposes that rearing experiments of PBT larvae can be conducted in small-scale tanks in which the rearing environment, such as water temperature and illumination, can be easily manipulated.

The growth of PBT larvae was not significant differences between different rearing tank shapes. Similar findings were reported in the seven-band grouper *Epinephelus septemfasciatus* and devil stinger *Inimicus japonicus*, for which the rearing tank shapes affected larval survival but not growth (Ruttanapornvareesakul et al., 2007). The growth of PBT larvae at 8 dph in the present study (about 4.1 mm TL) appeared to be smaller than that of larvae of the same age in previous studies (5.8–6.1 mm: Kurata et al., 2012, 2014). Growth measurements were conducted with formalin-fixed specimens in the present study and fresh specimens in Kurata et al. (2012, 2014). If it assume 10% shrinkage in the case of formalin-fixed larvae (Hay, 1982) to the previous studies (Kurata et al., 2012, 2014), body size of sample in the present study (4.1 mm) was still smaller than that of the previous studies (5.2–5.5 mm). The inferior growth of PBT larvae in this study may have been due to the smaller tank volume; thus, it should consider the larval density and water exchange when applying small-scale tanks for PBT larviculture experiments.

Sinking syndrome of PBT larvae occurs because larvae with higher specific gravity

than seawater sink to the bottom of the tank during dark periods due to low swimming activity (Tanaka et al., 2009; Takashi et al., 2006). Swimbladder inflation failure is one of the causes of sinking syndrome (Kurata et al., 2012, 2015, 2017; Ina et al., 2014; Woolley and Qin, 2013), as the swimbladder controls the buoyancy of fish by reducing specific gravity relative to that of surrounding water (Taylor et al., 2010; Phleger, 1998). However, Takashi et al. (2006) found that the specific gravity of PBT larvae was higher than seawater density during dark periods even in swimbladder-inflated larvae. In the present study, the swimbladder inflation rate varied among tanks but was not significantly different between tank shapes. It presumes that swimbladder inflation failure may not be the main cause of sinking syndrome. These results may be in agreement with the present finding that the survival rate in the CT was higher than that in the RT despite the same swimbladder inflation rates between tank shapes. It could not conclusively determine whether swimbladder inflation is directly connected to the cause of sinking syndrome; additional studies with more detailed observations of the diel movement of PBT larvae in the rearing tank will be needed.

### 3.4.2 Flow field in the cylindrical and rectangular tanks

Unfavorable flow in rearing tanks can cause mass mortality of marine fish larvae (Sakakura et al., 2019; Shiotani et al., 2003; Yamaoka et al., 2000; Backhurst and Harker,

1988). Flow field structures in tanks differ by tank size and shape (Sakakura et al., 2019; Moore and Prange, 1994). CTs have an axisymmetrical flow field pattern (Sumida et al., 2013), and the flow field provides a uniform current environment and facilitates the elimination of biosolids from the tank bottom (Masaló and Oca, 2016; Oca and Masalo, 2013; Timmons et al., 1998). In the case of RTs, 3-D complicated flow fields and low velocity areas where water velocity is lower than the swimming speed of Pacific bluefin tuna (PBT) larvae (8.8 mm/s) were observed in this study. Low velocity areas were larger in the RT, as shown in Figure 3.3. Moreover, the stagnate areas, where the water velocity is slower than the swimming velocity of rotifers (1.3 mm/s) and the eddy at the tank bottom coincided with the areas where rotifers were distributed at high density. Thus, the low velocity areas in RTs may be larger at the bottom than in CTs, leading to the sinking syndrome of PBT larvae because low velocities and poor mixing of water in RTs lead to the creation of stagnate areas, causing the accumulation of biosolids on the tank bottom (Oca and Masaló, 2007). These stagnate areas may have negative effects on larviculture (Sakakura et al., 2019).

The advantage of numerical modeling of the flow field in a larviculture tank is that the field in the rearing tank can be visualized without a flow meter or intensive labor, and that one established model can be expanded to similarly shaped tanks with different water volumes. Thus, the flow field in similar tank shapes can be easily estimated and the model

can help in designing the number and location of aerators and water inlets for the larviculture tank. The results of this study also demonstrated that comparison of rotifer densities at various sites in a rearing tank could approximately predict the stagnate areas that will cause the sinking syndrome of marine fish larvae.

In conclusion, the flow field in different larval rearing tank shapes affected the survival of PBT larvae in my experiments. The present study demonstrated that flow field patterns in small-scale CTs (50-l) at AR = 1.3 are more feasible for the survival of PBT larviculture experiments than those in RTs, and that improvement of aerators beyond a single air stone to decrease low velocity areas at the tank bottom should be developed for RTs.

# Chapter 4

# Effects of aerations on survival, growth and swim bladder inflation of Pacific bluefin tuna *Thunnus orientalis* larvae in rectangular tank

## 4.1 Introduction

Pacific bluefin tuna (PBT) *Thunnus orientalis* is an important species for aquaculture in Japan (Miyake et al., 2010). Recently, Sawada et al. (2005) reported that the life cycle of PBT was successfully completed under aquaculture conditions for the increasing demands of PBT seedlings. However, the population of PBT leads to decline in the wild due to the over fishing for its seed supply (Ottolenghi, 2008). Therefore, new technology is required to establish the sustainable development of tuna aquaculture without relying on natural resources.

The high mortality during 10 days post hatch (dph) observed in PBT larviculture due to the sinking of larvae to the tank bottom in nighttime (Tanaka et al., 2009) because the specific gravity of PBT larvae is higher than the seawater density and larval swimming activity is low during the night (Takashi et al., 2006; Tanaka et al., 2009; Nakagawa et al., 2011). The sinking of fish larvae could be reduced by aerations which create the vertical mixing of the rearing water (Tanaka et al., 2018; Masuma et al., 2011; Nakagawa et al., 2011). Manipulations of flow field also prevent the larval sinking death of PBT larvae

(Nakagawa et al., 2011). Nakagawa et al., (2011) revealed that aeration rates from 0 to >900 ml/min in night time in 500-l tanks increased larval survival by counteracting sinking of PBT larvae. Therefore, aeration is also one of the factors to consider for enhancing larval survival and growth of marine fish larvae.

Aerations are important factors to circulate the water flow which can disperse live feeds and artificial diets and insure oxygenation in rearing tank (Backhurst and Harker, 1988). However, the different aerator positioned in tank bottom were required to improve in order to achieve the flow criteria in rearing tank for various designs of tank although the long bar aerator in the flat-bottomed rectangular tank performed well (Backhurst and Harker, 1988). Moreover, different aeration design made a different result of marine fish larvae (Tanaka et al., 2009) and the rectangular tanks required to improve the aerators beyond a single air stone to decrease low velocity areas at the tank bottom (Aung Naing Win et al., 2020). Therefore, the improvement of aerations required to get the optimum flow in larval rearing tank for various tank designs.

This study hypothesizes that the aerations with different aerator types in small-scale tank may affect the survival, growth and swimbladder inflation of marine fish larvae. To test this hypothesis, I used a spherical aerator (5 cm in diameter) and long bar-shaped aerators (15, 30 and 60 cm long) to set at the bottom of 50-l rectangular tank (RT) which have three-dimensional complicated flow field patterns (Takakuwa et al., 2018).

The rotifer distribution was also examined to estimate the flow structures in tank.

### 4.2 Materials and methods

Six blue acrylic rectangular (60 cm × 30 cm × 35 cm depth) tanks containing a 50-l working volume were used in this study. The water surfaces of them were $1.8 \times 10^3$ $cm^2$ with 28 cm water depth. A spherical aerator (5 cm in diameter) was set at the center on the bottom of each three RT with 100 ml/min aeration rate as control for all experiment (Figure 4.1 (a)). The artificial seawater (Marine Art Hi, Tomita Pharmaceutical Japan) 32 part per thousand (ppt) was filled into each experimental tank. Light intensity at the water surface was 2000 lx with photoperiod at 24L:0D. The surviving larvae were counted to calculate the survival rate on the last day of experiments. Thirty larvae from each experimental tank were randomly sampled, and were anaesthetized with 200 ppm of MS222 (Tricaine; Sigma-Aldrich) and observed under a dissecting microscope with transmitted light to see whether the swimbladder was inflated by checking air bubbles in the bladder. Then, larvae were fixed with 5% formalin. The fixed larvae were measured the morphometric characters by using a digital microscope (VH-6300; Keyence, Osaka, Japan).

Super Chlorella V12 (Chlorella Industry Co., Fukuoka, Japan) was used to enrich the rotifers *Brachionus plicatilis* which were fed at 10 individuals/ml to larvae when the mouth opened (2 dph) and was added into experimental tank once daily to be green water,

adjusting the density to $5 \times 10^5$ cells/ml once daily. Water sample were collected from 27 stations (3 ml for each) using a pipet in the quarter segments of control tanks for all experiments and first experimental tanks and from 45 stations in the half of the 2 and 3 experimental tanks to see their distribution in tanks during experiment. The swimming speed of rotifers was referred from Yúfera et al. (2005), in which the swimming of rotifers was 1.3 mm/s, to estimate the stagnate areas in tanks. Since the rotifer numbers in the experimental tanks increased during the experimental period, I standardized the rotifer distribution in the tanks each sampling day using the following equations:

$$deviation\ value\ at\ station\ x\ on\ day\ i = \frac{rotifer\ density\ at\ station\ x - mean\ rotifer\ density\ on\ day\ i}{standard\ deviation\ of\ rotifer\ density\ on\ day\ i}$$

Physical environmental parameters during all experiments shown in Table 4.1.

**Table 4.1.** Physical environmental parameters during the experiments.

| Experiment | Temperature (ºC) | Salinity (ppt) | Dissolved oxygen (mg/l) | pH | Total ammonia ($NH_3$-$NH_4^+$) mg/l |
|---|---|---|---|---|---|
| 1 | 25.3-25.7 | 32.4-32.5 | 6.5-6.9 | 7.8-7.9 | 0.2-0.3 |
| 2 | 26.3-26.5 | 32.0-32.4 | 6.2-6.9 | 7.9-8.0 | 0.2-0.4 |
| 3 | 26.3-26.7 | 31.6-32.0 | 6.4-7.3 | 7.8-8.1 | 0.1-0.2 |

### 4.2.1 Experiment 1

Experiment 1 was conducted that two long aerators (30 cm) were place at the bottom of both narrow sides of three RT with 50 ml/min aeration rates for each aerators (Figure 4.1 (b)). Fertilized eggs were obtained from broodstock at Amami Experiment Station, the Fisheries Laboratory of Kinki University, transported by air Amami and Itami to Aquaculture Biology Laboratory, Nagasaki University in Japan on 4 July 2019. Eggs were firstly introduced into 100-l polycarbonate tank at 25ºC and 32 ppt. On 2 days post hatching (dph), larvae were distributed at 10 individuals/l into each experimental tank and were reared until 6 dph with static conditions.

### 4.2.2 Experiment 2

Long aerator (60 cm) was set at the bottom of long side of three RT adjusting 400-500 ml/min aeration rates (Figure 4.2 (a)). Hatching larvae were obtained from Seikai National Fisheries Research Institute, Nagasaki Prefecture, Japan on 18 July 2019. They were firstly introduced into 100-l polycarbonate tank at 25ºC and 32 ppt. Then, five hundred larvae were distributed into the each experimental tank. They were reared until 10 dph with static condition.

### 4.2.3 Experiment 3

Four baculiform aerators (15 cm) were set on the tank bottom under the edge of the bottom walls with 100 ml/min aeration rates for each aerator (Figure 4.2 (b)). Fertilized eggs were obtained from broodstock at Amami Experiment Station, the Fisheries Laboratory of Kinki University, transported by air Amami and Itami to Aquaculture Biology Laboratory, Nagasaki University in Japan on 4 September 2019. Five hundred eggs were directly distributed into each experimental tank. They were reared until 6 dph with static condition.

### 4.2.4 Statistical analysis

To determine the differences of survival, growth and swimbladder inflation rates of larvae between tanks with different aerators, Mann-Whitney *U* test was used for non-parametric test which a random variable of single or two group is not normally distributed by Shapiro-Wilk normality test. The rotifer distribution in tanks was standardized by deviation value on day *i* and determined by two-way analysis of variance followed by Tukey HSD test. R 3.4.1 software was used for all analysis, and a 5% level of confidence was used as a significant difference.

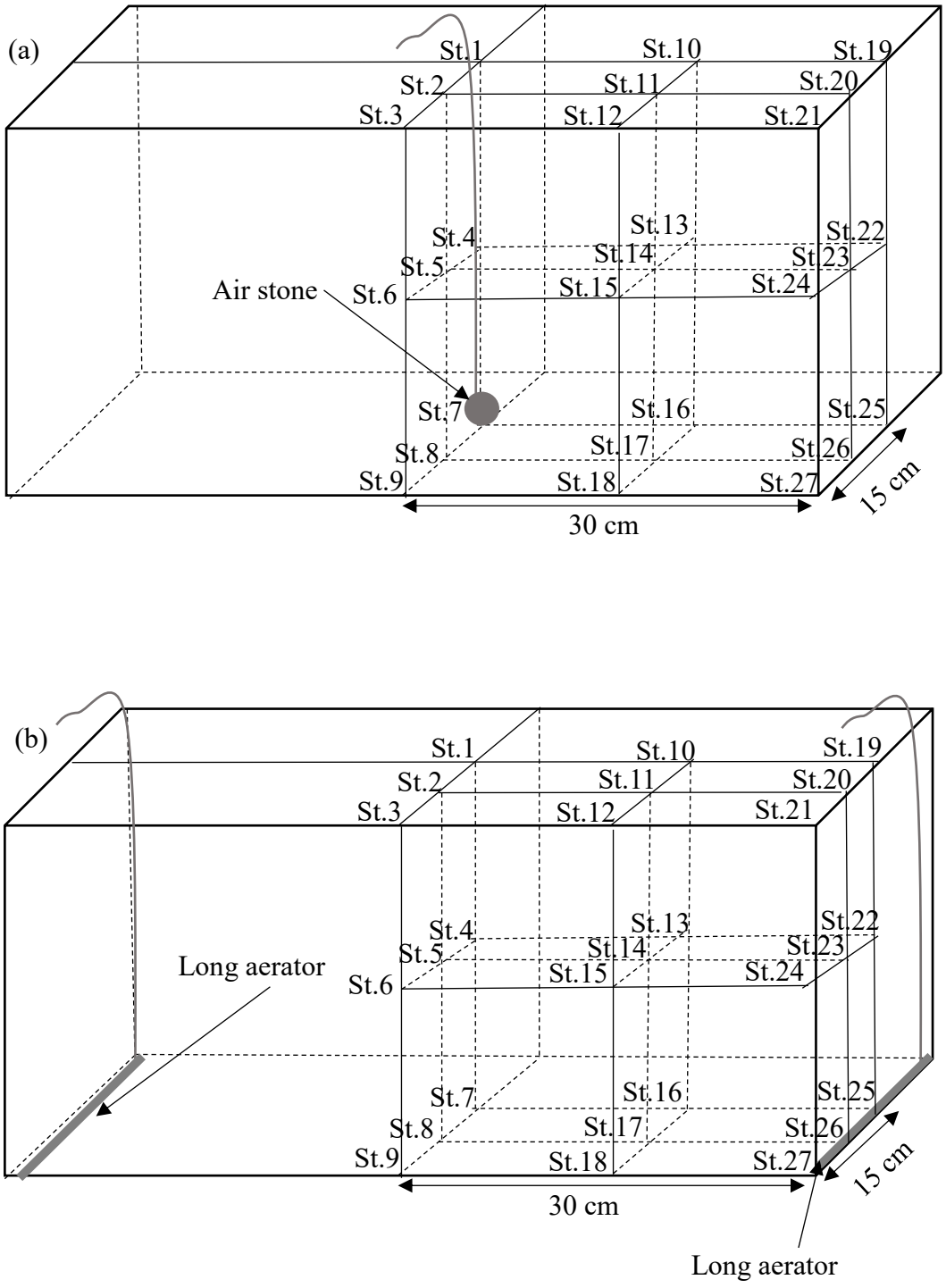



**Figure 4.1.** Sites for rotifer distribution in rectangular tank. (a) One spherical air stone was set at the center of the tanks bottom (b) two long aerators were set at the both narrows sides of tank bottom.

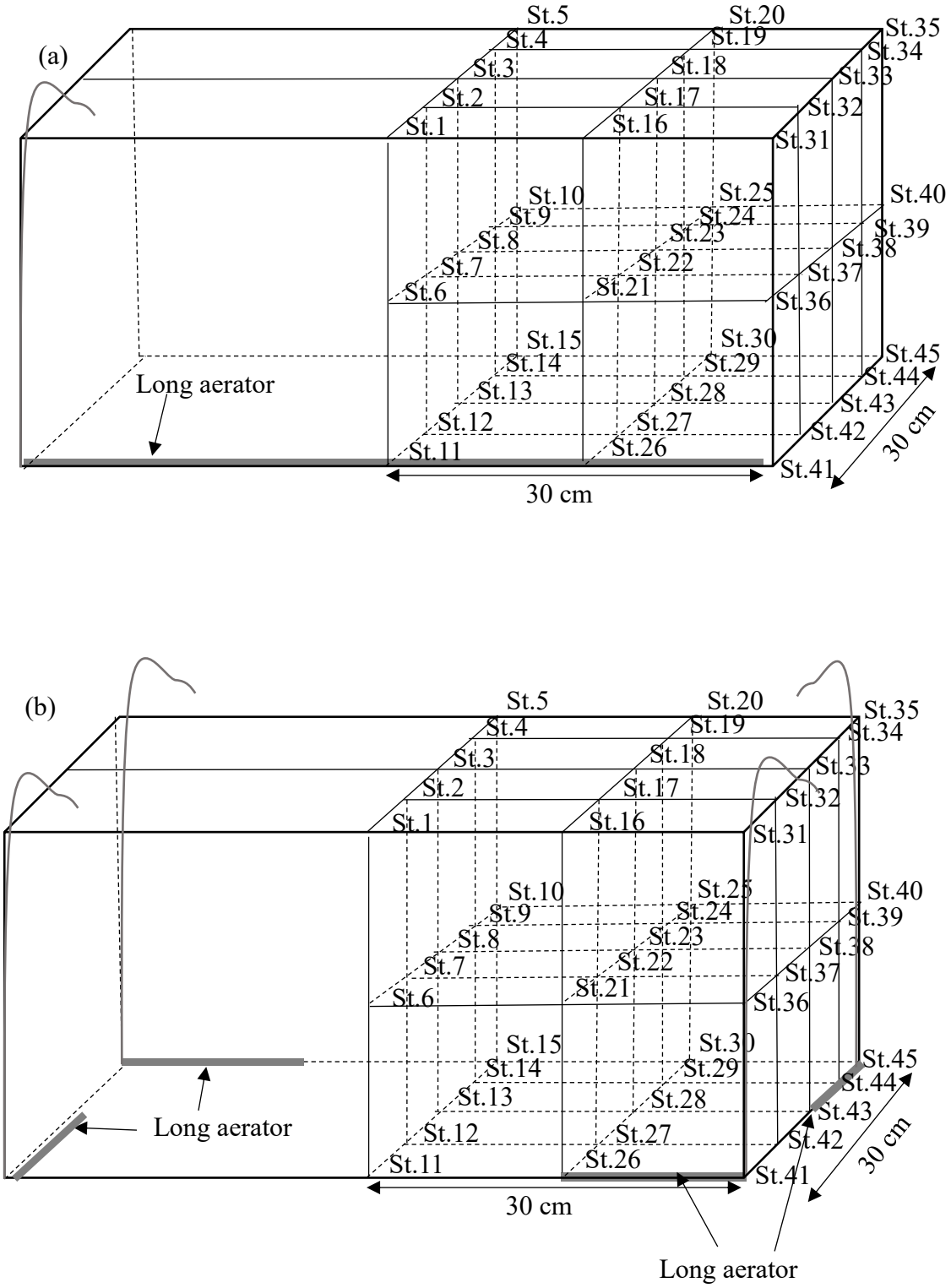



**Figure 4.2.** Sites for rotifer distribution in rectangular tank. (a) Long aerator was set at the long sides of the tank bottom (b) Four baculiform aerators were set at the edge of the bottom walls.

## 4.3 Results

Survival, growth and swimbladder inflation of Pacific bluefin tuna (PBT) larvae at 6 dph and 10 dph were similar or lower in the three experiments than control (Table 4.2, 4.3, 4.4). Morphological parameters were not significantly different (Table 4.5, 4.6, 4.7). Rotifer density increased during the experiments, reaching 20.7, 73.9 and 60.7 individuals/ml in control tanks (experiments 1, 2 and 3) and 13.0, 54.3, and 43.2 individuals/ml in experimental tanks (experiments 1, 2 and 3) during the culture period (n=4, 8) on the last day of experiments.

Rotifer distribution in control tank of the experiment 1 was different between stations (two-way ANOVA, $df$=26, $F$=2.0800, $p$=0.0032; Tukey HSD test, $p$<0.05, Figure 4.3 (a)) but not between days (two-way ANOVA, $df$=3, $F$=0.0060, $p$=0.9994). The stations and days had no interactions (two-way ANOVA, $df$=78, $F$=0.9690, $p$=0.5555). For the experimental tank of the experiment 1, rotifer distribution was also associated with stations (two-way ANOVA, $df$=26, $F$=3.6490, $p$<0.0001; Tukey HSD test, $p$<0.05, Figure 4.3 (b)) but not with days (two-way ANOVA, $df$=3, $F$=0.0140, $p$=0.9980). There were no interactions between stations and days (two-way ANOVA, $df$=78, $F$=0.9470, $p$=0.6000).

In the experiment 2, rotifer distribution in control tanks was different among stations (two-way ANOVA, $df$=26, $F$=1.9410, $p$=0.0043; Tukey HSD test, $p$<0.05, Figure 4.4 (a)) but it was not associated with days (two-way ANOVA, $df$=7, $F$=0.0040, $p$=1.0000).

Interactions between stations and days were not detected (two-way ANOVA, $df$=182, $F$=1.2210, $p$=0.0551). With the respect of experimental tank of the experiment 2, rotifer distribution was associated with stations (two-way ANOVA, $df$=44, $F$=30.1940, $p$<0.0001; Tukey HSD test, $p$<0.05, Figure 4.4 (b)) but not with days (two-way ANOVA, $df$=7, $F$=0.0050, $p$=1.0000). Interactions between stations and days were found (two-way ANOVA, $df$=308, $F$=1.3020, $p$=0.0032).

With the respect of the experiment 3, rotifer distribution in control tanks was associated with stations (two-way ANOVA, $df$=26, $F$=7.1590, $p$<0.0001; Tukey HSD test, $p$<0.05, Figure 4.5 (a)), although it was not affected by days (two-way ANOVA, $df$=3, $F$=0.0060, $p$=0.9993). There were no interactions between stations and days (two-way ANOVA, $df$=78, $F$=0.7380, $p$=0.9334). For the experimental tank, rotifer distribution was also different between stations (two-way ANOVA, $df$=44, $F$=11.0690, $p$<0.0001; Tukey HSD test, $p$<0.05, Figure 4.5 (b)) and was not associated with days (two-way ANOVA, $df$=3, $F$=0.0030, $p$=0.9997). There were no interactions between stations and days (two-way ANOVA, $df$=132, $F$=1.1640, $p$=0.1507).

**Table 4.2.** Survival, growth and swimbladder inflation rates of Pacific bluefin tuna *Thunnus orientalis* larvae at 6 dph in rectangular tank with different aerators in the experiment 1.

| Tank | Survival (%) | Total length (mm) | Swimbladder inflation (%) |
|---|---|---|---|
| Rectangular with one spherical air stone | 14.1±7.2 | 3.5±0.3 | 14.4±8.4 |
| Rectangular with two 30 cm long aerators | 0.1±0.2 | 3.3±0.0 | 0 |

Results are mean values ± SD (n=3).

**Table 4.3.** Survival, growth and swimbladder inflation rates of Pacific bluefin tuna *Thunnus orientalis* larvae at 10 dph in rectangular tank with different aerators in the experiment 2.

| Tank | Survival (%) | Total length (mm) | Swimbladder inflation (%) |
|---|---|---|---|
| Rectangular with one spherical air stone | 4.6±3.9 | 4.5±0.3 | 4.4±7.7 |
| Rectangular with one 60 cm long aerator | 0 | No data | No data |

Results are mean values ± SD (n=3).

**Table 4.4.** Survival, growth and swimbladder inflation rates of Pacific bluefin tuna *Thunnus orientalis* larvae at 6 dph in rectangular tank with different aerators in the experiment 3.

| Tank | Survival (%) | Total length (mm) | Swimbladder inflation (%) |
|---|---|---|---|
| Rectangular with one spherical air stone | 13.9±5.1 | 4.0±0.2 | 14.4±1.9 |
| Rectangular with four 15 cm long aerators | 0 | No data | No data |

Results are mean values ± SD (n=3).

**Table 4.5.** Morphological parameters of Pacific bluefin tuna *Thunnus orientalis* larvae at 6 dph in rectangular tank with different aerators in the experiment 1.

| Tank | SL (mm) | BD/SL (mm) | HL/SL (mm) | ED/SL (mm) |
|---|---|---|---|---|
| Rectangular with one spherical air stone | 3.3±0.3 | 0.2±0.0 | 0.2±0.0 | 0.1±0.0 |
| Rectangular with two 30 cm long aerators | 3.1±0.1 | 0.1±0.0 | 0.3±0.1 | 0.1±0.0 |

Results are mean values ± SD (n=3). Abbreviation: SL= Standard length, BD=Body depth, HL=Head length, ED= Eye diameter.

**Table 4.6.** Morphological parameters of Pacific bluefin tuna *Thunnus orientalis* larvae at 10 dph in rectangular tank with different aerators in the experiment 2.

| Tank | SL (mm) | BD/SL (mm) | HL/SL (mm) | ED/SL (mm) |
|---|---|---|---|---|
| Rectangular with one spherical air stone | 4.3±0.3 | 0.2±0.0 | 0.2±0.0 | 0.1±0.0 |
| Rectangular with one 60 cm long aerator | No data | No data | No data | No data |

Results are mean values ± SD (n=3). Abbreviation: SL= Standard length, BD=Body depth, HL=Head length, ED= Eye diameter.

**Table 4.7.** Morphological parameters of Pacific bluefin tuna *Thunnus orientalis* larvae at 6 dph in rectangular tank with different aerators in the experiment 3.

| Tank | SL (mm) | BD/SL (mm) | HL/SL (mm) | ED/SL (mm) |
|---|---|---|---|---|
| Rectangular with one spherical air stone | 3.8±0.2 | 0.2±0.3 | 0.2±0.0 | 0.1±0.3 |
| Rectangular with four 15 cm long aerators | No data | No data | No data | No data |

Results are mean values ± SD (n=3). Abbreviation: SL= Standard length, BD=Body depth, HL=Head length, ED= Eye diameter.

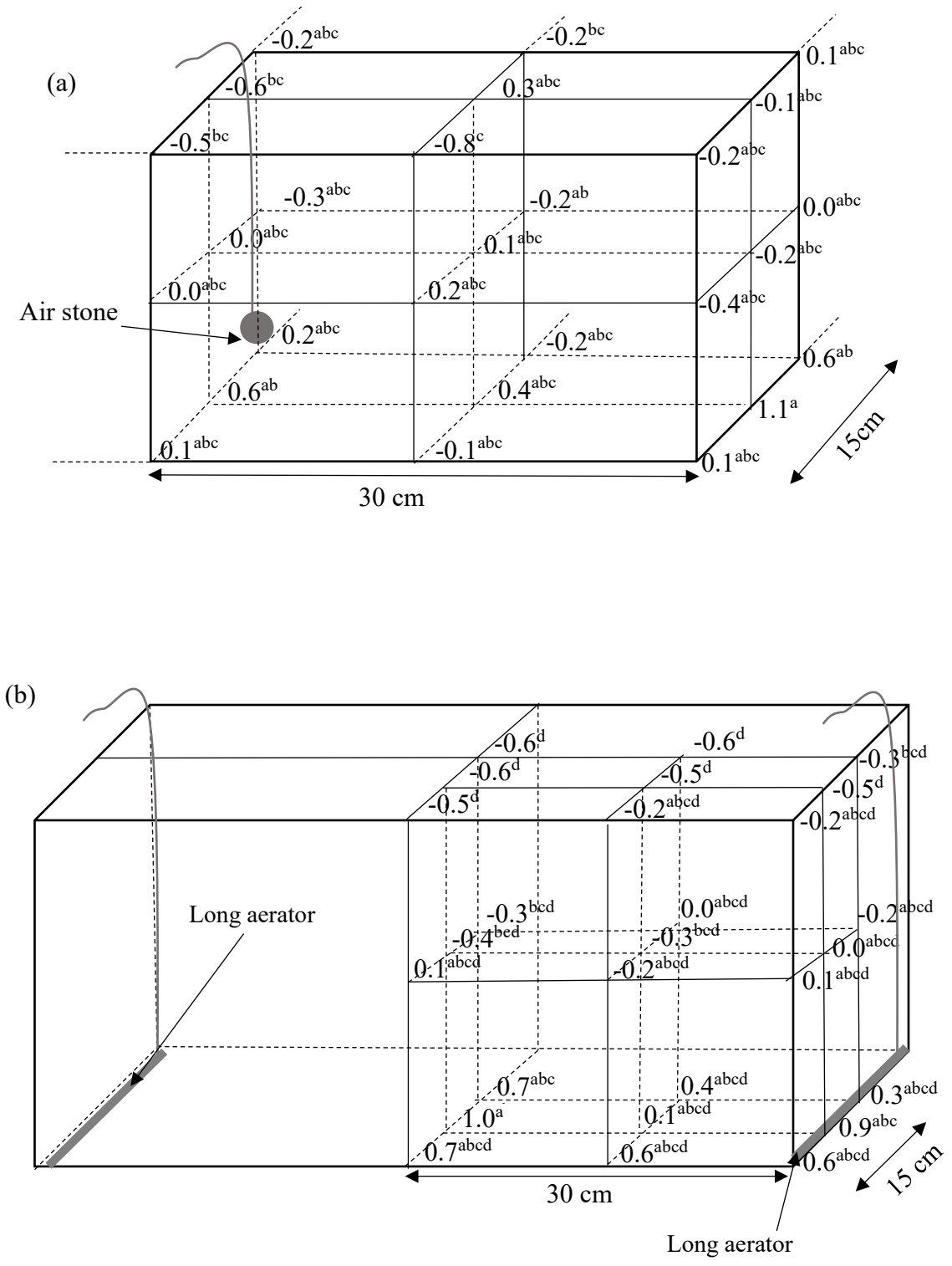



**Figure 4.3.** Rotifer distribution in quadrisection of the rectangular tank for the experiment 1. (a) Control tank and (b) experimental tank. Values are average of deviation values during culture period and alphabets in superscripts represent the significant differences between the stations that is one of the factors of two-way ANOVA (n=4, a>b>c>d, Tukey HSD, $p<0.05$).

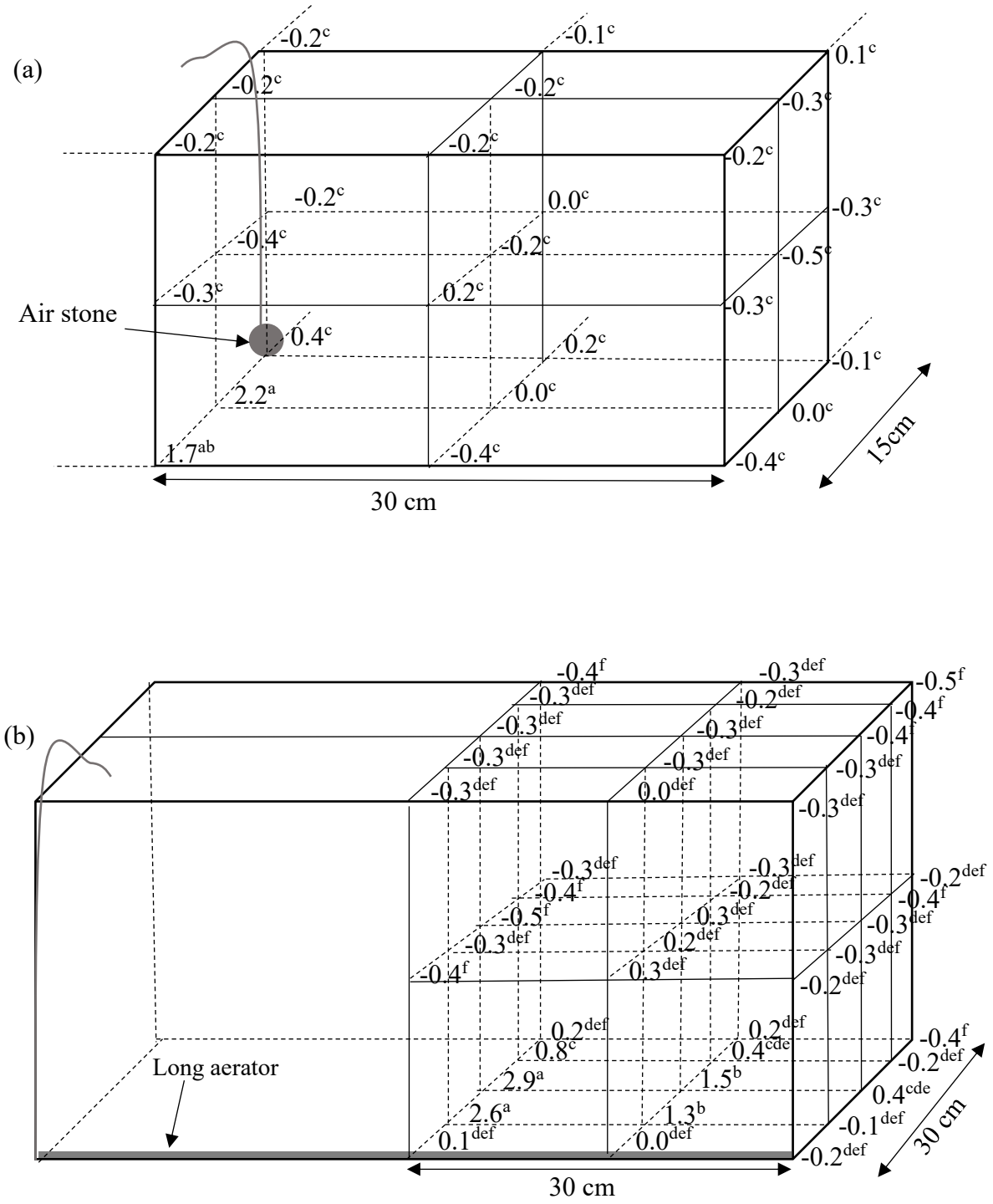



**Figure 4.4.** Rotifer distribution in the rectangular tank for the experiment 2. (a) Control tank and (b) experimental tank. Values are average of deviation values during culture period and alphabets in superscripts represent the significant differences between the stations that is one of the factors of two-way ANOVA (n=8, a>b>c>d>e>f, Tukey HSD, $p<0.05$).

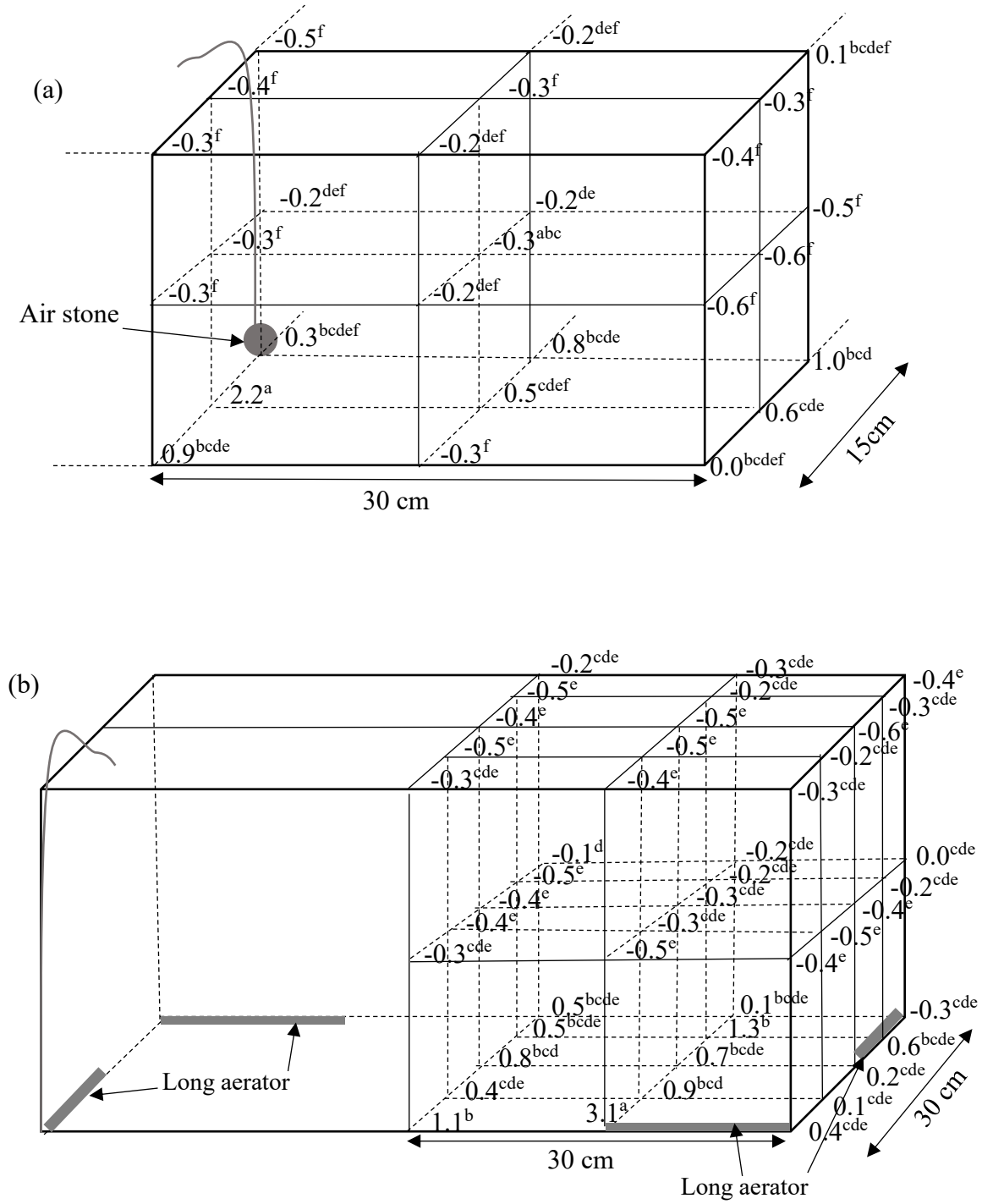



**Figure 4.5.** Rotifer distribution in the rectangular tank for the experiment 3. (a) Control tank and (b) experimental tank. Values are average of deviation values during culture period and alphabets in superscripts represent the significant differences between the stations that is one of the factors of two-way ANOVA (n=4, a>b>c>d>e>f, Tukey HSD, $p<0.05$).

## 4.4 Discussion

Rectangular tanks have three-dimensional complicated flow field patterns (Takakuwa et al., 2018), and stagnate areas in this tank may have negative effects on larviculture (Sakakura et al., 2019). Results in the present study showed that survival rates of Pacific bluefin tuna (PBT) larvae were not differed by the aerations in rearing tank. Survival rates of PBT larvae in all RT with different aerators types were similar or lower than all control tanks. The low velocity areas in RT may lead to the sinking syndrome of PBT larvae (Aung Naing Win et al., 2020) because low velocities and poor mixing of water in RT lead to the creation of stagnate areas, causing accumulation of biosolids on the tank bottom (Oca and Masaló, 2007).

Survival rates of PBT larvae in experiment 1 at 6 dph were not significantly different between different aerator types but survival rate (14.1±7.2%) of control tank showed higher trend than that (0.1±0.2%) in experimental tanks. Moreover, survival rates in control tank of experiment 1 was higher than that (4.6±3.9%) in control tank of experiment 2 at 10 dph, and similar to that (13.9±5.1%) in control tank of experiment 3 at 6 dph. However, these survival rates were comparable to the previous findings in the same tank and aeration systems (0.8%, Aung Naing Win et al., 2020), in which survival rates (52.7%) of PBT larvae in cylindrical tank with 100 ml/min aeration rates at 8 dph was higher than RT. Low survival rate of PBT larvae in RT may be the cause of sinking

syndrome of PBT larvae because PBT larvae with higher specific gravity sink to the bottom of the tank during the dark period due to low swimming activity and cause mortality at the bottom of rearing tanks (Takashi et al., 2006; Tanaka et al., 2009).

Moreover, the survival rates in control tanks in all experiments were lower than that 19.3% in 50 $m^3$ octagonal mass-scale tanks with water pump system under natural photoperiod at 8 dph (Tanaka et al., 2009). Then, this study was still lower than that (43.2 -48.6%) at 10 dph in 500-l cylindrical polycarbonate tanks with 300 ml/min day time aeration rates and 900 ml/min dark period aeration rates at 12L:12D (Nakagawa et al., 2011). In the case of the experimental tanks, survival rate of PBT larvae in these present study were similar and lower than control. Even an one spherical air stone at the bottom center of RT, complicated flow field and larger low velocity areas were observed in RT (Takakuwa et al., 2018; Aung Naing Win et al., 2020) and these low velocity areas may lead to the sinking syndrome of PBT larvae (Aung Naing Win et al., 2020). However, the present study examine to improve the aerations system with long bar-shaped aerators setting at the three patterns on the bottom of RT. As a result, the survival rates of PBT larvae were still low in RT with long aerators. Therefore, small-scale RT was still required to improve the aerations system beyond different aerator types to reduce the low velocity areas.

Growth of PBT larvae was not different between control and experimental tanks

in experiment 1 but there is not data for the experimental tanks in experiment 2 and 3. Compare with the growth of previous findings in the same tanks (Aung Naing Win et al., 2020), growth in control and experimental tank of experiment 1 was lower, and that in control tanks of experiment 2 and 3 were slightly longer and similar. Growth of PBT larvae was not affected by the aeration rates (Tanaka et al., 2018), however, the tank size may be an interactive factor due to density of larvae and water quality in tank for growth of marine fish larvae (Aung Naing Win et al., 2020).

Swimbladder inflation rates of PBT larvae in the present study were not different between control and experimental tanks in experiment 1. There are no results of swimbladder inflation for experiments 2 and 3. One of the cause of sinking syndrome is swimbladder inflation failure (Woolley and Qin, 2013; Ina et al., 2014; Kurata et al., 2012, 2015, 2017) due to the swimbladder controls the buoyancy of fish by reducing specific gravity relative to that of surrounding water (Taylor et al., 2010; Phleger, 1998). However, swimbladder inflation failure may not be the main case of sinking syndrome (Aung Naing Win et al., 2020) because the specific gravity of PBT larvae was higher than seawater density during dark periods even in swimbladder-inflated larvae (Takashi et al., 2006). Swimbladder inflation of this study makes it difficult to determine because larval survival was low in RT with different aerators types. Therefore, more detailed studies were needed to investigate whether sinking syndrome may be caused by the swimbladder inflation

failure, and whether the aerations may affect the swimbladder inflation of fish larvae.

The results of this study showed that the aerations with different aerators did not differ the survival, growth and swimbladder inflation of PBT larvae in RT. Thus, further detailed studies are required to improve the aeration system beyond the different aerators types in RT.

# Chapter 5

# General discussion

The present study demonstrated that the flow field generated by aerators in small (<50-l) tank with different shapes affect the survival, growth and swimbladder inflation of marine fish larvae. The velocity of flow fields was different not only by tank shapes but also by aeration with the same water volume and surface area. The high water velocity in tank may prevent larvae from attaching to both tank wall and water surface (Sakakura et al., 2019), and low flow areas (low velocity areas) in tank may save the energy for swimming of larvae. However, small-scale (<50-l) larviculture system was applicable for marine fish larvae with the present aeration system due to obtain the high survival rate.

Survival rates of red seabream larvae in CT and RT1AS were higher than that in RT2AS. The bubbles from the two air stones in small tank may stun the larvae more frequently and may have larvae contacting the tank walls than one air stone in tank. However, the viability of red seabream larvae was not different between different tank shapes with the same aeration system. It is assumed that flow field in small tank may not affect the quality of larvae. On the other hand, swimbladder inflation in CT showed lower trend than those in RTs. The swimbladder is an important internal organ for fish because it controls the buoyancy of them (Phleger, 1998; Taylor et al. 2010). Lacking the functional

swimbladder of fish larvae due to the failure of swimbladder inflation influences the negative effect in fingerling production, resulting not only in poor survival and/or growth in larviculture (Kitajima et al., 1981; Kurata et al., 2012). Swimbladder inflation of fish larvae is initiated by gaping air from the water surface and introducing it into swimbladder through the pneumatic duct after mouth opened (Kitajima et al., 1981). The low velocity areas (less than cruising speed of larvae) at the water surface of tank were observed along the sidewalls of the CT and RT1AS, and along the sidewalls and between two aerators (in the middle of tank) in RT2AS. These low velocity areas may support fish larvae to access the air through the water surface for swimbladder inflation. Further, average flow velocity at surface areas of CT (24.6 mm/s) was around 1.2 times higher than these in RT1AS and RT2AS (22.1 mm/s and 19.3 mm/s) in the present study. Therefore, flow velocity at surface area in CT may make fish larvae prevent from gaping air through water surface that will lead to the failure of swimbladder inflation.

In this study, survival of red seabream larvae was significantly different between different aeration systems but swimbladder inflation of larvae was not different between different tank shapes and aeration systems. It was assumed that swimbladder inflation failure may not affect the survival of red seabream larvae due to the absence of sinking syndrome. Sinking syndrome of PBT larvae can be caused by swimbladder inflation failure (Kurata et al., 2012, 2015, 2017), since swimbladder controls the buoyancy of fish by

reducing specific gravity relative to that of surrounding water (Phleger, 1998; Taylor et al., 2010). However, the specific gravity of PBT larvae was higher than seawater during dark periods even in swimbladder-inflated larvae (Takashi et al., 2006). In addition, swimbladder inflation failure may not affect the survival and/or sinking syndrome in small tanks (Chapter 2 and 3). Further detailed studies are required to observe that swimbladder inflation failure affect the survival, growth and/or sinking syndrome of marine fish larvae in different larval rearing tank sizes and/or shapes.

With the respect of PBT larviculture, the comparison between different tank shapes (CT and RT) was firstly examined with the same aeration system. The survival rate in CT was about 60-folds higher than that in RT. The plug-flow patterns, an irregular and unpredictable flow patterns, and poor water mixing conditions were observed in RT and these factors can influence the welfare of fish in several ways (Oca et al., 2004; Duarte et al., 2011). Low velocities and poor mixing of water in RT lead to the creation of stagnate areas, causing the accumulation of biosolids on the tank bottom (Oca and Masaló, 2007), and these stagnate areas may have the negative effects on the larviculture (Sakakura et al., 2019). Swimming speed of PBT larvae (8.8 mm/s) at 8 dph (4.2±0.1 mm in total length) was almost 2 times faster than that of red seabream larvae (4.6 mm/s) at 14 dph (4.8±0.2 mm in total length). However, swimming speed of PBT larvae with heavy specific gravity becomes lower at nighttime than in daytime, and sinking syndrome occurs (Takashi et al.,

2006). The low velocity areas, where the water velocity is slower than the cruising speed of larvae, were about 1.63 times larger in RT as shown in figure 3.3. Therefore, RT was required to improve the aeration beyond a single air stone to eliminate stagnate areas at bottom which may cause the sinking syndrome of PBT larvae.

The water velocity in rearing tanks can be estimated by the rotifer distribution at the various points in the tanks. The stagnate areas, where water velocity is slower than the swimming speed of rotifer (1.3 mm/s), coincided with high rotifer density in tank. Flow velocity was higher around the center than along the sidewalls at the surface areas in CT and RT1AS. It is similar with the points at low density of rotifer at the surface areas in CT and RT1AS. At the surface areas in RT2AS, flow velocity was higher around the center of the half of the tank than the middle and along the sidewalls of the tanks. The low density of rotifers at the surface areas of RT2AS were similar to the high velocity areas at the surface in this tank. Moreover, low velocity areas at the water surface were observed more in the downward streamlines areas than in upward flow from aerators and the areas where streamlines straighten toward the sidewalls before downward flow in both tanks.

To improve the aeration system for PBT larviculture, three different aeration rates (50, 100 and 300 ml/min) were preliminary tested with one spherical aerator (5 cm in diameter) at the bottom center of CTs and RTs. The survival in CTs were lower than the control aeration rates (100 ml/min), and the survival in RTs were similar to the control

aeration rates (100 ml/min). On the other hand, the survival in CT at 100 ml/min was highest among three different aeration rates in CTs and RTs. Then, three aeration systems generated by different aeration rates were tested with the long-bar shaped aerators in RTs. The results of all tested were similar or lower than the control tank, in which one spherical aerator set at the bottom center of tanks with 100 ml/min aeration rates. The aeration rates changed with the aerators. However, the appropriate aeration rate was not found in small RT for PBT larvae. For 50 ml/min aeration rate with two 30 cm long aerators at the both narrow side of bottom in RT, it is clear that stagnate areas are larger in bottom of RT because high rotifer density areas were observed in the middle of tank bottom. With the respect of 400-500 ml/min with one 60 cm long aerator at the long side of bottom in RT, it was high aeration rate but stagnate areas were also observed at the base of aerator, coinciding with high rotifer distribution. Four baculiform aerators at the edge of bottom walls of RT with 100 ml/min aeration rate for each may be circulate the water with high aeration rate but the stagnate areas were still observed at around near aerators and it coincided with the areas of high rotifer density. Therefore, this study suggested that RT still requires to improve the aeration beyond different aerator types to reduce the low velocity areas which can create the stagnate areas.

In the present study, flow field in the different small tank shapes and aeration systems affected the survival of marine fish larvae. The RT with one spherical air stone at

the bottom center should be applied for the red seabream larviculture with present aeration system because both survival and swimbladder inflation rates of larvae were quite high. With the respect of PBT larviculture, the CT with one spherical air stone at the bottom center is recommended due to the high survival rate. The difference between red seabream and PBT larvae was the occurrence of sinking syndrome in PBT. Hence, the small (<50-l) RT should not be applied for the marine fish larvae which shows sinking syndrome. Measuring the rotifer distribution at the various points in tanks was useful method to estimate the flow velocity in tanks, especially for the stagnate areas in tanks with higher rotifer densities than the average density.

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