水研センター研報，別冊第1号，83－90，平成16年
Bull. Fish. Res. Agen. Supplement No. 1, 83-90, 2004

An assessment of the beneficial roles of *Nannochloropsis oculata* in larval rearing of marine finfish

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Abstract From points of both water quality management by algal nutrient absorption and a good live food of rotifer, the role of *Nannochloropsis oculata* supplied to larval rearing water was examined by a larval rearing experiment in this study. Larvae of Japanese red sea bream, *Pagrus major*, were reared being fed rotifers and subsequently an artificial diet during the thirty-day experimental period. In the four test tanks (100-L capacity) each with 1,000 Japanese red sea bream larvae in addition to the rotifers, *N. oculata* was maintained at a density of $5\text{-}10\times10^5$ cells/mL while in the four control tanks, no *N. oculata* supplemented, but all the other experimental conditions were the same as the test tanks.

At the end of the experiment, the concentrations of inorganic nitrogen (NH_4-N, NO_2-N, and NO_3-N) and inorganic phosphate (PO_4-P) in the control tanks were 1.5-2.0 times higher than those in the test tanks. While, the number of rotifers with eggs in the test tanks was remarkably higher in comparison with that in the control tanks. Moreover, rotifers in the test tanks contained much more eicosapentaenoic acid (EPA) and n-3 highly unsaturated fatty acid (n-3 HUFA), so that their nutrient value is concluded as higher than that in the control tanks. From the results of this experiment, supplementation with *N. oculata* into the rearing water is concluded to produce beneficial effects on maintaining water quality and also enhancing the nutrient quality of the rotifers.

Key words: *Nannochloropsis oculata*, larval rearing, water quality management, *Pagrus major*, rotifer, *Brachionus*, food organism

Recently, the condensed freshwater *Chlorella* has been popularly used as a food organism for L-type and S-type rotifers, *Brachionus plicatilis* and *B. rotundiformis*, in Japanese hatcheries of marine fishes (Hirayama *et al.*, 1989; Maruyama *et al.*, 1989). Because condensed freshwater *Chlorella* is readily available from several private companies, it has contributed to much laborsaving and allowed the realization of the stable production of rotifers (Yoshimura *et al.*, 1997). While, the number of hatcheries which culture the microalga *Nannochloropsis oculata*, which has been used as the main food for rotifers for more than 20 years, has gradually reduced in Japan. However, the freshwater *Chlorella* has mainly three demerits as a live food. (1) *Chlorella* contains little n-3 highly unsaturated fatty acids (n-3 HUFA) (Maruyama *et al.*, 1986), which are essential fatty acids for marine fishes (Watanabe *et al.*, 1983), so its nutritive value has been considered as lower than that of *N. oculata*. (2) The alga does not survive long-term in sea water and readily decays within several days. The dead cells then induce water pollution. (3) The alga has a hard cell wall, so that it cannot be used as a food organism for almost all important aquacultured invertebrates.

2003年6月12日受理 (Received on June 12, 2003)
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From these reasons, small quantities of *N. oculata* are cultured in many hatcheries and used to enrich the HUFA content of rotifers, which are predominantly cultured with *Chlorella*, before feeding the rotifers to finfish larvae. Moreover, it is recognized empirically that the addition of *N. oculata* into the rearing water is useful to increase the activity and survival of larval fish (Eda *et al.*, 1990; Murashige *et al.*, 1991; Tamaru *et al.*, 1994). In this study, the effect of *N. oculata* additions to the rearing water was investigated from the point of view of both the quality of the food organisms rotifers and for water quality management in the larval rearing tanks.

Materials and Methods

Materials

Japanese red sea bream, *Pagrus major*, was selected as the material of this study, because the larval rearing method is fully established and larval production is carried out regularly in Japan. Eggs were obtained from a matured female, and were fertilized artificially. Prior to the experiment, the larvae were reared for 10 days after hatching in a 500-L polycarbonate tank with a continuous air-supply and water-exchange system. The larvae were fed with sufficient S-type rotifer during this period.

Nannochloropsis oculata collected from Gokasho Bay in 1986, and cultured in liquid Guillard F medium (Guillard and Ryther, 1962) was used in this experiment. This strain has been cultured using 100-mL flasks with periodic changes of medium under 20℃ and 50 μmol/m^2/s for more than ten years. During the experiment, the alga was produced using some 10-L glass carboys in batch style under conditions of 25 ℃ and 80 μmol/m^2/s. When the algal growth reached the stationary phase, it was added to the larval rearing water.

S-type and L-type rotifers were obtained from Mie Prefecture Fish Farming Center, Hamajima City. They could make few resting eggs in mass production. They mainly reproduced by parthenogenesis and produced few resting eggs in mass production. They were produced using some 500-L polycarbonate tanks fed with freshwater *Chlorella* at 25 ℃. The artificial diet for red sea bream larvae used in this study was purchased from a Japanese private company.

Larval rearing experiment

The experiment was conducted using eight 100-L transparent polycarbonate tanks each with 90-L filtered seawater. Four tanks were used as test trials and others were used as control. One thousand larvae at age 10 days were introduced into each tank. In the test tanks, both *N. oculata* and rotifers were put into the rearing water at densities of 10^6 cells/mL and 10N/mL, respectively, at the beginning of the experiment. They were kept at $5\text{-}10 \times 10^5$ cells/mL and 10-20/mL, respectively, during the experiment. In the control tanks, the density of rotifers was kept almost the same as that in the test tanks, however, *N.oculata* was not supplied. S-type and L-type rotifers were fed from day 10 to day 20 and from day 20 to day 30, respectively. Before being supplied to the larvae, the rotifers were fed with *N. oculata* sufficiently for one day to enhance the eicosapentaenoic acid (EPA) levels. From day 30 to the end of experiment (day 40), the appropriate quantity of artificial diet was fed in each tank.

The densities of *N. oculata* and rotifers in all tanks were estimated twice daily using a coulter counter and a stereoscopic microscope, respectively. Moreover, the densities were adjusted to the fixed densities by further additions or by draining and adding filtered seawater. The rearing water temperature was kept at 20-22 ℃ and aeration was continuously supplied at a rate of 400-500 mL/min per a tank. To maintain the water quality in each tank, about 30-L rearing water was exchanged once a day before feeding using thin vinyl tubes.

Counting of dead larvae and female rotifers carrying eggs

Dead larvae, which sank to the bottom of

each tank, were collected each day into a white beaker using thin glass tubes with a siphon effect. The average and standard deviation (±S. D.) were calculated from the number of dead larvae in the four test tanks and four control tanks, respectively. Moreover, four 10-mL samples were taken from each tank in order to cal culate the amictic (undergoing parthenogenesis) female ratio (%); (the number of rotifers carrying eggs/the number of rotifers) × 100. Because no resting eggs were observed in the mass culture tanks of S-type and L-type rotifers, all rotifers carrying eggs were regarded as amictic females in this experiment.

Water quality analysis

In this experiment, the contents of inorganic nitrogen (total ammonia; NH_4-N, nitrite nitrogen; NO_2-N, nitrate nitrogen; NO_3-N) and inorganic phosphate (PO_4-P) were selected as indicators of water quality. Water samples (about 500-mL) for their analysis were collected from each tank every day before changing the water. The samples were filtered using GF/C (Whatman). The filtered samples were put in 500-mL polyethylene bottle and preserved at −30 ℃ for later analysis. The total ammonia was measured using an ammonia electrode with digital ion analyzer (Orion Research, model 701A). Other nutrients were measured according to the methods of Strickland and Parsons (1972).

Measurement of the survival rate of larvae

At the end of the 30-day rearing experiment, the number of surviving larvae and the total body length of 100 randomly sampled larvae from each tank, were measured and their aver ages±S.D. in the test tanks and control tanks were calculated and analyzed by one-way ANOVA.

Fatty acid analysis of rotifers

On day 30, at the start of feeding the artificial diet, rotifers in the test and control tanks were sampled using a plankton net. They were washed with fresh water for about ten minutes, and were stored at −80 ℃ until analysis. Furthermore, rotifers enriched with *N. oculata* after being produced with freshwater *Chlorella* were also analyzed. Total lipids were extracted by chloroform-methanol method according to Folch *et al.*, (1957). The fatty acids of the total lipids were methyl-esterified and analyzed by gas-liquid chromatography.

Results

Survival and growth of larvae

Daily changes of the average number of dead larvae in the test and control tanks are shown in Fig. 1. The number of dead larvae per day in the control tanks was significantly higher than that in the test tanks after day 28 ($P<0.01$). The average total number of the conformed dead larvae in test tanks during this experiment was about 100, while that in the control tanks was about 198. The averages±S.D. of the total body length (mm) in the test and control tanks were 9.5±0.9 and 8.8±1.2, respectively. Thus, larvae in the test tanks were significantly larger ($P<0.05$) than those in the control tanks.

Rate of rotifers with eggs

Daily changes of the percentage of rotifers with eggs in the test and control tanks are shown in Fig. 2. Regardless of the rotifer type, the number of rotifers with eggs in the test tanks was clearly higher than that of the control tanks. More than 20 % of rotifers in the test tanks carried one or two eggs under their loricas, while rotifers with eggs in the control tanks were less than 10 %. During the experiment, no mictic reproduction was observed in any tank.

Fatty acid components of the rotifers

Percentage composition of the fatty acids of rotifers is shown in Table 1. The crude protein and crude lipid contents of them are also shown. The rotifers collected from the test tanks contained much EPA comparison with the rotifer from the control tanks.

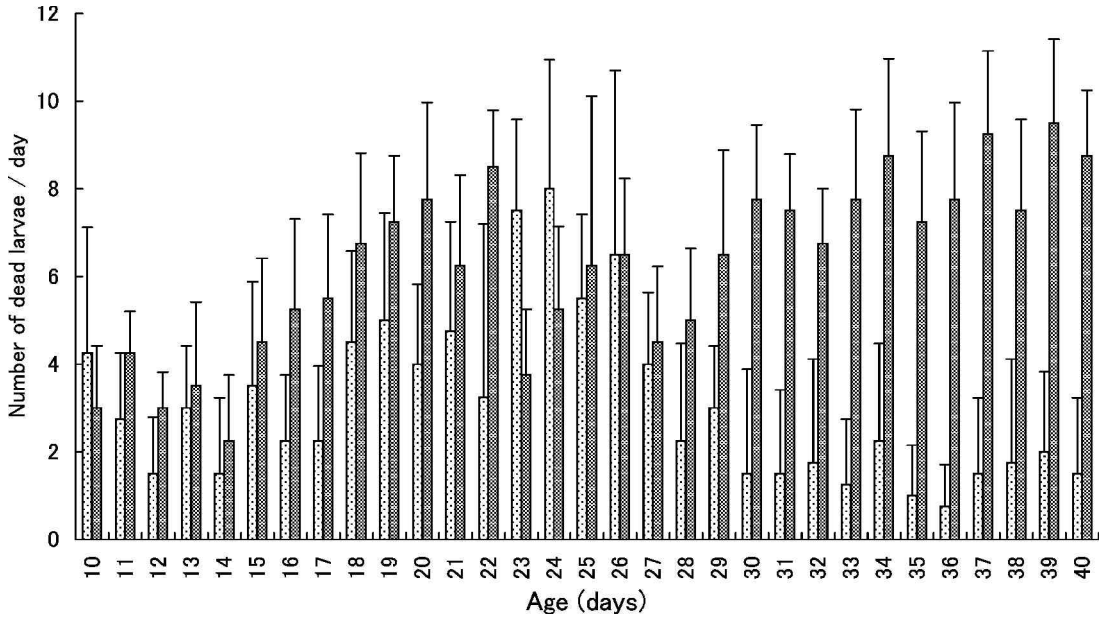

Fig. 1. Daily changes of the average number of dead larval Japanese red sea bream, *Pagrus major*, in the test tanks (Dotted bars ; ⬚) and the control tanks (Shaded bars ; ▩). Vertical lines indicate the standard deviations. X-axis shows the larval age, which is indicated by days after hatching.

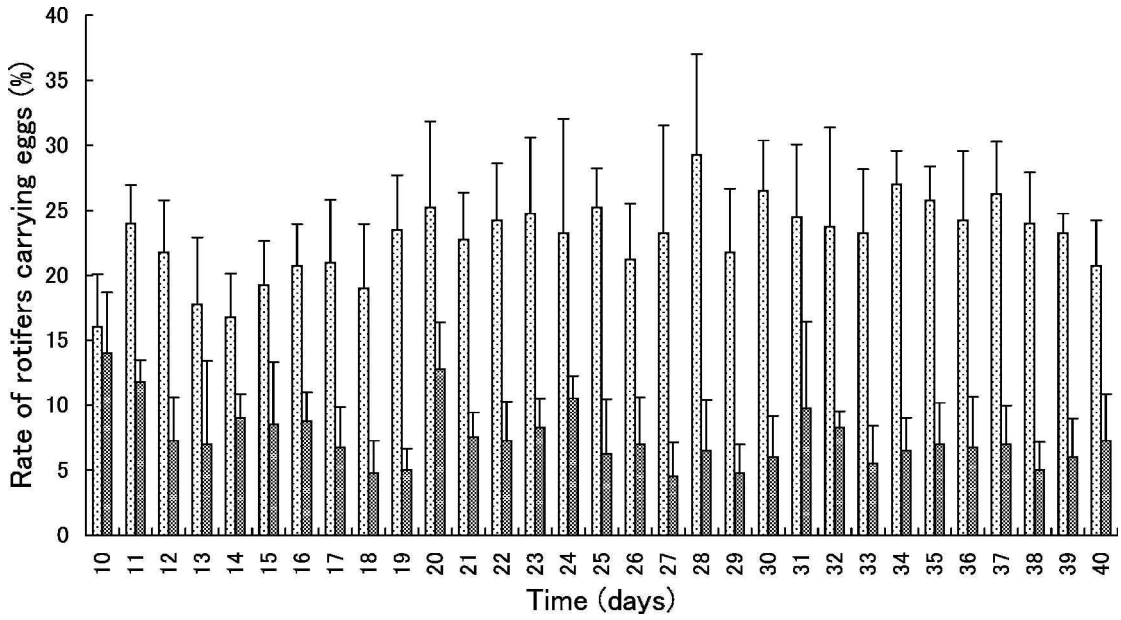

Fig. 2. Daily changes of percentage of rotifers with eggs in the test tanks (Dotted bars ; ⬚) and the control tanks (Shaded bars ; ▩). Vertical lines indicate the standard deviations. X-axis shows the elapsed time from the larval hatching out. In order to calculate the ratio of females carrying eggs, four 10-mL samples were taken from each tank and the number of rotifers with eggs was counted using a stereoscopic microscope.

Consequently, the content of n-3 HUFA contained in rotifers from the test tanks was significantly higher than that in rotifers from the control tanks. While, the fatty acid component of rotifers, which were secondarily enriched with *N. oculata* after production using freshwater *Chlorella*, was almost the same as that of rotifer from the test tanks. On the other hand, the protein content of rotifers from the control tanks was lower in comparison with those of rotifers from the test tanks.

Table 1. Percentage composition of the fatty acid components, percent total lipid and crude protein of rotifers collected from the test tanks and the control tanks are shown in the first and second rows. Moreover, these components of rotifers, which were secondarily enriched with *N. oculata* after production using freshwater *Chlorella*, are shown in the third row for comparison.

(%)

Fatty acid	Rotifers in the test tanks (with *Nannochloropsis*)	Rotifers in the control tanks (without *Nannochloropsis*)	Rotifer cultured with *Chlorella* (secondary enriched with *Nannochloropsis*)
14 : 0	5.5	5.2	5.8
16 : 0	15.2	15.6	16.4
16 : 1	18.8	11.1	19.8
16 : 4 n-3	0.6	1.2	0.8
18 : 0	2.6	2.2	2.8
18 : 1	8.2	9.6	10.5
18 : 2 n-6	4.5	5.6	4.7
18 : 3 n-6	0.5	0.7	0.4
18 : 3 n-3	0.5	0.2	0.8
18 : 4 n-3	0.9	0.8	0.1
20 : 1	3.7	2.7	4.7
20 : 2 n-9	0.2	0.2	0.3
20 : 3 n-6	0.4	1.1	1.0
20 : 4 n-6	3.2	2.7	4.7
20 : 4 n-3	0.5	0.6	0.5
20 : 5 n-3	18.9	5.8	20.8
22 : 1	1.2	2.0	1.1
22 : 3 n-6	0.1	0.3	0.1
22 : 5 n-6	1.6	Tr[*2]	0.1
22 : 5 n-3	2.6	1.2	5.6
22 : 6 n-3	0.4	0.2	0.4
24 : 1	0.3	0.2	0.4
∑n-6	10.3	10.4	11.0
∑n-3	24.0	10.0	29.0
∑n-3HUFA	28.7	12.5	33.0
Lipid (%) [*1]	2.5	2.1	2.9
Protein (%) [*1]	7.2	5.6	7.8

[*1] Wet matter basis; [*2] Tr indicates trale level

Results of water quality analysis

Daily changes of NH_4-N, NO_2-N and NO_3-N concentrations are shown in Fig. 3. After day 21, the concentration of NH_4-N in the test tanks was significantly lower than that in the control tanks ($P<0.01$), except on days 31 and 32. The average concentration of NH_4-N after day 32 in the test tanks was 1.6 mg/L, while that in the control tanks was 2.2 mg/L. In the case of NO_2-N, its concentration in the test tanks was significantly lower than that in the control tanks after day 30 ($P<0.01$), except on day 33. The average rate of increase per day from day 29 to day 40 in the test tanks was 0.064 mg/L, while that in the control tanks was 0.095 mg/L. The concentration of NO_3-N was about 10 times higher than that of NH_4-N and NO_2-N during the experiment in both test and control tanks. The difference between the concentration of NO_3-N in test tanks and that in the control tanks was significantly different after day 18 ($P<0.01$), except day 21. The average rate of increase per day after day 18 to day 40 was 0.545 mg/L in the test tanks and was 1.002 mg/L in the control tanks.

On the other hand, daily changes of PO_4-P concentration are shown in Fig. 3. No difference between its concentrations in the test tanks and those in the control tanks was detected from day 10 to day 29. However, the difference between them gradually became clear after day 30 ($P<0.01$). At the end of the experiment, the concentration of PO_4-P in the control tanks was about two times higher than that in the test tanks.

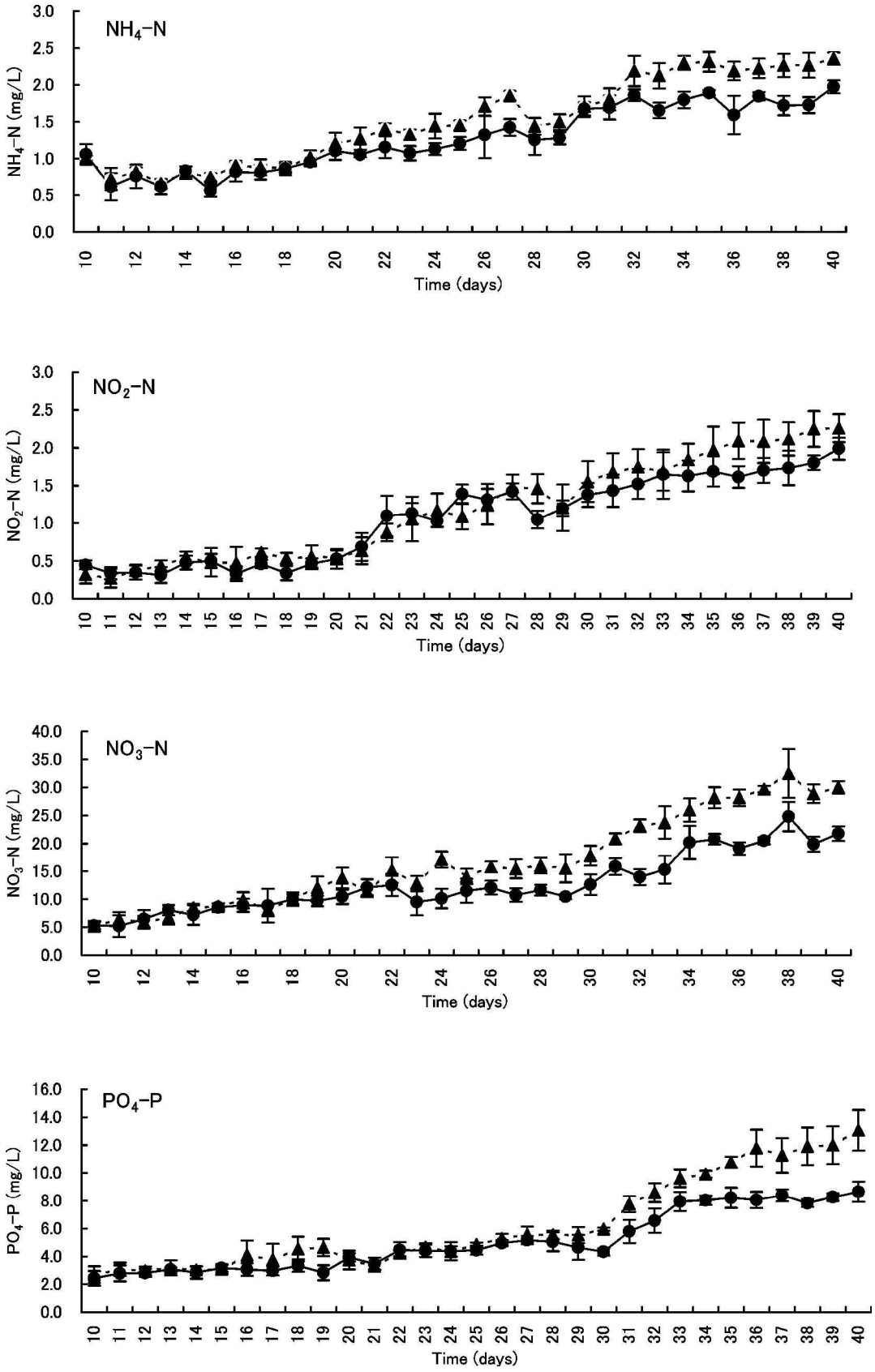

Fig. 3. Daily changes of the average contents of NH_4-N, NO_2-N, NO_3-N and PO_4-P in the test tanks (●) and the control tanks(▲). Vertical lines indicate the standard deviations. S-type rotifers were fed from day 10 to day 20, L-type rotifers were fed from day 20 to day 30, and an artificial diet were supplied from day 30 to the end of the experiment.

Discussion

Efficiency of *N. oculata* to enrich rotifers in larval rearing tanks

Regardless of the rotifer type, the nutritive value of rotifers collected from the test tanks was high comparison with that of rotifers from the control tanks. Especially, the EPA and n-3 HUFA contents of rotifers were reduced clearly in the control tanks. This is concluded to be caused by the deficiency of *N. oculata* in the control tanks. Both types of rotifer put in the control tanks and their newly hatched offspring were not supplied with alga and as such had minimal available food materials so that their n-3 HUFA and protein contents were reduced compared the test tank rotifers. Even if the larvae are fed such rotifers enough, their survival and growth rates will undoubtedly be lower. While, the rotifers in the test tank fed *N. oculata* enough, so that it's nutritive value was enhanced. Thus, the larvae in the test tanks grew well and their survival rate was high.

Moreover, the rotifers in the test tanks had a higher rate of egg carrying than that in the control tanks. The amictic reproduction of rotifers would be induced more frequently in the test tanks, because of the higher food availability in the tanks. Therefore, the addition of *N. oculata* seemed to be effective to produce both nutritive and reproductively active rotifers in the larval rearing water.

Water quality management by *N. oculata*

After day 30, the number of dead larvae in the control tanks became conspicuously higher than that in the test tanks. However, the same quantity of artificial diet was supplied to both the test and control tanks from day 30. Therefore, there was no difference in the food supplied to the test and control tanks. As the cause of larval death in the control tanks, two possible causes are apparent.

One is the sudden diminution of rotifers in the rearing water. No rotifers were supplied into both the test and control tanks after day 30. However, the number of rotifers only reduced gradually in the test tanks, because active reproduction occurred continuously. Thus, the larvae in the test tanks could eat not only the artificial diets, but also the rotifers. While, the larvae in the control tanks after consuming the remaining rotifer ate the artificial diet only. Therefore, the effect of the addition of *N. oculata* into the rearing water seemed to continue after even changing to the artificial diet.

Moreover, water pollution was a probable factor inducing larval mortality. After changing from rotifers to the artificial diet, inorganic nitrogen (NH_4-N, NO_2-N, and NO_3-N) and phosphate (PO_4-P) concentrations increased both in the test and control tanks. Especially, they increased rapidly in the control tanks. One of the reasons for the differences in concentrations of nitrogen and phosphate in the rearing water between in the test tanks and in the control tanks seemed to algal nutrient absorption. *Nannochloropsis oculata* absorbed dissoluble nitrogen and phosphate as nutrients in the test tanks. Additionally, many larvae died in control tanks, and their bodies, prior to being removed each day would also deteriorate the quality of the rearing water. Thus, water pollution seemed to be accelerating in a shot term. From this point of view, additive *N. oculata* was effective.

In an almost similar rearing experiment using mullet larvae, Tamaru *et al.*, (1994) reported that the NH_4-N content in the test tanks was significantly higher than the control tanks. They considered that the reason was contamination from the algal medium, because they used ammonium sulfate as a nutrient in the medium. In this experiment, sodium nitrate was used instead of ammonium sulfate, so that the toxicity of unionized ammonia was concluded as negligible. Moreover, *N. oculata* was put into the rearing water after the algal growth reached the stationary phase. At the stationary phase, almost all the nutrients in the medium were used for the algal growth. Thus, contamination from the algal medium would not raise the nitrate nitrogen level in the

larval rearing water.

A new role of *N. oculata* for larval rearing

From the results of this experiment, it was shown that *N. oculata* has main two roles in the larval rearing water. The addition of *N. oculata* into the rearing water was indispensable to enhance the fatty acid component of rotifers to a level suitable for the fish larvae. Moreover, the addition of the alga was effective to reduce the concentrations of the soluble nitrogen and phosphate. Especially, after feeding with the artificial diet, the concentrations of NO_3-N and PO_4-P increased remarkably. However, because the larvae still only had a weak swimming ability, we could not adopt a continuous flowing system with a high flow rate. When a semi-continuous changing water system was adopted, the alga seemed to be useful as absorbing potentially harmful excess nutrients and contribute to maintain the water quality management.

In the near future, various kinds of artificial diets will be used commonly in larval rearing. Moreover, freshwater *Chlorella* is now usually used in rotifer production. The characteristics of *N. oculata* have significant potential to enhance and stabilize production in finfish larval culture and as such for the effective utilization of this useful link in the food chain in hatchery production, further detailed research is required.

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