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Effect of combination feeding of *Nannochloropsis* and freshwater *Chlorella* on the fatty acid composition of rotifer *Brachionus plicatilis* in a continuous culture

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ABSTRACT: A continuous culture of rotifer was conducted to investigate the effect of combination feeding of both a high density of *Nannochloropsis oculata* (N) and condensed freshwater *Chlorella* (FC) on the fatty acid composition of L-type rotifer *Brachionus plicatilis* in a continuous culture system. The algal feeding of the rotifers was carried out in three successive steps: N-feeding → N+FC-feeding → FC-feeding. The culture was conducted at 24°C and 25–27 psu in a 2000 mL bottle with 50% of water exchanged daily. The combination N+FC-feeding was effective in increasing rotifer density. The rotifers fed on N+FC (N+FC-R) had more non-polar lipids than polar ones, similar to those on N (N-R), opposite to the rotifers fed on FC (FC-R). N+FC-R contained higher levels of 16:2, 18:2n-6 (linoleic acid [LA]) and 20:2n-6, but lower levels of 18:1, 20:4n-6 (arachidonic acid), 20:5n-3 (eicosapentaenoic acid [EPA]) and 22:5n-3 (docosapentaenoic acid [DPA]) compared with N-R. Whereas N+FC-R contained higher levels of 16:1n-7, EPA and DPA, but lower levels of 16:2 and LA compared with FC-R. N+FC-R had more DPA in polar lipids than in non-polar ones. The Σn-6/Σn-3 ratio in N+FC-R was 0.9–1.0, significantly different from those in N-R (0.4) and FC-R (6.6–8.4). Therefore, it is inferred that the fatty acid profile of the N+FC-R cultured in a continuous culture system was affected by both N and FC. Also, the combination N+FC-feeding may be effective in manipulating the Σn-6/Σn-3 ratio in continuously cultured rotifers.

KEY WORDS: ***Brachionus plicatilis*****, continuous culture, docosapentaenoic acid, eicosapentaenoic acid, fatty acid composition, *****Nannochloropsis oculata*****.**

INTRODUCTION

In recent years, much research has been carried out to develop microdiets for early life stages of marine organisms. However, so far, effective artificial microdiets have not been fully developed.[1–4] The requirement of rotifers as an initial food is therefore still indispensable.[5,6] As for the methods of rotifer culture, batch culture and semi-continuous culture are two conventional methods. Due to the need for reducing space and labor requirements, and improving the quality of rotifers, high density culture and continuous culture are increasingly being used in culture management.[6–8] In particular, since rotifers can be maintained in the logarithmic proliferation phase by consecutive feeding in a continuous culture system, quality improved rotifers with high activity and high nutritional value can be produced continuously, making them potentially continuously available to fish larvae.[6,9,10] In addition, it is known that the stability of rotifer culture is influenced by the difference in a species or a strain, and the continuous culture of S-type rotifer is easier to be stabilized than that of L-type rotifer.[11]

In a previous study,[10] continuous cultures of L-type rotifer *Brachionus plicatilis*, which seem to be more difficult to keep stable within production than that of S-type rotifer, were conducted for 30 days using condensed freshwater *Chlorella* and

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high density *Nannochloropsis* as food. It was found that the load of water in the *Nannochloropsis*-feeding rotifer culture was less than that in the freshwater *Chlorella*-feeding rotifer culture, and therefore the *Nannochloropsis*-feeding culture tended to be superior to the freshwater *Chlorella*-feeding culture in a long-term continuous culture of rotifers. As for the fatty acid profiles, it was found that docosapentaenoic acid (DPA) was contained in the rotifers cultured with *Nannochloropsis*, irrespective of the fact that DPA was not detected in the *Nannochloropsis*.

However, concern remains about whether *Nannochloropsis*-feeding culture can be maintained at a sufficiently high density to meet the practical requirements for seed production of fish. In continuous culture of S-type rotifer, it has been reported that a high density of *Nannochloropsis* or freshwater *Chlorella* was effective in increasing rotifer productivity.[12] However, to the authors' knowledge, reports on the effect of a combination feeding of *Nannochloropsis* and freshwater *Chlorella* for the high density culture of L-type rotifer have not been published to date.

In the present study, the algal feeding in a continuous rotifer culture was carried out in three steps: *Nannochloropsis*-feeding → *Nannochloropsis* + freshwater *Chlorella*-feeding → freshwater *Chlorella*-feeding, in order to examine the stability of the continuous culture during the successive change of foods, confirming the success of the continuous high density culture of rotifers by giving different foods (cell size) successively without having to restart the cultures. Moreover, the change in the fatty acid composition of the rotifers was also monitored. The effect of each food on the daily rotifer production ratio in each feeding treatment was also investigated. This is the first report on a continuous rotifer culture trial of successive feeding with different foods.

Furthermore in this study, *Nannochloropsis* culture was aimed to be a high density culture with an eicosapentaenoic acid (EPA)-rich production method by using a new jacket-type water temperature adjustment equipment. EPA is one of the essential fatty acids for early stages of marine organisms.[13]

MATERIALS AND METHODS

Culture of high density *Nannochloropsis*

The photobioreactor used for *Nannochloropsis oculata* culture was set up outdoors with a plain water tank (5.5 cm × 140 cm × 50 cm, volume 37 L) made of acrylic fiber. Sand filtered sea water, obtained from the Aburatsubo Marine Park (Kanagawa, Japan), was diluted to salinity 26 psu with tap water, and then was filtered through a hollow fiber membrane. *Nannochloropsis* was cultured under the conditions as shown in Table 1. The water temperature was adjusted to 20–25°C using a cooling/heating jacket installed under the tank. Aeration was set at 4 L/min via a diffuser on the bottom of the tank, mixed with carbon dioxide at a rate of 18 L/day.

Table 1 Conditions of *Nannochloropsis oculata* semi-continuous culture

Volume (L)	37
Harvest (L/3 days)	17[†]
Water temperature (°C)	20–25
Salinity (psu)	26
Aeration (L/min.)	4
CO_2 mixture (L/day)	18
Culture period (days)	15

[†]The same amount of fertilized sea water was added after the harvest. The composition of nutrients was 4.0 g of ammonium sulfate, 1.2 g of Na_2HPO_4 and 0.2 g of Clewat32 (Teikoku Chemical Industries, Itami, Hyogo, Japan).

The semi-continuous culture of *Nannochloropsis* was conducted for 15 days. Algae (17 L) was harvested every 3 days, and concurrently 17 L sea water with addition of the nutrients was added (Table 1). The algal density was monitored every day by counting in a Neubauer-counting chamber, and the fresh harvest of *Nannochloropsis* was used as food for the continuous rotifer culture.

Continuous rotifer culture

The conditions of the continuous culture of rotifer *Brachionus plicatilis* are shown in Table 2. The continuous culture system consisted of a culture tank, a harvest tank (both made of white polyethylene, 2000 mL), and a feeding tank (made of glass, 2000 mL). Aeration in each tank was set at 1 L/min via a Pasteur pipette. In this system, filtered water containing algal food was continuously supplied from the feeding tank into the culture tank at a rate of 0.7 mL/min via a tube pump, and the same amount of culture water was transferred through the projecting tube into a harvest tank, and a significant biomass of rotifer was obtained. The temperature of the culture and harvest tank was constantly maintained at 24°C by using a water bath. Light conditions were kept under the natural indoor light conditions (50–1000 lx).

The culture trial of the rotifers (*Brachionus plicatilis,* L-type rotifer, strain Ishikawa) was started

Table 2 Design of the successive feeding for the continuous culture of the rotifer *Brachionus plicatilis*

	Treatment		
	N[†]-feeding	N[†] + FC[‡]-feeding	FC[‡]-feeding
Culture period (day)	Day 1–7	Day 8–14	Day 15–21
Culture (mL)	2000	2000	2000
Water temperature (°C)	24	24	24
Salinity (psu)	26	27	25
Daily water exchange (%/day)	50	50	50
Daily ration (cells/day)	230×10^9 (N)	210×10^9 (N) + 45×10^9 (FC)	130×10^9 (FC)
Food provision to the harvest tank (cells/day)	38×10^9 (N)	Sea water 200 mL only	8×10^9 (FC)

[†]N, *Nannochloropsis oculata* (190–210 × 10^6 cells/mL). [‡]FC, Commercial freshwater *Chlorella* (15 × 10^9 cells/mL).

with an initial density of 500 individuals/mL, which had been fed with *Nannochloropsis* for 20 days in advance. The feeding experiment was conducted in three successive steps: first, a *Nannochloropsis*-feeding period (N-feeding period), then a *Nannochloropsis* + freshwater *Chlorella*-feeding period (N+FC-feeding period), and finally a freshwater *Chlorella*-feeding period (FC-feeding period); 7 days for each period. During the N-feeding period, 1000 mL cultured *Nannochloropsis* (190–210 × 10^6 cells/mL) was added to the feeding tank every day. Cultured *Nannochloropsis* (1000 mL) (190–210 × 10^6 cells/mL) with the addition of 3 mL condensed freshwater *Chlorella* (Concentrated Chlorella Solution; Nihon Chlorella, Kunitachi, Tokyo, Japan) was added to the feeding tank for the N+FC-feeding period. During the FC-feeding period, 1000 mL sea water mixed with 8 mL condensed freshwater *Chlorella* was added to the feeding tank each day. In the harvest tank, 200 mL *Nannochloropsis* was prepared each day during the N-feeding period; 200 mL sea water during the N+FC-feeding period, and 200 mL sea water with an addition of 0.5 mL condensed freshwater *Chlorella* during the FC-feeding period.

The rotifer density and the egg number in the culture and harvest tank were measured twice a day. The rotifers harvested on 4, 5, 6 and 7 days after the start of each feeding periods were pooled into the three respective samples.

Analysis of lipid contents and fatty acid profiles

Lipid contents and fatty acid profiles of the cultured *Nannochloropsis*, the commercial freshwater *Chlorella* used in the experiment, and the harvested rotifers cultured with different food were analyzed.

The *Nannochloropsis* suspension was concentrated by centrifugation (6200 ×*g*, 20°C, 10 min) and the rotifers collected from the harvest tank by a plankton net (40 μm) were cryopreserved at –80°C prior to analysis. The commercial freshwater *Chlorella* was analyzed without a pretreatment. Determination of crude lipid contents was analyzed by chloroform-methanol (2:1 v/v) extraction according to the method of Folch *et al.*[14] In addition, the fractionation of non-polar and polar lipids was accomplished using a Sep-Pak Silica Cartridge (Waters Corp., Millford, MA, USA). For fatty acid profile analysis, non-polar and polar lipids from each crude lipids were saponified using 50% KOH-ethanol to prepare methylesters with 7% boron trifluoride in a methanol solution (BF_3-methanol), and the fatty acid profile was determined using gas liquid chromatography (HP6890GC; Hewlett-Packard, Palo Alto, CA, USA) as described in the previous study.[10]

Statistical analysis

Data in the daily rotifer production ratio were analyzed by analysis of variance (ANOVA). Differences between the daily rotifer production ratio were assessed by the Tukey's multiple range test at a 5% significance level.

RESULTS

Culture of *Nannochloropsis*

The result of the high density *Nannochloropsis* culture is shown in Figure 1.

Starting with an inoculation density of 120 × 10^6 cells/mL, a five-times harvest was accomplished in the density of nearly 200 × 10^6 cells/mL throughout the 15 days culture. The semi-continuous culture of *Nannochloropsis* was maintained at a high density, though 45% of the volume of culture water was removed every 3 days.

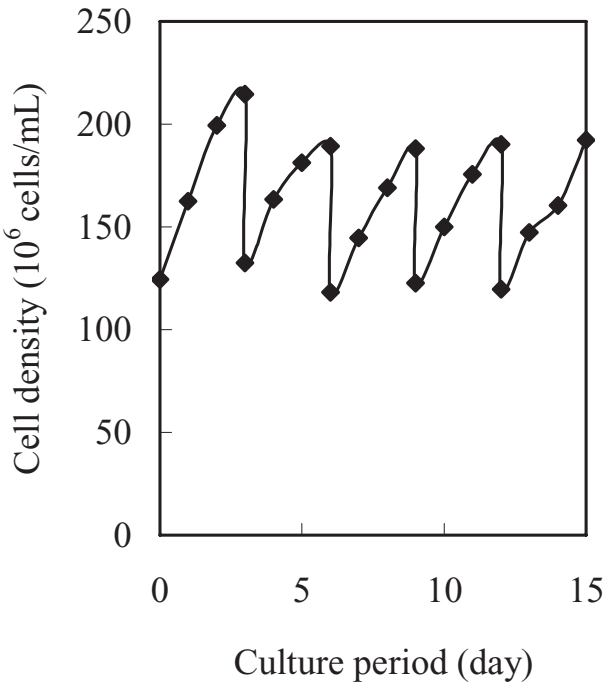

Fig. 1 Semi-continuous culture of *Nannochloropsis oculata*.

Continuous rotifer culture

The result of the continuous rotifer culture is shown in Figure 2 and the productivity of rotifers is summarized in Table 3.

The rotifer density of 460–700 ind./mL (600 ± 93 ind./mL; mean ± SD) was maintained in the culture tank during the N-feeding period. In the harvest tank, a density range from 310 to 840 ind./mL (510 ± 160 ind./mL) was observed, resulting in a rotifer production of (400–1000) × 10^3 ind./day ([610 ± 190] × 10^3 ind./day) during the N-feeding period. The egg rate (no. eggs/no. rotifers) was between 22 and 67% (51 ± 13%) in the culture tank, and between 22 and 56% (46 ± 11%) in the harvest tank.

During the N+FC-feeding period, the rotifer density in the culture tank was maintained between 630 and 1050 ind./mL (850 ± 190 ind./mL), in the harvest tank between 430 and 740 ind./mL (560 ± 110 ind./mL), and harvested rotifers of (520–890) × 10^3 ind./day ([670 ± 130] × 10^3 ind./day) were produced. The egg rate was between 23 and 73% (52 ± 16%) in the culture tank, and between 21 and 59% (36 ± 13%) in the harvest tank.

During the FC-feeding period, the rotifer density in the culture tank was maintained between 750 and 1000 ind./mL (900 ± 90 ind./mL), in the harvest tank between 520 and 700 ind./mL (590 ± 67 ind./mL), and rotifers of (620–840) × 10^3 ind./day ([700 ± 80] × 10^3 ind./day) were harvested. The egg rate was between 39 and 58% (47 ± 6%) in the culture tank, and between 30 and 51% (37 ± 8%) in the harvest tank.

Moreover the daily rotifer production ratio (no. rotifers in harvest tank/no. rotifers in culture tank the day before) was highest in the N-feeding period, at an intermediate level during the N+FC-feeding period, and lowest in the FC-feeding period ($P < 0.05$).

Chemical analysis of *Nannochloropsis* and rotifers

Crude lipids and fatty acid composition of the commercial freshwater *Chlorella,* the cultured *Nannochloropsis* and harvested rotifers are given in Table 4.

Lipid contents (%, on dry basis) in the *Nannochloropsis*, the freshwater *Chlorella*, the harvested rotifers during N-feeding (N-R), the harvested rotifers during N+FC-feeding (N+FC-R) and the harvested rotifers during FC-feeding (FC-R) were 21.4 (non-polar/polar; 14.6/6.8)%, 9.5 (3.0/6.5)%, 13.3 (8.6/4.7)%, 13.0 (9.1/3.8)% and 10.7 (3.7/7.0)%, respectively. The fatty acid profiles from the analysis indicated that both non-polar and polar lipid fractions in *Nannochloropsis* were particularly rich in palmitic acid (16:0), 16:1n-7 and EPA (20:5n-3), additionally some amounts of 18:1, linoleic acid (LA; 18:2n-6), alpha-linolenic acid (LNA; 18:3n-3) and arachidonic acid (AA; 20:4n-6) were also present. Freshwater *Chlorella* was rich in 16:0, 16:2 and LA, and 16:3n-6 and LNA were also contained. Reflecting the fatty acid composition of *Nannochloropsis* as food, N-R was rich in 16:0 and EPA, and 16:1n-7, 18:1, LA, LNA and AA were also contained. Moreover, 20:1 and DPA (22:5n-3) were also present though these two fatty acids were hardly contained in *Nannochloropsis*. The proportion of DPA in the polar lipid was higher than that in the non-polar lipid. N+FC-R was rich in 16:0, LA and EPA, but 18:1, AA, EPA and DPA tended to decrease, while 16:2, LA and 20:2n-6 tended to increase compared with N-R. FC-R was especially rich in 16:0, 16:2 and LA, but 16:1n-7, EPA and DPA decreased more markedly than that in N+FC-R. The Σn-6/Σn-3-values in *Nannochloropsis*, freshwater *Chlorella*, N-R, N+FC-R and FC-R were 0.3, 5.7–5.8, 0.4, 0.9–1.0 and 6.6–8.4, respectively.

DISCUSSION

The semi-continuous culture of *Nannochloropsis* in the present study was accomplished at a high

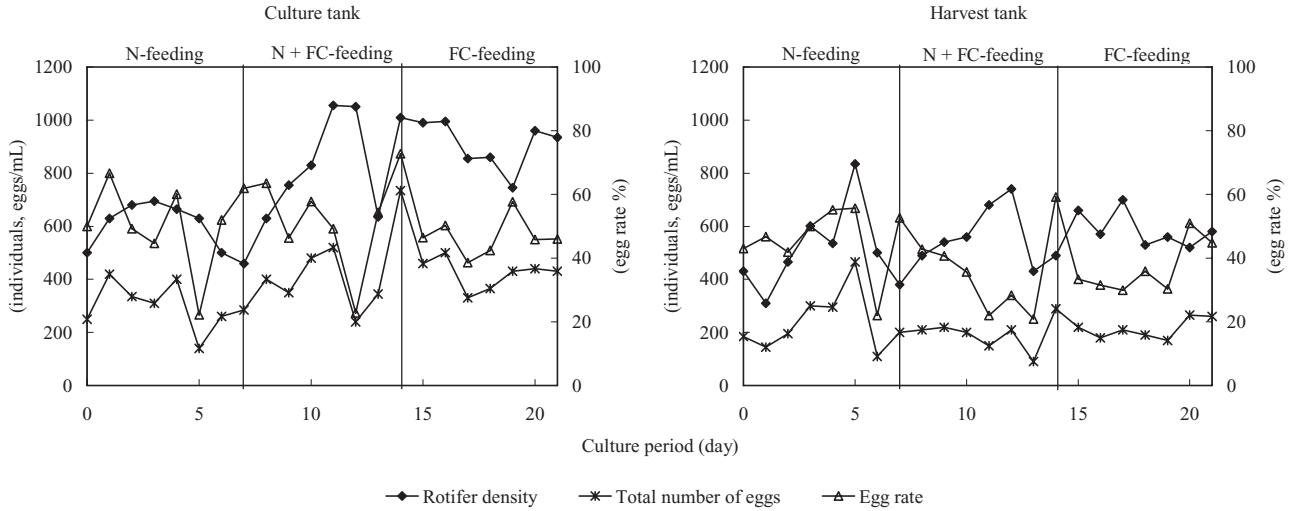

Fig. 2 Continuous culture of rotifer *Brachionus plicatilis*. Details of parameters for *Nannochloropsis oculata* (N)-feeding, N+condensed freshwater *Chlorella* (FC)-feeding, and FC-feeding are presented in Table 2.

Table 3 Productivity of the rotifer *Brachionus plicatilis* in continuous culture

	Culture tank			Culture tank		
	N-feeding	N+FC-feeding	FC-feeding	N-feeding	N+FC-feeding	FC-feeding
Rotifer density (individuals/mL)	600 ± 93[†]	850 ± 190	900 ± 90	510 ± 160	560 ± 110	590 ± 67
Rotifer production (10^3 individuals/day)	–	–	–	610 ± 190	670 ± 130	700 ± 80
Daily production ratio (%)[‡]	–	–	–	50 ± 12[a]	46 ± 13[a,b]	39 ± 3[b]
Egg rate (%)[§]	51 ± 13	52 ± 16	47 ± 6	46 ± 11	36 ± 13	37 ± 8

[a,b]Values not sharing a common superscript in daily production ratio are significantly different ($P < 0.05$).
[†]Mean ± SD.
[‡]Number of rotifers in harvest tank/number of rotifers in culture tank the day before.
[§]Number of eggs/number of rotifers.
FC, Commercial freshwater *Chlorella*; N, *Nannochloropsis oculata*.

density of 200×10^6 cells/mL (Fig. 1), approximately 6–10 times of the density in usual open pond culture by using the jacket-type temperature equipment, similar to the result obtained in the previous study by using the tube-type equipment.[10] Both non-polar and polar lipids in the high-density cells contained more than 25% of EPA (Table 4). It has been demonstrated that *Nannochloropsis* produced by aseptic culture does not have essential vitamins for rotifers, but culture water cultured by outdoors contains them.[15] The achievement of the continuous rotifer culture suggests that the culture water including the cells also contained vitamin B_{12}, A, D and E which are essential for rotifers.[15–19] Therefore, these *Nannochloropsis* cells are considered to be feasible and available in practice for rotifer culture.

Continuous rotifer culture by the successive feeding with different foods (N→N+FC→FC) was achieved (Fig. 2). Since the previous study demonstrated that *Nannochloropsis* and freshwater *Chlorella* showed the same feed efficiency on the basis of the absolute quantity of feeding,[10] the rotifer density in this study also changed in proportion to the absolute quantity of feeding. The available rotifer density in a practical continuous culture system for L-type rotifers is required to be 1000–1500 individuals/mL, therefore it will be necessary to maintain the stability of such a high density of rotifers either by feeding condensed *Nannochloropsis* or by using condensed freshwater *Chlorella* together with *Nannochloropsis*. From the results of the present study, it was suggested that the continuously cultured rotifers had adaptation ability to the combination feeding smoothly from the single feeding, and the combination feeding of *Nannochloropsis* and *Chlorella* was also effective in increasing rotifer density. N-feeding treatment showed a higher daily rotifer production ratio than the other two treatments, especially a significantly

Table 4 Crude lipid contents (%) and fatty acid compositions (area%) of non-polar and polar lipids in microalgae and harvested rotifers

	N		FC		N-R		N+FC-R		FC-R	
Moisture (%)	–		–		85.3		86.2		88.1	
Crude lipid (d.b.%)†	21.4		9.5		13.3		13.0		10.7	
NL (d.b.%)	14.6		3.0		8.6		9.1		3.7	
PL (d.b.%)	6.8		6.5		4.7		3.8		7.0	
Fatty acid (area%)	NL	PL	NL	PL	NL	PL	NL	PL	NL	PL
12:0	0.5	0.2	0.5	0.1	0.2	0.1	0.2	0.1	0.2	0.1
14:0	4.6	3.6	0.7	0.5	3.8	2.5	3.4	2.9	2.3	1.4
16:0	20.3	21.3	12.6	13.6	16.0	18.6	15.4	20.4	14.2	15.1
16:1n-7	17.5	14.4	1.3	3.3	13.8	7.5	11.2	7.5	3.1	1.7
16:2	0.7	1.2	26.5	22.3	0.4	0.2	3.3	1.8	15.0	5.9
16:3n-6	0.3	0.2	6.3	11.1	0.5	0.4	1.1	1.5	1.2	2.6
16:3n-3	2.5	3.7	1.2	0.2	0.9	2.6	0.6	1.5	0.1	1.2
18:0	0.6	0.3	0.9	0.9	1.9	2.4	2.4	2.6	2.7	4.2
18:1	7.4	7.6	1.1	1.7	11.8	8.0	8.6	5.7	4.1	3.6
18:2n-6 (LA)	5.7	7.6	33.1	33.3	5.9	7.8	15.8	18.6	37.0	43.5
18:3n-6	0.2	0.3	nd	0.2	0.3	0.3	0.2	0.3	0.1	0.1
18:3n-3 (LNA)	6.7	5.8	5.7	7.5	3.0	3.6	2.5	2.9	3.8	4.4
18:4n-3	0.1	nd	nd	nd	0.1	0.1	tr	0.3	0.1	0.1
20:0	0.1	0.1	nd	tr	0.1	0.1	0.2	0.1	0.2	0.2
20:1	0.2	0.2	nd	0.2	2.9	3.4	2.8	1.9	1.4	2.6
20:2n-9	0.1	0.1	3.7	0.6	0.3	0.2	0.3	0.3	0.2	0.2
20:2n-6	0.1	0.1	nd	0.6	0.5	0.2	1.4	0.7	5.3	2.3
20:3n-9	0.1	0.1	nd	nd	0.2	nd	0.3	0.4	0.4	0.6
20:3n-6	0.1	0.2	nd	nd	0.6	0.7	0.9	1.1	1.5	2.7
20:4n-6 (AA)	2.7	3.0	nd	nd	4.1	2.9	3.4	2.0	0.4	1.1
20:3n-3	nd	nd	nd	nd	0.3	tr	0.3	0.1	0.6	0.3
20:4n-3	tr	nd	nd	nd	0.6	1.2	0.6	1.2	0.5	1.1
20:5n-3 (EPA)	25.8	26.7	nd	0.1	25.2	20.7	18.5	14.7	0.2	0.7
22:0	0.1	0.1	nd	nd	0.1	nd	0.1	0.1	0.1	0.1
22:1	0.1	0.1	nd	nd	0.9	0.7	0.7	0.7	0.3	0.9
22:4n-9	0.1	nd	nd	nd	0.1	nd	0.1	0.1	nd	tr
22:4n-6	nd	0.3	nd	nd	0.3	0.5	0.2	0.3	nd	0.1
22:5n-6	tr	nd	nd	nd	nd	nd	nd	nd	nd	tr
22:5n-3 (DPA)	0.1	nd	nd	nd	3.5	7.2	2.7	4.8	0.1	0.1
22:6n-3	0.1	0.1	nd	nd	0.3	nd	nd	nd	nd	0.1
Others‡	3.1	2.8	6.4	3.7	1.6	8.1	2.7	5.4	4.8	2.9
Σsaturates	28.0	27.3	16.8	17.1	23.6	25.8	23.6	29.1	22.0	22.9
Σmonoenes	25.2	22.2	2.5	5.2	29.4	19.7	23.4	15.8	8.9	8.8
Σn-6	9.2	11.6	39.3	45.3	12.1	12.7	23.0	24.5	45.6	52.5
Σn-3	35.3	36.4	6.9	7.8	33.9	35.4	25.2	25.6	5.4	8.0
Σn-6HUFA	3.0	3.5	0.0	0.6	5.4	4.2	5.9	4.1	7.2	6.3
Σn-3HUFA	26.0	26.8	0.0	0.1	29.9	29.1	22.1	20.9	1.4	2.3
Σn-6/Σn-3	0.3	0.3	5.7	5.8	0.4	0.4	0.9	1.0	8.4	6.6

†On dry matter basis.
‡Containing *trans*-fatty acids.
AA, arachidonic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FC, commercial freshwater *Chlorella*; HUFA, highly unsaturated fatty acid; LA, linoleic acid; LNA, alpha-linolenic acid; N, *Nannochloropsis oculata*; nd, not detected; NL, non-polar lipids; PL, polar lipids; R, rotifer; tr, trace (<0.05).

higher than FC-feeding treatment (Table 3), similar to the results of the previous work.[10] Therefore, it suggested that the rotifer production ratio by N-feeding tends to be higher than that by FC-feeding.

As for the fatty acid profiles, N-R contained more than 25% EPA in non-polar and 20% in polar lipids (same as the previous study[10]), and 3.5% and 7.2% DPA in non-polar and polar lipids, respectively (Table 4). That is, N-R had more DPA in the polar lipid fraction. Although the rotifers formed DPA in the n-3 series cascade according to Kayama,[20] the metabolism to docosahexaenoic acid (DHA; 22:6n-3) has not been observed. It is known for

some species of phytoplankton and trout that the metabolism to DHA follows the steps of LNA→20:3n-3→20:4n-3→EPA→DPA, and then via 24:5n-3→24:6n-3 to 22:6n-3 (DHA).[21–23] These results revealed that the metabolism of n-3 series fatty acids in *Nannochloropsis* reached to EPA, while in N-R to DPA. Further studies are needed to clarify the differences in the effect of DPA-rich rotifer and DHA-rich rotifer on fish larvae. In fact, it will be difficult to produce healthy larval marine fish (with vitality and normal schooling behavior) by sole feeding of N-R without DHA. Moreover, since polar lipids usually exist in the biomembranes and cell membranes, and DPA content increases in starved rotifers,[10] DPA was supposed to be accumulated in phospholipids. This has been confirmed in some reports on highly unsaturated fatty acid (HUFA) including DPA.[24–29]

The detected high levels of 16:2, LA and 20:2n-6 in N+FC-R were found because of the addition of freshwater *Chlorella* to the *Nannochloropsis* for feeding to the rotifers. This revealed that N+FC-R ingested freshwater *Chlorella* together with *Nannochloropsis*. In contrast, 18:1, AA, EPA and DPA showed a tendency to decrease (Table 4). N+FC-R showed an intermediate fatty acid profile, reflecting the fatty acid profiles of both *Nannochloropsis* and freshwater *Chlorella*. Moreover, the Σn-6/Σn-3 ratio, which in general shifts to either >1 or <1 due to the feeding on *Nannochloropsis* or freshwater *Chlorella*,[30] approximately equals 1 in the N+FC-R (Table 4). Therefore, the combination N+FC-feeding may be effective in manipulating the Σn-6/Σn-3 ratio in continuously cultured rotifers. This N+FC-R may also serve as an effective food to freshwater and brackish water species which do not require n-3HUFA so much.[31]

As for FC-R, the effect of solely feeding freshwater *Chlorella* that leads to high concentrations of 16:0, 16:2 and LA, as indicated in the fatty acid profile (Table 4), is identical to a previous study.[10]

In conclusion, the results of this study demonstrated that the continuously cultured rotifers had adaptation ability to the combination feeding smoothly from the single feeding, and the combination feeding was also effective in increasing rotifer density. Moreover, N+FC-R ingested both *Nannochloropsis* and freshwater *Chlorella*, and contained high contents of the fatty acids from both foods. Furthermore, a high density (200×10^6 cells/mL) culture of *Nannochloropsis* was achieved by using a new jacket temperature control system as well as the tube temperature control system, thus the new microalgal cultivation system was established. Future studies focusing on not only *Nannochloropsis* and rotifer culture, but also the treatment and recycling use of waste water in rotifer culture, may help to improve the efficiency of this cultivation system. The resulting nutrient-rich (NH_4-N, PO_4-P) water after rotifer harvest may be used again for *Nannochloropsis* culture, by the implementation of a water recirculating culture system.

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