Nitrogen Flow in a Chemostat Culture of the Rotifer *Brachionus plicatilis*

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Nitrogen flow in a chemostat culture of the rotifer *Brachionus plicatilis* was measured. It was found that a chemostat could keep the rotifer in a stable condition and were suitable for studying the effect of algal density. The rotifer egested 70–80% of the ingested nitrogen as particulate organic nitrogen (PON) and about 80% of the remaining assimilated nitrogen was utilized for reproduction. Low net growth efficiency (K_2) at high algal densities proved “superfluous feeding”. 20% of PON egested was incorporated in the rotifer again by bacteriivory, and 13% of that was remineralized to NH_4^+ by bacteria. The PON was accumulated as a suspended or attached form in the culture vessel, which might cause an unstable environment. For a safety culture of the rotifer, removal of PON is an effective counter plan.

Key words: rotifer, nitrogen flow, chemostat culture, bacterial function

Since Ito¹⁾ found that the rotifer *Brachionus plicatilis* is available as live food for fish larvae, the rotifer have been produced as an important initial feed of fish or shellfish larvae. However an unexpected sudden decrease of density or suppressed growth occasionally occurs during mass production, causing serious problems during seed production of fish or shellfish. Toxicity of un-ionized ammonia^{2,3)} and accumulation of waste products⁴⁾ are proposed as possible causes. Also, certain bacteria inhibit rotifer growth,⁵⁾ while others enhance the growth by producing vitamin B₁₂, an essential material for the rotifer.^{6,7)} The mechanism as to how water quality changes in the rotifer culture medium is not well studied.

Continuous culture of rotifers have been applied to study population dynamics or energy flow^{8–18)} and these were reviewed by Droop and Scott¹⁹⁾ and Walz.²⁰⁾ A chemostat can keep the rotifer at a steady state and is suitable for the study of population dynamics and energy flow, variables easily affected by food conditions. In our previous tracer experiment,²¹⁾ it was confirmed that the rotifer egested 80% of the ingested nitrogen as particles. In the rotifer culture medium, the presence of large number of bacteria and their activities can not be ignored. The objective of this paper is to measure the nitrogen flow including the function of bacteria in a rotifer culture. Further, the mechanism which makes the rotifer culture unstable is discussed.

Materials and Methods

Organisms

The strains of the rotifer *Brachionus plicatilis* and the algae *Nannochloropsis oculata* were described previously.²¹⁾ The algae was grown in a modified Yashima medium²¹⁾ and harvested during an exponential phase. The concentrated algae was stored at -30°C . Prior to experiments, the stocked algae was thawed, washed twice by centrifugation, and resuspended in seawater. It was observed that this algae did not grow and absorb nutrients.

Experimental Design

Two experiments were designed to measure the nitrogen budget in the rotifer chemostat culture. In the first experiment, the effect of food concentration on growth and metabolic factors was examined. The one-stage chemostat system consisted of an algal reservoir (1,000 ml/ erlenmeyer flask) and a rotifer reactor (2,000 ml/ beaker). The algae was supplied from the reservoir to the reactor by a dual feeding microtube pump, and the reactor medium was drawn out simultaneously through another tube using the same flow rate. Tubes were made of silicon with 1 mm inner diameter. The rotifer culture was kept in the dark, mixed uniformly by aeration, and maintained at $30 \pm 0.5^\circ\text{C}$ in a water bath. Seawater used for the cultures of both algae and rotifers were diluted with tap water to 20 salinity. These conditions are similar to those commonly used in hatcheries for practical indoor mass cultures.

When the system dilution rate D (=medium flow rate/ reactor volume) is the same as the specific growth rate (μ) of the rotifer, conditions in the reactor should be in a steady state. In our chemostat, when the dilution rate was less than 0.021/h, the rotifer density in the outflow became significantly lower than in the reactor. Therefore, the dilution rate had to be fixed more than 0.021/h, so that the steady state culture at lower growth rates ($\mu < 0.021/\text{h}$) will not be achieved. The washout continuous culture, where the dilution rate is larger than the rotifer growth rate, could be applied to evaluate variables of the rotifers under the low growth rates.

In the control without rotifer, algal density was kept constant. It was therefore assumed that the algal concentration decreased only due to the feeding of the rotifers in the reactor. Fifty ml of each sample was collected from the reactor and the algal reservoir by siphoning every 8 hours, and the same volume of seawater was replaced in the reactor. Rotifer and egg densities were counted with a binocular microscope and for the algal density, a hemocytometer was used. Our chemostat system was not designed to be germfree, thus, seawater was boiled for 10 min and filtered

through a Whatman GF/F glass-fiber filter to prevent protozoa contamination.

In the second experiment, the nitrogen budget in the steady state rotifer chemostat was measured. A three-necked flask sealed was used as the reactor in place of the beaker in the first experiment. Ammonia which escaped into the air from the culture medium was trapped by 0.1N H₂SO₄, and the amount was determined. Water sample was collected from the flask and the algal reservoir three times every 4 h. Nitrogen was measured as described below and the average of the 3 samples was used to estimate the nitrogen pools. When a chemostat is in a steady state, the nitrogen difference between the reactor and the algal reservoir reflects consumption and/or production by the rotifer. Generally in the rotifer culture, bacteria are abundant and have various effects on nitrogen flow. Therefore, 2 more experiments of batch culture were conducted to understand the contribution of bacteria to the nitrogen budget. First, bacterial remineralization of organic nitrogen was directly measured. Whole culture medium was drawn out from the steady state reactor and the rotifer was removed with a 40 μ m mesh. It was incubated at different batches, and changes in nitrogen concentration and bacteria density were monitored. The bacteria number was determined by direct counts with DAPI under an epifluorescence microscope.²²⁾ At the same time, nitrogen excretion rate of the rotifer was also measured by the water bottle method.²³⁾ Next, the amount of bacteria cleared by the rotifer was measured. The rotifer were inoculated again at 300, 600 or 900 individ./ml into the reactor medium which had been left for 6 h after the rotifer was removed. The decrease in the number of bacteria was measured in duplicate experiments by direct counting, and the clearance rate (F) was calculated using the following equation.

$$F = -\ln(C_t - C_o)/(t \cdot R)$$

where: C_t and C_o are the final and the initial concentrations of bacteria, respectively; t , the incubation time; and R , the rotifer density.

Analyses

Nitrogen and carbon contents of both the rotifer and the algae were measured by a CHN analyzer (Yanagimoto Co., Tokyo, Model MT-2). Fifty ml of the reactor medium was left for 5 min to allow the faeces to sink, and the rotifer were then washed quickly using a 40 μ m mesh and collected on a GF/F-filter previously combusted at 400°C for 4 h. The algae were washed by centrifugation and collected on a GF/F-filter and the nitrogen content was determined.

Nitrogen of the water sample was analyzed as particulate organic nitrogen (PON), total dissolved nitrogen, urea, NH₄⁺, NO₂⁻, and NO₃⁻. To distinguish the rotifer from other particulate matter such as the algae, PON was separated into two fractions by a 40 μ m mesh. Each fraction was filtered onto a GF/F-filter and analyzed by a CHN analyzer. Samples for total dissolved nitrogen, urea, NH₄⁺, NO₂⁻, and NO₃⁻ were filtered through the GF/F-filter. Analyses followed that of Parsons *et al.*²⁴⁾ (NH₄⁺ was analyzed by an indophenol method). Dissolved organic nitrogen (DON) was determined from the difference between the total dissolved nitrogen and the sum of inorganic

nitrogen and urea.

Results

Nitrogen Contents of the Rotifer and the Algae

The amount of nitrogen and C/N ratio (weight base) of the algae and the rotifer are shown in Table 1. The size distributions of rotifers varied with the egg ratio. Boraas¹⁰⁾ similarly reported a change in the size distribution of the rotifer with D in a chemostat. Although it was impossible to distinguish the nitrogen content of the egg from the body, a linear relation was observed between nitrogen contents of the rotifer and egg ratio (Fig. 1).

Relationships between Food Density and Variables of the Rotifer

Various factors were calculated as shown in Table 2. The growth rate of the rotifer (μ) as a function of algal density is shown in Fig. 2. It began to arise hyperbolically

Table 1. Nitrogen content and C/N ratio of the rotifer, *Brachionus plicatilis* and algae, *Nannochloropsis oculata*

	<i>Brachionus plicatilis</i>	<i>Nannochloropsis oculata</i>
N (g atom N/individ.)	$(6.88 + 5.12E) \times 10^{-10}$	2.9×10^{-14}
C/N ratio (weight base)	4.6 ± 0.5	7.9 ± 0.2

E = egg ratio (numbers of eggs per rotifer).

Fig. 1. Relationship between nitrogen content and egg ratio of the rotifer.

Table 2. Equations used to determine variables of the rotifer in the rotifer chemostat

Variables	Units	Equation
Growth rate (μ)	/h	$D + (\ln R_t - \ln R_o)/t$
Ingestion rate (I)	g atom N/individ./h	$D \cdot (A_a - A_r)/R_t$
Clearance rate (F)	ml/individ./h	$1/A_r$
Gross growth efficiency (K_2 , Yield)		$\mu \cdot W/1$

D , dilution rate (/h); R_t and R_o , final and initial rotifer density (individ./ml) (in a steady state chemostat, $R_t = R_o$, i.e. $\mu = D$), respectively; t , interval measuring the rotifer density (h); A_a and A_r , the algal density in the reservoir and rotifer reactor, respectively (g atom N/ml); W , nitrogen amount of the rotifer (g atom N/individ.).

Fig. 2. Specific growth rate, μ , of the rotifer as a function of food concentration. Food concentration is plotted on logarithmic coordinate (A), and linear coordinate (B), respectively.

Fig. 3. Ingestion rate (I) and clearance rate (F) of the rotifer as a function of food concentration.

at 2.1×10^5 cells/ml, and attained a maximum of 0.058/h at density over 10×10^5 cells/ml. This relation fit well to the following modified Michaelis-Menten equation ($R^2=0.92$):

$$\mu = \mu_{\max} \cdot \frac{(A_r - A_o)}{(K - A_o) + (A_r - A_o)},$$

where: $\mu_{\max}$, the maximum growth rate (0.067/h); K , saturation constant (4.8×10^5 cells/ml); and A_o , the threshold of algal density for rotifer growth (2.1×10^5 cells/ml).

The ingestion rate and the clearance rate in relation to algal density are shown in Fig. 3. The ingestion rate linearly increased and became constant at densities over 20×10^5 cells/ml. As for the clearance rate, it decreased sharply with the food concentration, and approached 2 to 1 $\mu\text{l}/\text{indiv.}/\text{h}$ at high food densities. In contrast to the ingestion rate, the growth rate (Fig. 2) attained maximum at lower food concentrations, and at over the algal density which induced the saturation of growth rate the ingestion rate continuously increased. The curve expresses the gross growth efficiency (K_1) as follows: it arose at A_o ($=2 \times 10^5$ cells/

Fig. 4. Gross growth efficiency, K_2 , as a function of food concentration.

ml) and increased up to the maximum of 0.32 at 10×10^5 cells/ml, and then decreased and tended to be constant of about 0.20 (Fig. 4).

Table 3. Nitrogen concentration and budgets of the steady state chemostat

	PON				NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N	Urea	DON	Total N
	40 μ<		40 μ>							
	Algae* ¹	others	Rotifer* ²	others						
Chemostat 1)										
X _a	1620	0	0	0	84.9	1.91	19.5	3.22	10.8	1740
X _r	19.5	34.0	432	107	402	2.22	33.0	57.6	42.7	1130
B	-66.7	1.42	18.0	4.46	13.2	0.013	0.563	2.28	1.34	-25.4
Chemostat 2)										
X _a	2220	0	0	0	43.2	3.52	36.6	2.45	36.8	2340
X _r	60.0	148	420	990	406	7.16	58.2	50.3	110	2250
B	-97.2	6.66	18.9	44.6	16.3	0.164	0.972	2.15	3.29	-4.05

X_a and X_r, nitrogen concentration (μg atom N/l) in the algal reservoir and the rotifer reactor; B, the nitrogen budgets (μg atom N/h/l) in the rotifer reactor.

In chemostat 1) D (=μ), 0.042/h; algal density in the rotifer reactor, 0.96 × 10⁶ cells/ml; rotifer density, 501/ml; and egg ratio, 34%, respectively.

In chemostat 2) D (=μ), 0.045/h; algal density in the rotifer reactor, 2.60 × 10⁶ cells/ml; rotifer density, 440/ml, and egg ratio, 52%, respectively.

*¹ In each chemostats, algae used was from different culture and the nitrogen contents per cell was different.

*² The rotifer nitrogen contents was calculated by the equation of Table 1.

Nitrogen Flow in the Rotifer Culture

Nitrogen budgets (B, μg atom N/h/l) in the rotifer reactor were calculated by the following equation:

$$B = D(X_r - X_a)$$

where, X_r and X_a are the nitrogen concentrations in the rotifer reactor and the algae reservoir (μg atom N/l), respectively.

Nitrogen concentration and the nitrogen budgets in two steady state chemostats having different algal densities are shown in Table 3. In the reactor, algae was consumed by the rotifer and waste materials were accumulated as PON, NH₄⁺ or urea. In chemostat 1), however, PON accumulation was not much. It was thought that PON rapidly attached to the wall of the reactor and removed by the outflow of culture medium, which may bring the budget of total nitrogen to negative. After the experiment, the attached PON (AON) was collected and measured. Aeration or culture period influenced the amount, which ranged widely from 2.98 to 24.2 μg atom N/h/l (means ± SD = 12.1 ± 7.3 μg atom N/h/l, n=8), comparable to the total nitrogen budget of the chemostat. The diffusion of ammonia into the air was negligible.

Changes in nitrogen concentrations in the medium incubated after removing rotifers are shown in Fig. 5. During the first 6 h, DON linearly decreased, NH₄⁺ increased in contrast and the total bacteria increased. After that, however, there was no remarkable changes both in nitrogen and bacteria. The constancy of PON may reflect the bacterial production and decomposition of detritus. These phenomena indicate that some kind of organic nitrogen in the rotifer medium is quickly remineralized by bacteria, but others remain without decomposition. The remineralization rate into NH₄⁺ was calculated from the increase of ammonia concentration during the first 6 h (Fig. 5). It ranged from 3.7 to 4.7 μg atom N/h/l. Nitrogen excretion rate of the rotifer by the water bottle method was 1.35 ± 0.17 × 10⁻⁵ μg atom N/indiv./h (means ± SD, n=8) as NH₄⁺-N and 0.19 ± 0.08 as urea. These values were constant within the range of 1 to 3 × 10⁶ cells/ml of algae.

No significant variation was observed in the clearance

Fig. 5. Change in the nitrogen concentration and bacterial density in the chemostat medium incubated without the rotifer.

*DON other than urea.

rate (F) among different rotifer densities. The clearance rate was 1.68 ± 0.41 μl/h. (means ± SD, n=6). Small coccus passing through a GF/F-filter (0.7 μm) did not decrease, showing that the rotifer did not ingest particles smaller than 0.7 μm. Rothhaupt²⁵ reported that ingestion of particles by rotifers was size dependent. The nitrogen fraction which passed through a Nuclepore filter (3.0 μm pore size) and trapped on a GF/F-filter, was used to calculate the ingestion rate of bacterial-size particles. The clearance rates on bacteria was the quarter to the half of those on *N. oculata*, and bacterial-size nitrogen ingested was about 20% of those on algae (Table 4).

From the above results, nitrogen flow models in the chemostat 1) and 2) are drawn in Fig. 6. It was assumed that attached PON (AON) is equal to the total nitrogen budgets in the chemostat. Bacterial mineralization for

Table 4. The clearance rate (F: $\mu\text{l/h/indiv.}$) and ingestion rate (I: 10^{-10} g atom N/h/indiv.) of the rotifer in chemostat 1) and 2) relationg to Table 3

food	bacteria		algae	
	F	I	F	I
Chemostat 1)	1.68	0.29	6.82	1.33
Chemostat 2)	1.68	0.44	3.68	2.21

Fig. 6. Nitrogen flow in the rotifer chemostat.

Numbers beside are the nitrogen budget B ($\mu\text{g atom N/l/h}$) indicated in Table 3. *These values were not measured directly, but calculated by balance method. Dashed lines are other possible pathway not measured in our experiment.

DON was calculated to be proportionate to DON. Fig. 6 shows that the largest nitrogen pool in a chemostat is PON (including AON), followed by, rotifer and ammonia. Even though a part of ammonia was nitrified, the speed of nitrification was very slow in comparison with ammonia accumulation.

Table 5. Ecological efficiencies (%) of the rotifer in the steady state chemostat.

	Chemostat 1)	Chemostat 2)
Assimilation efficiency	31.7	22.0
Gross growth efficiency (K_1)	22.2	16.2
Net growth efficiency (K_2)	70.0	73.6

In chemostat 1), algal density was 0.96, and in 2), 2.6×10^6 cells/ml, respectively. (see Table 3).

Ecological efficiencies in the chemostat are shown in Table 5. K_1 was lower than that in Fig. 4, because of the nitrogen recycling through bacterivorous feeding of the rotifer. Although Chemostat 2), where food density was thrice higher than in chemostat 1), had lower values both in assimilation efficiency and K_1 than chemostat 1), similar K_2 was obtained. It is deduced that rotifer had superfluous feeding at high food densities.

Discussion

Growth rates and ecological efficiencies in rotifers are known to be governed by biochemical composition of food algae.⁹ In our one-stage chemostat system, food algae was cultured in advance in a batch and stored together till the experiment, hence the nutritional quality is less variable than in the two-stage chemostat which has an algal chemostat as the first stage.

Another advantage of chemostat culture is postulated in Fig. 7, where the dilution rate was fixed at 0.05/h and algal density of the algae reservoir was adjusted at 0 time in order for the algal density of the reactor to decrease from 2 to 0.5×10^6 cells/ml. Growth rate of the rotifer became stagnant 8 to 16 h after the food adjustment. This lag time and stagnant growth can be explained by the following two observations obtained: a) at around 30°C, an egg takes 8–10 h before hatching (unpublished data); b) the rotifer utilizes the assimilated nitrogen immediately for reproduction,²¹ leading to reduction of egg-laying soon after the beginning of food deficiency. It is clear that physiological conditions affecting growth rate are very sensitive to food quality and a chemostat, which can keep rotifers under constant food conditions, is a suitable system for studying the ecophysiology of rotifers.

In Fig. 4, the gross growth efficiency (K_1) at low food density rose as algal concentration increased, and attained its maximum. After the peak, it decreased slowly and tended to be flat. There are some reports on K_1 relating growth rate in a continuous culture.^{8,10,13,15} Droop and Scott⁸) got an inverted U-shape curve, and the other three reported that the efficiency changed hyperbolically. The difference can be explained by the range of food concentration used. In the latter three reports, they might not have observed the decrease at high food density. In contrast, the curve obtained by Walz²¹) is similar to ours when yield ($=K_1$) is expressed in relation to specific ingestion rate. Table 5 shows that the decrease of K_1 depends on assimilation efficiency, and not on K_2 . It is deduced that the rotifer feeds superfluously at high food densities as Conover²⁶) reported. A food density of 26.0×10^5 cells/ml is much higher than the natural standing stock of phytoplankton. While in aquaculture, algal density at the initiation of rotifer massculture is higher than 50×10^5 cells/ml.²⁷)

In our chemostat culture, the rotifer egested 70–80% of ingested nitrogen as PON, and about 80% of the remaining assimilated nitrogen was utilized for reproduction. The low assimilation efficiency and high K_2 is consistent with the results of our previous tracer experiment²¹) (Table 6). This is because the algal C/N ratio was higher than the rotifer, and nitrogen was the limiting element.²¹)

Nitrogen egested by the rotifer as PON diverges into 2 main ways. One is remineralization by bacteria into NH_4^+ ,

Fig. 7. Rotifer and algal density in a chemostat.
Arrows indicates the time when algal density of the reservoir was adjusted.

Table 6. Ecological efficiencies of chemostat 2) and tracer experiment.

	Chemostat 2)	The tracer experiment*
C/N ratio (weight base)		
algae	7.9	7.8–15.0
rotifer	4.4	5.5–9.1
Ecological efficiency		
assimilation efficiency	22.0	22.8
K ₁	16.2	17.4
K ₂	73.6	77.5

In chemostat 2), algal density was 2.6, and in tracer experiment, $3\text{--}6 \times 10^6$ cells/ml, respectively.

* Aoki *et al.*²⁰; ¹⁵N was used as a tracer.

which is comparative to the excretion of the rotifer. Another is reincorporation to the rotifer by bacteriivory. Starkweather and Gilbert,²⁸⁾ Hino and Hirano,²⁹⁾ and Rothhaupt²⁵⁾ reported that *Brachionus* ingests bacteria-size particles less. In this study, clearance rate on bacteria also ranged from a quarter to a half of that on food algae (Table 4). While nitrogen content of bacteria in rotifer culture medium (1.7×10^{-15} g atom N/cell) is significantly larger than that in natural aquatic zone (0.4×10^{-15} g atom N/cell³⁰⁾), the ingested bacteria-size nitrogen was only about 20% of algae (Table 4).

As to DON other than urea, excretion by the rotifer and exudation of algal plasma through mastication are possible sources.¹⁷⁾ Although we could not distinguish them, algal plasma which exudated but non-assimilated in digestive channel of rotifer may have been a major pathway, because DON was higher in chemostat 2) where there was superfluous feeding.

In our chemostat, $\mu_{\max}$ decreased from 0.055/day to 0.033/day when rotifer density changed from 500 indiv./ml to 5,000 indiv./ml. Rothhaupt¹²⁾ reported that over at 10^7 cells/ml of algae, μ began to decrease. This suppression of growth rate might be due to physical interference with grazing by high-density particles. Kirk³¹⁾ and Hanson³²⁾ observed, however, that both growth and ingestion rate of rotifers were not affected by particles.

Through the microscopic observation, the suppressed rotifer did not show any changes in swimming and grazing behaviors. In addition, unionized ammonia concentration was under toxic levels reported by Schlüter³⁾ and Yu.²⁾ Other possible suppression may be due to influences of water quality caused by accumulation of PON. Yu *et al.*⁵⁾ found a bacteria strain with strong toxicity to the rotifer. PON and AON provide different microbial environment inside the aggregated flocs, and this may affect the ecosystem and their products. Accordingly, for stable mass culture of the rotifer, prevention of the accumulation of particles either suspended (PON) or attached (AON) is thought essential. An adsorptive filter commonly used in hatcheries is thought to be effective in removing PON, considered to be a major source of NH_4^+ or a substratum of undesirable microbes.

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