Measurements of the Nitrogen Budget in the Rotifer *Brachionus plicatilis* by Using ^{15}N

Shigeru Aoki,^{*1} Jota Kanda,^{*2} and Akinori Hino^{*1}

^{*1}Fisheries Laboratory, Faculty of Agriculture, The University of Tokyo, Bentsujima, Hamana, Shizuoka 431-02, Japan

^{*2}Faculty of Liberal Arts, Shizuoka University, Ohya, Shizuoka 422, Japan

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The nitrogen budget of the rotifer, *Brachionus plicatilis*, fed on algae (*Nannochloropsis oculata*) was examined using a ^{15}N tracer technique. It was found that this technique could be used to measure the nitrogen budget of zooplankton using the difference in the turnover time between egestion and excretion. When food was unlimited, the assimilation efficiency, the gross growth efficiency (K_1), and the net growth efficiency (K_2) were 22.8, 17.4 and 77.5% respectively. Therefore, approximately 80% of the ingested nitrogen was egested into the culture medium and about 80% of the assimilated nitrogen was utilized for reproduction. The turnover time of egestion and excretion were about 20 min and 2.5 h, respectively. The obtained short turnover time for excretion is consistent with the hypothesis that assimilated nitrogen is used immediately and primarily for reproduction and the excess is stored in body tissue when food is abundant.

Key words: rotifer, nitrogen budget, ^{15}N labeling technique, ecological efficiency

The rotifer *Brachionus plicatilis* is produced as live food for finfish or shellfish larvae all over the world.^{1,2)} Occasionally, an unexpected sudden decrease of density or suppressed growth occurs during mass production. Although possible causes include high levels of un-ionized ammonia^{3,4)} and accumulation of waste products,⁵⁾ the mechanism of how water quality influences the rotifer is not well understood. Measuring the nitrogen budget in the rotifer is important to understand the regulatory mechanism of water quality in rotifer-culturing tanks. In contrast to the carbon or energy budget,^{6,7)} few reports exist on the nitrogen budget due to the lack of a convenient labeling technique such as using the radio isotope, ^{14}C . Moreover, in the balance methods, the presence of bacteria in seawater interferes with the measurements of nitrogen budget of the rotifer, resulting in poor sensitivity. Recently, a technique for measuring the less hazardous stable isotope, ^{15}N , has become readily available. This paper investigates the use of the ^{15}N isotope to determine the nitrogen budget and ecological efficiencies of the rotifer.

Materials and Methods

The rotifer *B. plicatilis* was isolated from a fishpond at Songkhla, Thailand, in 1987 and maintained at 25°C in diluted seawater of 20 salinity. This strain has a smaller adult lorica, with length ranging from 150 to 195 μm , and a faster growth rate than rotifers commonly used in Japanese aquaculture. Precultures were grown at 30°C in diluted seawater of 20 salinity in darkness, the condition commonly used for practical indoor mass cultures in hatcheries. The algae *Nannochloropsis oculata* was also isolated from the same fishpond and grown in a modified Yashima 1 medium:⁸⁾ 0.1 g ammonium sulfate, 0.01 g urea, 0.015 g calcium superphosphate, and 0.0075 g Clewat 32 (mixture

of chelated metals, Teikoku Kasei Sangyo Co., Osaka, Japan) in 1 liter of seawater diluted with tap water to 20 salinity. Algae cultures were kept in a 5,000 ml erlenmeyer flask at 25°C under continuous illumination from fluorescence lamps and with constant aeration. Growth of algae was monitored daily through microscopic enumeration using a hemocytometer, and the cells were harvested during the exponential phase. The algae was collected and washed twice with filtered seawater by centrifugation. The harvested algae was stocked in a -30°C freezer until use; the storage period was less than one month. Labeled algae was grown in the same medium composed of 99.7 atom% ^{15}N -ammonium sulfate, harvested, and stocked; it was previously observed that the frozen algae did not absorb nutrients and its isotope-ratio was stable during the experiment. The uniformity of labeling was effective for measuring the assimilation efficiency in zooplankton.⁹⁾ The ^{15}N -ratio of labeled algae was larger than 80% (Table 2), which means that the nitrogen of the algae was mostly replaced with ^{15}N .

Seawater used for experiments was diluted to 20 salinity with tap water, then boiled for 15 minutes to prevent contamination with protozoans. Particulate matters were removed with GF/F-filters.

Rotifer samples were washed well with seawater in a 40 μm mesh, then collected on a GF/F-filter previously combusted at 500°C for 4 hours. After freeze-drying, the amount of nitrogen and ^{15}N -ratio were measured with a quadrupole mass spectrometer.¹⁰⁾ The amount of nitrogen and ^{15}N -ratio of algae were also measured as above. Samples were taken in triplicate.

The budgets of nitrogen and ^{15}N in the rotifer during incubation were described by the following equations:

$$W_a = W_b + t\{F - (E_{cx} + E_{eg})\}, \quad (1)$$

or

$$P = F - E_{ex} - E_{eg},$$

$$= A - E_{ex},$$

where W_a and W_b are nitrogen contents of the rotifer after and before incubation respectively; F , grazing rate; E_{ex} , excretion rate; E_{eg} , egestion rate; P , net production rate; A , assimilation rate; and t , incubation time. Because of the difficulty in distinguishing the excretion and egestion of zooplankton in the strict sense, the following definition is practical in the present investigation: egestion is the undigested matter egested as faeces, while excretion is the metabolic by-products. The turnover time of these two variables is different, as shown below:

$$W_a I_a = W_b I_b + t(F I_f - E_{ex} I_{ex} - E_{eg} I_{eg}), \quad (2)$$

where I_a and I_b are ^{15}N atom % of the rotifer after and before incubation, respectively; whereas I_f , I_{ex} and I_{eg} are the ^{15}N atom % of the algae, excretion, and egestion, respectively.

Initial concentrations of the algae and the rotifer were 6×10^6 cells/ml and 200 individuals/ml, respectively. The algae was maintained at a level of more than 3×10^6 cells/ml through the addition of new batches. Within this range ingestion and growth rates are known to be stable and at the maximum,¹¹ a condition used for actual rotifer rearing in hatcheries.

Results

Assimilation Rate

The first experiment was designed to determine the assimilation rate using the method modified from Holm *et al.*¹² Rotifers were fed with labeled algae for 0.5, 1, 1.5, 2.0 and 3.0 hours, and allowed to exchange their gut content for 30 minutes in seawater which suspended unlabeled algae. The results of the experiment are shown in Fig. 1. Assimilated ^{15}N increased linearly with duration of feeding up to 2 hours, but became stagnant thereafter. The excretion of ^{15}N was thus thought to begin 2.5 hours (2 hours feeding with labeled algae plus 30 minutes to clear gut) af-

ter feeding was initiated. It was assumed that I_{ex} was equal to the natural ^{15}N ratio before the beginning of the excretion of ^{15}N .

Due to the short duration of the experiment, it was assumed that there was no increase in the amount of nitrogen in the rotifer, (i.e. $W_a = W_b$). Denoting the ^{15}N -ratio in the rotifer fed with labeled algae for t hours as I_t , and designating the assimilation rate as $A (= F - E_{eg})$, equation (2) can be rewritten as follows:

$$W_b I_t = W_b I_o + t(A I_f - E_{ex} I_o), \quad (3)$$

where I_o is ^{15}N atom% of the rotifer at zero time (0.56 atom%) and I_f is ^{15}N atom% of the labeled algae (83.2 atom%). From $A I_f \gg E_{ex} I_o$,

$$A/W_b = \{(I_t - I_o)/t\}(1/I_f).$$

The increase rate of the ^{15}N -ratio from 0 to 120 minutes was 2.82%/h, thus,

$$A/W_b = 0.034.$$

The assimilated nitrogen of the rotifer was 3.4% body N per hour.

Egestion and Excretion Rate

The second experiment measured both the egestion and excretion rates. The rotifer was provided with labeled algae for 2 hours and then transferred to seawater suspending unlabeled algae. Rotifer samples were then taken at 0, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0 hours and the ^{15}N -ratio measured. The results are shown in Fig. 2. During the initial 20 minutes, the ^{15}N -ratio of the rotifer decreased rapidly because of egestion of labeled algae. The egestion rate was determined from the slope of the line between 0 and 10 minutes. Assuming that there was no change in the nitrogen content of the rotifer body ($W_a = W_b$), equation (1) is rewritten as,

$$F = E_{ex} + E_{eg}. \quad (4)$$

From the previous experiment, the excretion of ^{15}N had not yet begun, so equation (2) can be written as,

$$W_b (I_a - I_b) = t \{(F - E_{ex}) I_o - E_{eg} I_f\}. \quad (5)$$

Fig. 1. Relationship between assimilated ^{15}N in the rotifer and duration of feeding on labeled algae. Bars represent ± 1 S.D. ($n=3$).

Fig. 2. Changes in ^{15}N ratio of the rotifer after transfer into unlabeled condition.

The rotifer fed on labeled algae for 2 hours prior to the experiment. Bars represent ± 1 S.D. ($n=3$).

where I_o and I_f are the same as in equation (3). From equation (4), $(F - E_{ex})$ can be substituted for E_{eg} , as follows:

$$E_{eg}/W_b = (I_a - I_b)\{t(I_o - I_f)\}.$$

From the values obtained, $I_a - I_b$, 9.5%/h, the above equation gives,

$$E_{eg}/W_b = 0.115.$$

This means that the egested nitrogen of rotifers was 11.5% body N per hour.

The decrease of ^{15}N slowed down after 20 minutes, suggesting that the egestion of labeled algae had finished and only the excretion of ^{15}N continued. The time required to pass the digestive channel is thought to be less than 20 minutes.

The decrease lasted up to 120 min and the excretion of ^{15}N finished by then. The excretion rate was calculated from the slope of the line between 30 and 120 minutes. Substitution of the assimilation rate ($A = 0.034W_b$) in the first experiment equations (1) and (2) gives the following:

$$W_a = W_b + t(0.034W_b - E_{ex}), \quad (6)$$

$$W_a I_a = W_b I_b + t(0.034W_b I_o - E_{ex} I_f). \quad (7)$$

It was assumed that the ^{15}N -ratio of excretion was equal to that of the labeled diet (see discussion). Therefore, from equations (6) and (7),

$$E_{ex}/W_b = [I_b - I_a + 0.034t(I_o - I_a)]/[t(I_f - I_a)]. \quad (8)$$

The slope of the regression line in Fig. 2 between 30 and 120 minutes was 0.75%/h and the excretion rate was calculated to be 0.8% body N per hour. After 120 minutes, no excretion of ^{15}N was observed.

Net Production Rate

In the third experiment, net production of the rotifer was measured directly by a long-term experiment in which the accumulation of tracer in the rotifer was monitored. The nitrogen budget of the rotifer fed on labeled or unlabeled algae was compared (Fig. 3). The rotifer fed on the unlabeled algae had been cultured in labeled algae for 3 days prior to the experiment. As the rotifer reproduces exponentially, nitrogen and ^{15}N budgets are described as follows:

$$W_a = W_b \exp(pt), \quad (9)$$

$$W_a I_a = W_b I_b + (W_a - W_b)I_i, \quad (10)$$

where t is time and p is net production rate, the same as specific growth rate. Other symbols are the same as for equations (1) and (2). From equations (9) and (10),

$$I_a = I_i + (I_b - I_i) \exp(-pt). \quad (11)$$

From the values between 3h and 9h in Fig. 3, p for the rotifer fed on labeled diet or unlabeled each was 3.5 ± 0.1 (avg. $\pm$ s.d.) or $4.5 \pm 0.2\%$ body N per hour. Using p , the changing rate of ^{15}N -ratio between 30 and 120 minutes in Fig. 3, and equations (1) and (2), the assimilated nitrogen was calculated to be 4.3 or 6.0% body N per hour for each rotifer.

There are some reports that the nitrogen excretion of zooplankton is isotopically lighter than the diet fed¹³⁾ and that the $\delta^{15}\text{N}$ in herbivorous zooplankton is usually higher

Fig. 3. Changes in ^{15}N ratio of the rotifer fed on labeled (○) or unlabeled algae (●).

The rotifer which was fed on the latter had been cultured in labeled algae for 3 days prior to the experiment. Bars represent ± 1 S.D. ($n=3$).

Table 1. Nitrogen budget (% body N per hour) and ecological efficiencies (%) of *B. plicatilis* fed on *N. oculata*

Experiment #*1	1	2	3*2
Nitrogen budget (% body N per hour)			
Assimilation rate (A)	3.4		4.3 or 6.0
Egestion rate (E_{eg})		11.5	
Excretion rate (E_{ex})		0.8	
Ingestion rate (F), $A + E_{eg}$	14.9		
Net production rate (p), $A - E_{ex}$	2.6		3.5 or 4.5
Ecological efficiency (%)			
Assimilation efficiency, A/F	22.8		
Gross growth efficiency (K_1), p/F	17.4		
Net growth efficiency (K_2), p/A	76.5		81.4 or 75.0

*1 Described in the text.

*2 For the rotifer fed labeled or unlabeled algae, respectively.

than that found in phytoplankton.¹⁴⁾ In the third experiment, however, the effect of isotope selection was found to be not significant within the range of precision in this experiment.

The nitrogen budget and efficiencies are shown in Table 1.

Discussion

Bidigare¹⁵⁾ reported that a large portion of ingested nitrogen is used for growth or reproduction when food is abundant and the ratio of excreted nitrogen to ingested nitrogen is low. We found that the rotifer egested about 80% of grazed nitrogen into the culture medium as faeces, and most of the assimilated nitrogen (avg. 77.5%) was used for growth and reproduction when the rotifer was fed *N. oculata*. The passing time in the digestive channel was 20 minutes, similar to the results of Starkweather and Gilbert¹⁶⁾ in *B. calyciflorus*. This finding indicates that the 30 minutes which was used for gut clearance in the first experiment was reasonable. The turnover time of assimilated nitrogen was determined to be 2.5 hours (see results of the first experiment) where the increase of assimilated ^{15}N

slowed down at 2 hours of feeding (Fig. 1). This is further supported by results of the second experiment where the decreasing slope of ^{15}N ratio leveled off 2–3 hours after feeding started on unlabeled algae (Fig. 2). This fast metabolism proves that assimilated nitrogen is used immediately for growth and reproduction. Studies on energy allocation of zooplankton in *Daphnia*,^{17,18)} revealed that assimilated energy is used for reproduction directly or stored in the body tissue for maintenance. Our results show that this metabolic mechanism is common to both *Daphnia* and the rotifer, further elucidating the ecological characteristics of these plankters which increase rapidly under an eutrophic environment.

A comparison of the ecological efficiencies in our studies is difficult, since such values depend on the chemical composition of the diet^{19–21)} and more importantly, on the method of measurement. Our results are similar to those of Doohan⁶⁾ who observed undigested diet in the faeces of rotifer supplied an excess of food, and derived an assimilation efficiency and K_2 of 19.4 and 57% in calories, respectively. On the other hand, Droop and Scott⁷⁾ obtained a higher assimilation efficiency and a lower K_2 . In Doohan's experiment, assimilation rate was estimated as the sum of respiration and egg production rate, and in our case, it was directly measured by a labeling technique; however, in Droop and Scott's experiment, it was indirectly determined by computing the difference between the ingestion and egestion rate. Rotifer faeces are easily broken and resuspended in culture media, which might cause overestimation of the assimilation rate and underestimation of K_2 .

In the second experiment, the high egestion rate was estimated from the rapid decrease of the isotope-ratio during the initial 20 minutes (Fig. 2). A possible explanation for this steep slope is stimulation caused by transferring. A similar phenomenon in the third experiment (Fig. 3), however, did not support this hypothesis. The high ingestion rate in Fig. 3 almost coincided with the one calculated in Table 2, indicating that transferring did not interfere with the feeding and egestion of the rotifer.

Gardner and Scavia²²⁾ distinguished two kinds of nitrogen excretion in *Daphnia*: a food dependent release (release from digested and metabolized material) and an endogenous release. If the rotifer also had these two types of nitrogen excretion and if the turnover time of the latter was longer, then the ^{15}N ratio of excretion in equation (7) should be smaller than that of the labeled diet, and the calculated excretion was only that of the food dependent type. Even in this case, the evaluation resulted in decreases of K_1 and K_2 by 10%, because the ratio of excretion reported is 3:1.

Differences in the chemical composition of food may account for the variety of ecological efficiencies in our studies. In *B. rubens*, Schlüter *et al.*²¹⁾ proved that the ammonia-excretion rate is low and nitrogen K_2 is high when nitrogen in food is limited. In our study, the C/N ratio of the rotifer was lower than the diet (Table 2), which means that nitrogen was a limiting element, thus K_2 became high. In these cases, a different metabolism may occur in the rotifer. In our experiments many oil droplets, indicative of an active fat metabolism, were observed outside of the gut. The utilization of fat can assist in the assignment of food proteins much more for reproduction

Table 2. Chemical compositions of the rotifer and the algae in our experiment

Experiment #*	1 & 2	3	
		labeled algae	unlabeled algae
Rotifers			
C/N ratio	9.1	6.5	5.5
Algae			
C/N ratio	15.0	11.1	7.8
Nitrogen contents ($\times 10^{-14}$ g atom N/cell)	1.96	3.28	2.94
^{15}N -ratio (atom%)	83.2	81.7	0.56

* Described in the text.

than for maintenance. The assimilation rates shown in Table 1 are inversely proportional to the C/N ratios of food algae, contrary to the nitrogen contents (Table 2). As for net growth efficiency (K_2), no particular relationship was found (Table 2). We conclude from these results that the rotifer can not assimilate limited elements selectively, but assimilates both carbon and nitrogen in proportional to the observed ratios in the food and selectively utilizes the limited element, nitrogen, for reproduction.

We have proven that the ^{15}N tracer technique may be used to measure the nitrogen budget of zooplankton using the difference in the turnover time between excretion and egestion. The stable isotope tracer technique is safe and sensitive, and it will become a very useful method to estimate the ecological efficiencies of zooplankton.

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