Geochimica et Cosmochimica Acta Vol. 48, pp. 1135–1140
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0016-7037/84/$3.00 + .00

# NOTE

## Stepwise enrichment of $^{15}N$ along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age

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(*Received July* 19, 1983; *accepted in revised form January* 24, 1984)

**Abstract**—The isotopic composition of nitrogen was measured in marine and fresh-water animals from the East China Sea, The Bering Sea, Lake Ashinoko and Usujiri intertidal zone. Primary producers, showed average $\delta^{15}N$ *versus* atmospheric nitrogen of +5.0‰ (+3.4 to +7.5) in the Bering Sea and Lake Ashinoko, and +6.8‰ (+6.0 to +7.6) in Usujiri intertidal zone. Blue green algae from the East China Sea show an average −0.55‰ (−0.8 to +1.2). All consumers, zooplankton, fish and bird exhibited stepwise enrichment of $^{15}N$ with increasing trophic level. The $^{15}N$ enrichment at a single feeding process ranged from +1.3 to +5.3 averaging +3.4 ± 1.1‰. This isotopic fractionation seems to be independent of habitat.

The effect of age in animals was obtained by analyzing two marine mussels. The soft tissue nitrogen showed +2.0‰ enrichment relative to that of primary producers, and the magnitude was almost constant with shell ages ranging from 0 to 8 years.

A similar $^{15}N$ enrichment occurs in all Molluscs, Crustaceans, Insecta, Amphibia, Fish, Ave and Mammal species regardless of the difference in the form of excreted nitrogen and in laboratory cultured fish, brine shrimp and mice (+2.9 to +4.9‰). The excreted ammonia from guppy was sufficiently light to balance the concentration of $^{15}N$ to animal body.

### INTRODUCTION

RECENT ANALYSES of food web have used naturally occurring stable carbon and hydrogen isotope ratios to study food sources (HAINES, 1976; DENIRO and EPSTEIN, 1978; FRY *et al.*, 1977; RAU *et al.*, 1981; DESMARAIS, 1980). These studies have been based upon the general concept that the isotopic composition of these elements should be conserved during animal feeding process.

Previous data have shown that nitrogen isotopes in animal tissue are highly fractionated during the feeding process and more than those of carbon and hydrogen. Most previous data show a significant enrichment of $^{15}N$ in the animal body (GAEBLER *et al.*, 1966; MIYAKE and WADA, 1967; WADA *et al.*, 1974; STEELE and DANIEL, 1978). DENIRO and EPSTEIN (1981) reported that the deviation of $^{15}N$ between animals and diet for some terrestrial animals which were raised in the laboratory varies over a wide range. Few studies of marine and fresh water animals collected in the field have been reported (WADA and HATTORI, 1976; PANG and NRIAGU, 1977; SCHOENINGER *et al.*, 1983) and the relationship between $\delta^{15}N$ of animal and its diet has not been carefully investigated.

If the isotopic fractionation of nitrogen during feeding occurs in general, and the magnitude is common to many animals, the nitrogen isotope study might provide useful information about the trophic level of animals and the food web structure. With this in mind, we have determined here the nitrogen isotope ratio of phytoplankton, zooplankton and fish collected from several kinds of ecosystems, and have attempted to determine the relationship between animals and their $^{15}N$ content. We have also studied the $^{15}N$ distribution in aquatic biota in an intertidal ecosystem where the food web structure is already well known.

The isotope ratio of an animal undergoes both the food source effect and any *in vivo* metabolic effects. A recent report suggests that in fish $\delta^{15}N$ increases with their weight (RAU *et al.*, 1981). It is presumed that both effects may vary with the age and the physiological condition of an individual animal. In order to make clear this relationship between the nitrogen isotope ratio of an animal and its age, we have investigated two kinds of field-grown marine mussels whose age is known.

### MATERIALS AND METHODS

Plankton and fish were collected by filtering or netting from the surface water of the East China Sea, the Bering Sea and Lake Ashinoko. Large zooplankton and the predominant species of marine algae were sorted by hand. Fish samples were taken by 800 meter oblique towing (ORI or IKMT type net sampler). Fish, mussels, sea weed and other ecological samples were obtained from an intertidal area on the Usujiri coast in Hokkaido, Japan. The sampling site borders the Northern Pacific Ocean, and has numerous tidal pools where macrophytes are the primary producers. Suspended organic debris in the surrounding water was taken by water filtration through glass fiber filters. Some terrestrial animals were collected at a paddy field at the Central Agricultural Experiment Station at Konosu, Saitama Prefecture, Japan.

In laboratory experiments, guppy (*Lebistes* sp.), brine shrimp (*Artemia* sp.) and a mouse (*Mus musculus*) were fed on controlled diets with constant nitrogen isotopic compo-

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sition. Guppies were raised up collectively for one-half to two months on a single type of nutrient (Tetramin). Food was supplied so as to be always available, and any remaining debris and feces were removed from the aquarium every day. After hatching, the brine shrimp were grown on a diet of suspended yeast, for a period of 10 to 25 days, after which they were separated from the medium by serial washing. Mice were cultured on a commercial diet (Oriental yeast).

Parts of muscles of large animals like octopi or mice were analyzed. Most small samples were, however, totally analyzed except mixed plankton samples which were not sorted. A few pieces of feather from sea gull and parts of sea weeds were analyzed. Marine mussels were collected at Usujiri pier and intertidal area on July and November 1981, and were separated into shell and soft tissues as soon as possible after collection.

All biological samples were frozen for storage, freeze-dried, and digested by the Kjeldahl method. The resulting ammonia was distilled by steam, concentrated into sulfuric acid and converted to nitrogen gas using the potassium hypobromine. Aliquots of the distillate were taken to determine the amount of nitrogen by calorimetry. After the purification of nitrogen gas as described by WADA and HATTORI (1976), the nitrogen isotope ratio was measured by Hitachi RMU-6R mass spectrometer fitted with a dual inlet system and double collector. The analytical precision was ±0.3 per mil. All data are presented as $\delta^{15}N$ values using the atmospheric nitrogen as a standard, where

$$\delta^{15}N = \{(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{air} - 1\} \times 1000\ (‰).$$

## RESULTS AND DISCUSSION

### *(1) The nitrogen isotopic composition of natural samples from the Bering Sea, the East China Sea, Lake Ashinoko and a paddy field*

Analytical results of $\delta^{15}N$ of organisms are presented in Fig. 1. All organisms were assigned to one of three trophic levels based on consideration at feeding habits. All primary producers show relative low $^{15}N$ content in all of the ecosystems studied. Most phytoplankton except those from the East China Sea ranged from 5 to 7‰ and were consistent with previously reported values (WADA and HATTORI, 1976).

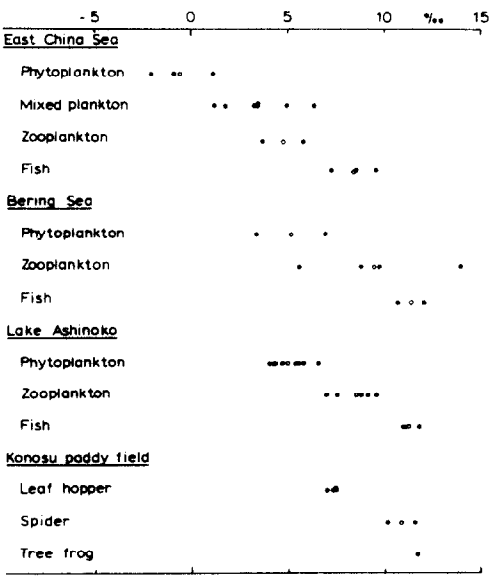


FIG. 1. The $\delta^{15}N$ of animals collected from marine, freshwater and land ecosystems. Closed and open circles represent individual measurements and average values. Sampling locations are as follows: 23°59'N 125°03'E and 27°29'N 125°01'E in the East China Sea, 56°58'N 167°08'E and 57°03'N 176°56'W in the Bering Sea area.

Phytoplankton in the East China Sea were collected from a red tide composed of *Tricodesmium* sp., a marine nitrogen fixing blue green alga, and showed the lowest $^{15}N$ content of −2.1‰. Biological nitrogen fixation causes less than 1.003 fractionation as reported so far (HOERING and FORD, 1960; WADA and HATTORI, 1976; MARIOTTI *et al.*, 1980). The lower $\delta^{15}N$ value obtained here suggests that the primary producer in this oligotrophic sea depends mainly on atmospheric nitrogen fixation. Zooplankton and fish, collected in the surrounding waters during the red tide also shows lower $^{15}N$ content than those of other ecosystems.

The $\delta^{15}N$ of phytoplankton (mainly diatom) in the Bering Sea represented almost the same range of $\delta^{15}N$ of phytoplankton from the western North Pacific Ocean, +5.6 ± 1.7‰ reported by MIYAKE and WADA (1967). The isotopic fractionation in assimilation of inorganic nitrogen by phytoplankton has been revealed to go up to 1.015 according to the growth rate (WADA, 1980). The $\delta^{15}N$ of dissolved nitrogen in this area is not available. If it is the same as +10‰ of the surface water in the western North Pacific Ocean (WADA, 1980), these $\delta^{15}N$ of phytoplankton suggest that the isotopic fractionation associated with the nitrate uptake may be less than 1.005 in this sea. The $\delta^{15}N$ of zooplankton in Fig. 1 consists of Copepoda (+5.6‰), Decapoda (+8.8‰), Sagita (+9.7‰) and Euphausia (+14.0‰). We think that this wide variation reflects a relatively complicated food chain in the boreal eutrophic sea. Each of these zooplankton might originally belong to different trophic levels.

Phytoplankton in Lake Ashinoko consist of diatoms with $\delta^{15}N$ from +3.8 to +7.5, average +5.0‰. Average $\delta^{15}N$ of zooplankton and fish are respectively +8.1‰ (+6.8 to +9.4) and +11.1‰ (+10.7 to +11.6). Additional data on herbivores and carnivores from a paddy field show average +7.4‰ (+7.1 to +7.7) and +11.2‰ (+10.2 to +12.4), respectively.

In summary, these data suggest that the nitrogen in field animals is strongly affected by the isotopic composition of the food source and consequently the ultimate nitrogen source. On the other hand, the $^{15}N$ content of animals depends on the trophic level. Studies of nitrogen isotopes along the food chain are now investigated in more detail by analyzing individual animals in a specific ecosystem to help understand the complexities of these systems.

### *(2) The nitrogen isotopic composition of marine organisms from an intertidal ecosystem*

Figure 2 shows the $\delta^{15}N$ of typical sea weed and animals collected at Usujiri intertidal area on July 28, 1981. The primary producer, sea weed, shows the lowest $\delta^{15}N$ value, +6.8 ± 0.6‰. Detritus including phytoplankton and organic debris of macrophytes showed a similar value of $\delta^{15}N$ of +6.8 ± 0.5‰.

Animals collected here are classed into three groups

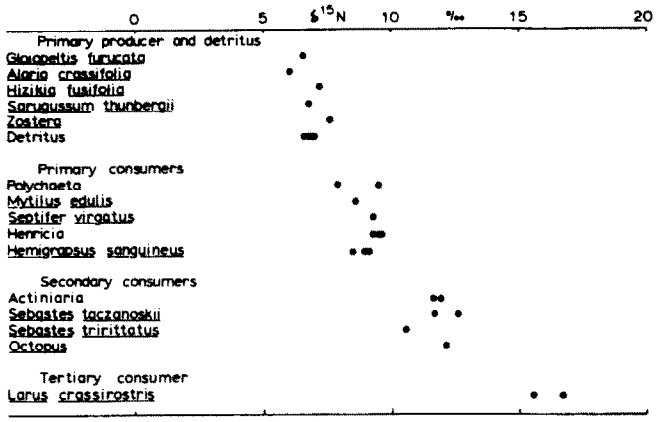


FIG. 2. The $\delta^{15}N$ of biota from Usujiri intertidal zone. The average for adults is shown for *Mytilus edulis* and *Septifer virgatus*.

as determined by trophic level. They are herbivores (the primary consumer), carnivores (the secondary consumers) who feed predominantly on herbivores, and the higher carnivores (the tertiary consumers) who feed mainly on carnivorous animals. The $\delta^{15}N$ range of these animals is distinct for each of the three groups. Mussels, shore crabs, sand worms and starfish are ecologically classed as primary consumers, and show the same level of $^{15}N$ content, ranging from +8.4 to +9.5‰. The $\delta^{15}N$ of these herbivores shows a significant enrichment of about 2 to 3 per mil relative to the plant values. The secondary consumer group consists of sea anemones, octopuses and fish, and exhibited $\delta^{15}N$ values of from +10.6 to +12.7 average +11.7‰ and +3.0‰ enrichment. The tertiary consumer is a sea gull, *Larus crassirostris*, with +16.2‰ (+15.6 to +16.8). This bird is known to migrate along the northern Japanese coast and to feed on small fish and insects near the shore (ISHIZUKA, 1966). Its diet therefore is not necessarily confined to foods which originate in an intertidal ecosystem. They stay in this area about half of a year and contribute to the food chain in this community. They are 4.4‰ enriched relative to the second consumer.

These results show that the $\delta^{15}N$ of animals composing an intertidal ecosystem is gradually enriched from +6.8 to +16.2‰ according to the trophic level, and suggests that the highest value of $\delta^{15}N$ of ecological samples should be found in the highest trophic level, assuming that the original nitrogen sources are all equivalent.

### *(3) The relationship between $\delta^{15}N$ of marine mussels and their age*

To clarify the relationship between animal $\delta^{15}N$ and age, we investigated two kinds of marine mussels. *Septifer virgatus* were collected from the same intertidal area studied above and *Mitylus edulis* were obtained from off the pier in Usujiri harbor, which is exposed to the open ocean. Both types of mussels are sessile after their adhesion to rocks during the juvenile stage, and do not change their feeding habits once they attach. Their food source is limited to the organic detritus suspended in the surrounding water. The results are presented in Fig. 3. The soft tissue of individual bivalves were mainly analyzed since the $\delta^{15}N$ of nitrogen contained in the shell is influenced by clinging organisms.

In the case of *Septifer virgatus*, the nitrogen inventory in the soft tissue appears to reach a maximum in samples four or more years old. It is therefore presumed that the nitrogen uptake to soft tissue almost balances the nitrogen excretion of the mussel. The $\delta^{15}N$ of mussels belonging to this age group nevertheless shows significantly constant values of $+9.0 \pm 0.6$‰. A slight variation can be distinguished only in the early stages. *Mytilus edulis* shows a similar trend in the relation of $\delta^{15}N$ to shell size. Its nitrogen balance has not reached the steady state, and still the $\delta^{15}N$ appears to remain constant ($+8.7 \pm 0.3$‰) with age except during early stages. The enrichment value of these two mussels showed almost the same ranges, +2.1 and +1.9‰ for *Septifer* and *Mytilus* respectively, despite the large difference of growth rate.

From these results it is concluded that the $\delta^{15}N$ of marine mussels is independent of both their age and the nitrogen mass balance in the body. In the early stage, when the nitrogen inventory in the animal is small, the nitrogen isotopic composition may vary because of fluctuations in the source material. Recent work by RAU *et al.* (1981) reported a positive correlation between $\delta^{15}N$ of Dover sole and the weight of the individual. This differs from the result for marine mussels for the following possible reason: the food of fish may change with age because of the development of organs for feeding or perhaps change of habitat. The physiological differences between fish and molluscs may be a less important factor of this, because a type of nitrogen metabolism seems essentially to be independent on the ultimate fractionation factor of nitrogen as described later. Furthermore, recent experiments on a fish (Tilapia) which was raised on monotonous diets does not show any trend of $\delta^{15}N$ with age (unpublished data).

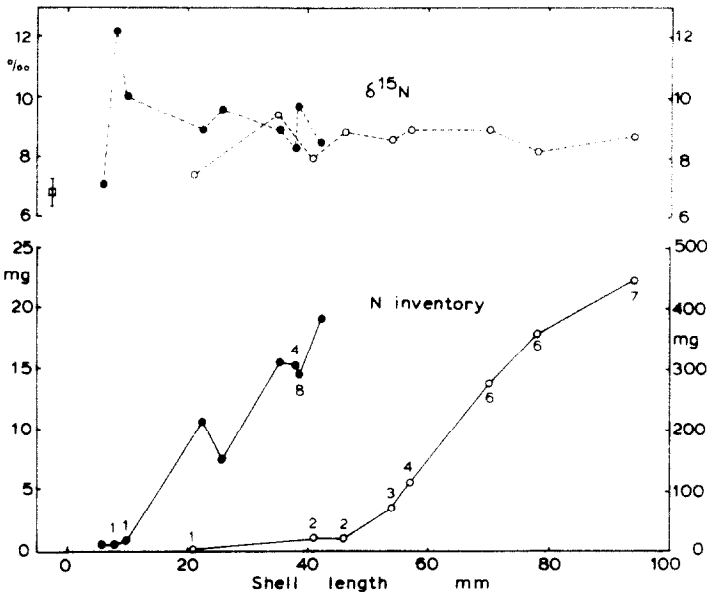


FIG. 3. The relationship between $\delta^{15}N$ of mussels and their ages. The $\delta^{15}N$ and the nitrogen inventory for *Septifer virgatus* and *Mytilus edulis* are shown as closed circles (left scale) and open circles (right scale), respectively. An open square shows the $\delta^{15}N$ of their foods. The age (year) estimated from the line number of shells is also shown by each circle.

### *(4) Evaluation of the utility of nitrogen isotopic composition in food web analysis*

Every animal reported here exhibits a stepwise enrichment of $^{15}N$ in the body tissue with increasing trophic level. We have attempted to verify this enrichment by culturing animals in the laboratory. The results presented in Table 1 show the enrichment of $^{15}N$ in mice, guppies and brine shrimp relative to the $\delta^{15}N$ of the individual's diet. The extent of this fractionation is approximately the same as the data obtained from the field.

Analytical results of $\delta^{15}N$ of excreted ammonia from cultured water for guppies presents further evidence for the isotopic discrimination generated in the nitrogen metabolism of animals. The $\delta^{15}N$ of excreted ammonium seems to be sufficiently light to interpret the observational enrichment of $^{15}N$ in the animal tissue. There is a possibility that the incubation stimulated an abnormal metabolism in fish or bacterial degradation arose in the medium. Supposing that these effects are negligible, the isotope discrimination may be generated by the excretion of lighter nitrogen. STEELE and DANIEL (1978) also reported that the $\delta^{15}N$ of urine from steer and cows show values lighter than those of their blood and diet. Probably the $\delta^{15}N$ of urine is related to the nitrogen mass balance of the animal body.

General scheme of nitrogen metabolism in animals is: protein introduced into the digestive tract is severed amino acid and suffers from deamination prior to entering the metabolic recycling system. Nitrogen generated from this process is excreted as ammonium for most aquatic animals, or uric acid or urea in the case of more developed animals. It may therefore be important to examine whether the enrichment of $^{15}N$ in animals depends on the excretion mechanism and the urine form. To examine this question, available data concerning the $^{15}N$ enrichment in various animals are listed in Table 2 with the form of excreted nitrogen. The enrichment value for culturing animals is estimated by the difference of $\delta^{15}N$ between animals and

Table 1: $^{15}N$ enrichment in cultured animals

| Animals | Diets | $\delta^{15}N$ of body tissue (A) | $\delta^{15}N$ of diet (B) | Enrichment (A)-(B) | Excreted nitrogen |
|---|---|---|---|---|---|
| Lebistes | Tetramin | +11.9 ± 1.1 (6) | + 8.7 ± 0.3 (2) | + 3.2 | - 10.3<br>- 21.0 |
| Mus musculus | Oriental yeast NMF | + 9.0 ± 0.6 (2) | + 6.1 ± 0.0 (2) | + 2.9 | |
| Artemia | Yeast | + 7.7 ± 0.5 (3) | + 2.8 ± 0.3 (2) | + 4.9 | |

Numbers in parenthesis are the number of samples.
Errors are standard deviations.

Table 2: $^{15}N$ enrichment into animals, with relation to the class and the chemical form of their excreted nitrogen

| Animal | Class | $\Delta\delta^{15}N$ (Animal-food)‰ | Main form of N in excreta | Source: Field or Lab | Habitat | Ref. |
|---|---|---|---|---|---|---|
| round worm | Nematoda | 2.8 | $NH_3$, Amino acid | Lab | Land | a |
| sand worm | Polychaeta | 1.6 | $NH_3$ | Field | Marine | e |
| sea anemone | Anthozoa | 3.0 | $NH_3$ | Field | Marine | e |
| starfish | Asteroidea | 2.7 | $NH_3$ | Field | Marine | e |
| snail | Mollusca | -0.1 | $NH_3$ | Lab | Land | a |
| marine mussel | Mollusca | 2.0 | $NH_3$ | Field | Marine | e |
| octopus | Mollusca | 3.3 | $NH_3$ | Field | Marine | e |
| brine shrimp | Crustacea | 9.2 | $NH_3$ | Lab | Brakish | a |
| brine shrimp | Crustacea | 4.9 | $NH_3$ | Lab | Brakish | e |
| Copepoda | Crustacea | 5.3 | $NH_3$ | Field | Marine | e |
| Decapoda | Crustacea | 2.1 | $NH_3$ | Field | Marine | e |
| spider | Araneida | 4.1 | $NH_3$ | Field | Land | e |
| milk weed bug | Insecta | 2.8 | Uric acid | Lab | Land | a |
| blow fly | Insecta | 1.3 | Uric acid | Lab | Land | a |
| house fly | Insecta | 4.0 | Uric acid | Lab | Land | a |
| moth | Insecta | 4.0 | Uric acid | Lab | Land | a |
| weevil | Insecta | 5.1 | Uric acid | Lab | Land | a |
| grasshopper | Insecta | 0.5 | Uric acid | Lab | Land | a |
| tree frog | Amphibia | 4.5 | $NH_3$, Urea | Field | Fresh | e |
| rock fish | Osteichthyes | 3.4 | $NH_3$ | Field | Marine | e |
| guppy | Osteichthyes | 3.2 | $NH_3$ | Lab | Fresh | e |
| rainbow trout | Osteichthyes | 3.0 | $NH_3$ | Field | Fresh | e |
| dolphinfish | Osteichthyes | 5.1 | $NH_3$ | Field | Marine | e |
| sea gull | Aves | 4.4 | Uric acid | Field | Land/ Marine | e |
| rat | Mammalia | 3.6 | Urea | Lab | Land | b |
| mouse | Mammalia | 2.9 | Urea | Lab | Land | a |
| mouse | Mammalia | 2.9 | Urea | Lab | Land | e |
| cow | Mammalia | 4.0 | Urea | Field | Land | c |
| steer | Mammalia | 3.2 | Urea | Field | Land | d |

$\Delta\delta^{15}N$ were obtained by subtracting $\delta^{15}N$ of foods from $\delta^{15}N$ of an animal. The main chemical form of the excreted N was referred to Prosser and Brown Jr. (1950). Quated results are from a: DeNiro and Epstein (1981), b: Gaebler et al. (1966), c: Kreitler (1975), d: Steele and Daniel (1978) e: This study

used diet. The ecologically estimated diets or preys which were taken from the animal's stomach in some cases were used to estimate the enrichment value for the field-grown animals.

The nitrogen isotope fractionation ranges from +1.3 to +5.3‰ average +3.4 ± 1.1‰ for animals except snail, grasshopper and one brine shrimp. The following factors may be responsible for the scatter of the enrichment values: the exact food which has been digested by field-grown animals is unknown, the nitrogen balance in the body and the isotope ratio may be changeable depending upon environmental and biochemical conditions. Certain animals, for example, snail, blow fly and grasshopper show results exceptionally different from the average value. We guess that the digested food for them might not be exactly the same with the material which was analyzed as their food, because such a small animal can digest only a part of foods. Biochemical differences, like the existence of a urea cycle, the production of uric acid, or differences in habitat, do not seem to influence the isotopic fractionation. It can thus be presumed that similar nitrogen isotopic fractionations occur in most animals and probably before the final treatment of ammonium in the body.

GAEBLER *et al.* (1966) reported that many amino acids extracted from cultured rats have variable $^{15}N$ contents during the transaminating process. If nitrogen undergoes fractionation sometime during the transamination or other deamination process, the $\delta^{15}N$ of nitrogen accumulated in animals could be expected to increase with time. However the results of marine mussels do not indicate such age trends. This suggests that the earlier steps of the digestion may limit the ultimate enrichment of $^{15}N$, even if a part of the nitrogen suffers from further fractionation in the internal metabolic process.

In summary, the enrichment of $^{15}N$ is widespread among most animals collected from several different kinds of ecosystems, even if they belong to different trophic levels. Isotopic enrichment of +3.4 ± 1.1‰ occurs independently of habitat, form of nitrogen excreted and growth rate. The nitrogen isotopic composition of these animals is influenced however by the

ultimate source of the fixed nitrogen. Furthermore, it has been shown that the $\delta^{15}N$ of soft animal tissues is almost constant during the life span of two marine mussels. Consequently it appears that nitrogen isotopes can be used as a tracer not only for dietary analysis, but also for determining the trophic level of given animals.

*Acknowledgements*—We are grateful to M. Nakamura of Teikyo University, H. Motoh in Marine Ecology Research Institute, Japan, Y. Ito in Hokkaido Cultural Fisheries Center, and H. Fujimoto in the Mitsubishi-Kasei Institute of Life Sciences for presenting or classifying animal samples, and R. Shibata, K. Karasawa and Y. Kabaya for their assistance in analyzing organic nitrogen. We thank the officers and crew members of RV Hakuho Maru and the scientist aboard the KH 75-4 Cruise for their cooperation and assistance to collect pelagic organisms. We are indebted to M. DeNiro of the Department of Earth and Space Sciences at University of California Los Angeles for his critical readings and B. Barraclough of the Institute of Geophysics and Planetary Physics at University of California of Los Angeles for his helpful review of the manuscript.

This study was supported partly by a grant from the Japanese Ministry of Education, for Special Research on Environmental Science during 1979, 1980 and 1981.

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