

Simultaneous Determinations of Nitrification and Nitrate Reduction in Coastal Sediments by a ^{15}N Dilution Technique

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Nitrification, the oxidation of ammonia to nitrite and nitrate, and nitrate reduction by bacteria in coastal sediments of Mangoku-Ura and Odawa Bay were simultaneously determined by a ^{15}N dilution technique. In muddy sediments of Mangoku-Ura, nitrate reduction proceeded at a rate of 10^{-2} to 10×10^{-2} μg -atoms of N/g per h. Nitrification was far less intensive. Denitrification, or N_2 production from nitrate, accounted for about 30% of the nitrate reduction. A simultaneous occurrence of nitrification and nitrate reduction with a similar rate of 10^{-2} μg -atoms of N/g per h was demonstrated in sandy sediment collected from a *Zostera* bed of Odawa Bay.

Investigation of denitrification, the reduction of nitrate to gaseous nitrogen, in lake sediments is often part of eutrophication studies (5). In coastal sediments rich in organic materials, denitrification is controlled by the supply of nitrate and nitrite (14). In highly reduced sediments, the overlying waters and ground waters are probably the only sources of nitrate and nitrite. In aerobic sediments, however, nitrate and nitrite are produced in situ by oxidation of ammonia by nitrifying bacteria (2).

The extent of nitrification in sediments has been estimated from changes in nitrate or ammonia yielded when the sediments were incubated under simulated conditions for a rather long period (3, 11). Jørgensen (4) demonstrated the occurrence of bacterial sulfate reduction within reduced microniches of oxidized marine sediments. Nitrate reduction will likewise occur in apparently aerobic sediments. If so, the application of this conventional technique for estimation of nitrification will be unsatisfactory. Billen (2) proposed a new method to determine nitrification in sediments. $^{14}\text{CO}_2$ uptakes in the dark were measured in the presence and absence of N-Serve (Dow Chemical), a potent inhibitor of bacterial nitrification, and the difference was used as an index of nitrification activity.

We applied, with success, a ^{15}N dilution technique for simultaneous determinations of nitrification and nitrate reduction. This paper describes the experimental procedures and some results obtained with coastal surface sediments from Mangoku-Ura and Odawa Bay.

MATERIALS AND METHODS

Sediment sampling. Sediment samples were collected with a Plexiglas tube by divers. The location

and characteristics of the sediments and waters are given in Table 1. The color of sediments indicated that the oxidized layer extended from the surface to 1 cm at station 7 and to 0.5 cm at station 10 of Mangoku-Ura. At other locations (station 3 of Mangoku-Ura and Odawa Bay), the sediments were sandy and were effectively aerobic. Samples of the sediments were frozen immediately after collection to minimize possible biochemical changes. The interstitial waters were later extracted with Reeburgh squeezers (9).

Experimental procedures. Within a few hours after sampling, 5-g (wet weight) samples of top sediments (0 to 2 cm) were placed in 10 glass bottles (250 ml in volume). A 50-ml amount of sterile seawater containing 10 μg -atoms of N per liter of ^{15}N -nitrate (50% ^{15}N) and 50 (for sandy sediment) or 100 (for muddy sediment) μg -atoms of N per liter of unlabeled ammonia was added to each bottle. The sediment and seawater were mixed thoroughly and incubated near in situ temperatures (Table 1) under normal atmosphere without shaking. The sediments in the incubation bottles were about 1.5 mm thick on average.

At the times indicated, the incubated samples, two bottles at a time, were filtered through Whatman type C glass fiber filters (47 mm), and the filtrates (50 ml) were frozen and saved for later analyses. The sediments collected on the filters were dried at 60°C and weighed.

Analysis of ^{15}N in nitrate. Carrier nitrate (10 μg -atoms of N) was added to 20 ml of the filtrates, and nitrate was reduced to nitrite by a Cu-Cd column method, following the procedure described by Strickland and Parsons (13). A peristaltic pump provided a constant flow rate through the column. After each sample was run through the column, the column was washed twice with 15 ml of 3% NaCl, and the washes were combined with the sample. The recovery of nitrate as nitrite was >98%. The pH of each sample was adjusted to 9.5 by the addition of 4 M KOH, and the volume was then reduced to about 1 ml in a rotary evaporator. The nitrite was then converted to N_2 in vacuo by the addition of 2 ml of 100 mM sulfamic acid

TABLE 1. Data describing coastal sediments (0 to 2 cm) used in nitrification and denitrification experiments

Location	Sampling date	Depth (m)	Temp (°C)	Texture	Interstitial waters (μg-atoms of N/liter)			Overlying waters (μg-atoms of N/liter)		
					NO ₃ ⁻	NO ₂ ⁻	NH ₄ ⁺	NO ₃ ⁻	NO ₂ ⁻	NH ₄ ⁺
Mangoku-Ura (38°25'N, 141°24'E)										
Station 7	1 Sept. 1976	4	21	Mud	61.8	0.77	1,090	2.10	0.00	8.7
Station 10	1 Sept. 1976	1	21	Mud	1.6	0.70	400	1.63	0.05	8.7
Station 3	11 Nov. 1976	2	15	Sand	27.8	6.44	845	2.83	0.32	ND ^a
Odawa Bay (35°13'N, 139°36'E)										
	20 Nov. 1976	5	18	Sand	55.4	27.6	810	ND	ND	ND

^a ND, Not determined.

containing 0.15 ml of 6 N H₂SO₄. ²⁹N₂/²⁸N₂ ratios of N₂ were determined with a Hitachi RMU-6 mass spectrometer fitted with a double collector and dual inlet system. Precision of the mass spectrometry was ±0.0004 atomic % in ¹⁵N content. The ¹⁵N content of nitrogen in the sulfamic acid was separately determined, after Kjeldahl digestion and conversion to N₂, by the reaction with KOB₂ (10).

Nitrite is reduced to N₂ with sulfamic acid: NO₂⁻ + NH₂SO₃H → N₂ + H₂SO₄ + OH⁻. Therefore, the ²⁹N₂/²⁸N₂ ratio (*R*) of the N₂ gas produced can be represented by

$$R = \frac{pq' + p'q}{pp'} \quad (1)$$

$$p + q = 1, p' + q' = 1$$

where *p* and *q* are the atomic fractions of ¹⁴N and ¹⁵N in nitrite-N, respectively, and *p'* and *q'* are those in sulfamic acid-N, respectively. The ¹⁵N atomic percentage (*A*) of nitrite can be written as

$$A_{\text{NO}_2^-} = \frac{q}{p + q} \times 100 \quad (2)$$

Thus, we obtain

$$A_{\text{NO}_2^-} = \frac{100 \left(R - \frac{q'}{p'} \right)}{1 + \left(R - \frac{q'}{p'} \right)} \quad (2')$$

The ¹⁵N atomic percentage of nitrate should be the same as *A*_{NO₂⁻}, because nitrate is quantitatively converted to nitrite.

The reproducibility of the present method was examined using standard samples of nitrate with known ¹⁵N content (product of Hikari Kogyo Co., Tokyo). Triplicate determinations yielded almost identical ²⁹N₂/²⁸N₂ ratios, and the obtained values for ¹⁵N content in nitrate coincided within the limit of experimental error with the original values (Table 2).

From the ²⁹N₂/²⁸N₂ ratios determined, the ¹⁵N content in nitrate plus nitrite in experimental samples was

TABLE 2. ¹⁵N content in nitrate determined by the proposed method^a

¹⁵ N atomic % given	Measured	
	²⁹ N ₂ / ²⁸ N ₂	¹⁵ N atomic % ^b
1.820	2.174 × 10 ⁻²	1.775
	2.177 × 10 ⁻²	1.778
	2.177 × 10 ⁻²	1.778
1.095	1.451 × 10 ⁻²	1.072
	1.454 × 10 ⁻²	1.075
	1.454 × 10 ⁻²	1.075
0.730	1.093 × 10 ⁻²	0.720
	1.093 × 10 ⁻²	0.720
	1.093 × 10 ⁻²	0.720

^a Standard ¹⁵N-nitrate (10 μg-atoms of N) dissolved in 20 ml of nitrate- and nitrite-free seawater was reduced to nitrite by a Cu-Cd column (13) and treated with sulfamic acid as described in the text.

^b Calculated from measured ratios and measured ¹⁵N content in sulfamic acid (0.366%), using equation 2'.

calculated using the concentrations of nitrate and nitrite in the samples and carrier nitrate, the ¹⁵N content of carrier nitrate (0.365%), and equation 2'. The ¹⁵N content in nitrite was not measured separately. The combined ¹⁵N content in nitrate plus nitrite will simply be referred to as the ¹⁵N content in nitrate.

The rate of production or reduction of nitrate was estimated from changes with time in the ¹⁵N content and amount of nitrate (see below) and expressed on the basis of sediment dry weight.

Denitrification assay. Experimental procedures for determining ¹⁵N-N₂ production in sediments were similar to those described previously (6). The step involved with replacement of dissolved gases with N₂ was omitted.

Chemical analyses. Nitrate and nitrite were determined with a Technicon Autoanalyzer. The Cu-Cd method (13) for reduction of nitrate to nitrite and the method of Bendschneider and Robinson (1) for nitrite determination were applied with minor modifications. Ammonia was determined manually by the indo-phenol method (12).

RESULTS

Nitrate, nitrite, and ammonia in interstitial waters. Except for the sediment from station 10 of Mangoku-Ura, the nitrate in sediment interstitial water ranged from 30 to 80 μg -atoms of N per liter and ammonia ranged from 400 to 1,000 μg -atoms of N per liter (Table 1). At stations 3 and 7 of Mangoku-Ura, the nitrate in interstitial water was one order of magnitude higher than that in overlying water. The sediments apparently act as a source of nitrate in this water-sediment system. Nitrite concentrations were also high in sandy sediments from station 3 of Mangoku-Ura and Odawa Bay.

Nitrification and nitrate reduction. Figures 1A and 2A show the nitrate and nitrite concentration changes with incubation time and their combined ^{15}N content. During the first 24 h, nitrate decreased almost linearly. During the later period of incubation, however, an increase in nitrate was sometimes observed (Fig. 1A). The ^{15}N content in nitrate remained unchanged during the first 12 to 24 h and then it decreased.

If only nitrification and nitrate reduction are responsible for the nitrate change in sediments, their rates can be calculated by using the following equations:

$$N_2 - N_1 = Z - Y \quad (3)$$

$$N_2 X_2 - N_1 X_1 = Z \bar{X}_a - Y \bar{X} \quad (4)$$

where Y is the consumption of nitrate by bacterial nitrate reduction (microgram-atoms of N per liter); Z is the production of nitrate by nitrification (microgram-atoms of N per liter); N and X are the concentrations of nitrate (microgram-atoms of N per liter) and its ^{15}N content (percent), respectively; and \bar{X} and \bar{X}_a are the average ^{15}N content of nitrate and ammonia between observations t_1 and t_2 , respectively. Subscripts 1 and 2 refer to the observation times.

Averages of duplicate determinations were used for N and X . A value of 0.37% (natural ^{15}N abundance) was assigned for \bar{X}_a , because the amount of natural ammonia present in the sediment samples was very large compared with that of ^{15}N -ammonia possibly produced by reduction of ^{15}N -nitrate. Initial rates of nitrate reduction in two muddy sediments from stations 7 and 10 of Mangoku-Ura were almost identical and were calculated to be 3×10^{-2} μg -atoms of N/g per h on the basis of sediment dry weight (Fig. 1B and 2B). Nitrate reduction proceeded at rates several times greater than those for nitrification. The increase of nitrification observed during the later period of incubation (Fig. 1B and 2B) can be attributed to a rise of redox potential caused by the prolonged exposure of

the sediment to oxygen-rich water. Time courses different from those for muddy sediments were observed for sandy sediments from station 3 of Mangoku-Ura (Fig. 3). Nitrate increased linearly with time, whereas ^{15}N content in nitrate decreased. The rate of nitrification calculated using equations 3 and 4 (4×10^{-3} μg -atoms of N/g per h) was identical to that estimated from the increase in nitrate ($Y = 0$ in equation 3). Nitrate reduction was not detected.

In sandy sediment from Odawa Bay, the ^{15}N content in nitrate also decreased, but the nitrate concentration did not change substantially (Fig. 4B). Nitrate reduction proceeded at a rate of 2×10^{-3} μg -atoms of N/g per h, a value similar to that for nitrification (3×10^{-3} μg -atoms of N/g per h) (Fig. 4B). The simultaneous occurrence of nitrification and nitrate reduction by bacteria in this sediment is thus evident. The rates of nitrate reduction were one order of magnitude

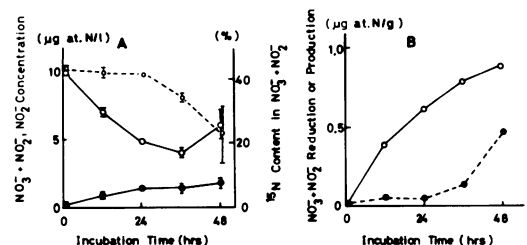


FIG. 1. Nitrate reduction and nitrification in muddy sediments from station 7 of Mangoku-Ura (1 September 1976). (A) Changes with time in nitrate and nitrite (\circ), nitrite (\bullet), and ^{15}N content in nitrate and nitrite (\circ). Averages of duplicate determinations are given, and standard errors are shown by vertical bars. (B) Nitrate reduction (\circ) and nitrate production (\bullet) were calculated using equations 3 and 4 (see text).

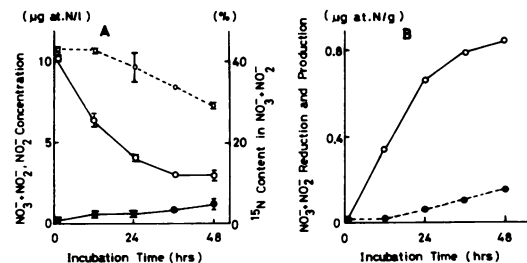


FIG. 2. Nitrate reduction and nitrification in muddy sediments from station 10 of Mangoku-Ura (1 September 1976). (A) Changes with time in nitrate and nitrite (\circ), nitrite (\bullet), and ^{15}N content in nitrate and nitrite (\circ). Averages of duplicate determinations are given, and standard errors are shown by vertical bars. (B) Nitrate reduction (\circ) and nitrate production (\bullet) were calculated using equations 3 and 4 (see text).

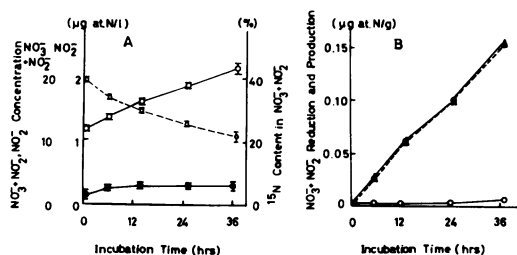


FIG. 3. Nitrate reduction and nitrification in sandy sediments from station 3 of Mangoku-Ura (11 November 1976). (A) Changes with time in nitrate and nitrite (○), nitrite (●), and ^{15}N content in nitrate and nitrite (○). Averages of duplicate determinations are given, and standard errors are shown by vertical bars. (B) Nitrate reduction (○) and nitrate production (●) were calculated using equations 3 and 4, and nitrate production (Δ) was calculated using equation 3 (see text).

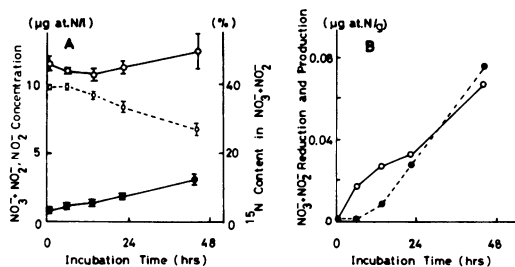


FIG. 4. Nitrate reduction and nitrification in sandy sediments from Odawa Bay (20 November 1976). (A) Changes with time in nitrate and nitrite (○), nitrite (●), and ^{15}N content in nitrate and nitrite (○). Averages of duplicate determinations are given, and standard errors are shown by vertical bars. (B) Nitrate reduction (○) and nitrate production (●) were calculated using equations 3 and 4 (see text).

lower than that observed for the organisms in muddy sediments from Mangoku-Ura.

Nitrate reduction and denitrification. Figure 5 shows the time courses of denitrification, or production of N_2 from nitrate by bacteria, in sediments from stations 3, 7, and 10 of Mangoku-Ura. The average rate of N_2 production during the initial 12 h or less was one-third that of nitrate reduction (Table 3). The results agree well with our previous observations that about 30% of nitrate reduced by bacteria in muddy sediments of Mangoku-Ura is evolved as N_2 and the rest is further reduced to ammonia and organic forms of nitrogen (6). However, the possibility of N_2O and NO evolutions cannot be discounted.

The isotope dilution method used for nitrate reduction measurements is less sensitive by two orders of magnitude than the method used for N_2 production. Therefore, nitrate reduction in

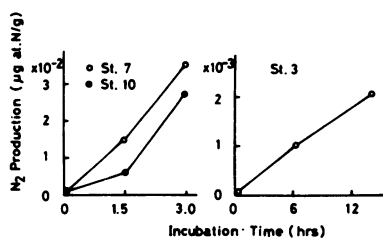


FIG. 5. Time courses of N_2 production from nitrate in Mangoku-Ura sediments. Ten microgram-atoms of N per liter of ^{15}N -nitrate (50% ^{15}N) was used.

TABLE 3. N_2 production and nitrate reduction in Mangoku-Ura surface sediments

Location	Date	$\mu\text{g-atoms of N/g per h}$	
		N_2 production ^a	NO_3^- reduction ^b
Station 7	1 Sept. 1976	1.1×10^{-2}	2.7×10^{-2}
Station 10	1 Sept. 1976	7.6×10^{-3}	2.9×10^{-2}
Station 3	11 Nov. 1976	1.5×10^{-4}	Not detected

^a Obtained by the ^{15}N -tracer method of Koike and Hattori (6).

^b Calculated using equations 3 and 4.

sandy sediments from station 3 of Mangoku-Ura was not detected by the former method, but its occurrence was clearly demonstrated by the latter method (Table 3).

DISCUSSION

On the basis of the features of inorganic nitrogen metabolism, three types of coastal sediments can be distinguished: type 1 sediment, in which nitrate reduction predominates, as represented by the sediments from stations 7 and 10 of Mangoku-Ura; type 2 sediment, in which nitrification predominates, as represented by the sediment from station 3 of Mangoku-Ura; and type 3 sediment, in which bacterial nitrate reduction and nitrification occur simultaneously, as represented by the Odawa Bay sediment.

The texture of sediments seems to be an important factor. In muddy sediments rich in organics, the oxygen supply is probably restricted to the surface of particles in the sediment, as suggested by Jørgensen (4), and this favors anaerobic metabolism, such as denitrification. Sandy sediments, however, favor nitrification. In addition to the high ambient concentration of ammonia, an ample supply of oxygen and the presence of sand particles allow nitrifying bacteria to flourish. The Odawa Bay sediment is also sandy, but it was collected in a *Zostera* bed. The supply of organic detritus from the seagrass and animals associated with it may contribute enough organic matter to maintain reduced microniches in otherwise aerobic sediments. The

^{15}N dilution technique reported in this paper is most effective in distinguishing type 3 sediments.

The concentrations of nitrate and ammonia used in our experimental bottles were several times lower than their in situ concentrations, except for the sediment at station 10 of Mangoku-Ura. Denitrification in coastal sediments has been reported to be half-saturated at 30 to 40 μg -atoms of N per liter of nitrate (7). Assuming a linear relationship between nitrate reduction rate and nitrate concentration, the in situ rates of nitrate reduction were calculated to be 10^{-2} to 10×10^{-2} μg -atoms of N/g per h for the muddy sediments of Mangoku-Ura and 2×10^{-2} μg -atoms of N/g per h for the Odawa Bay sediment. The effect of ammonia concentrations on nitrification occurring in coastal sediments is unknown, but nitrification by *Nitrosomonas* is approximately proportional to ammonia concentrations up to approximately 1 mg-atom of N per liter (8). The in situ rates of nitrification estimated in a similar way are 5×10^{-2} μg -atoms of N/g per h for sandy sediments of Mangoku-Ura and 2×10^{-2} μg -atoms of N/g per h for the Odawa Bay sediments. These values fall in the same range as those observed by Billen (2) using coastal sandy sediments of the North Sea and an N-Serve technique.

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