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Annual Pattern of Denitrification and Nitrate Ammonification in Estuarine Sediment

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The seasonal variation and depth distribution of the capacity for denitrification and dissimilatory NO_3^- reduction to NH_4^+ (NO_3^- ammonification) were studied in the upper 4 cm of the sediment of Norsminde Fjord estuary, Denmark. A combination of C_2H_2 inhibition and ^{15}N isotope techniques was used in intact sediment cores in short-term incubations (maximum, 4 h). The denitrification capacity exhibited two maxima, one in the spring and one in the fall, whereas the capacity for NO_3^- ammonification was maximal in the late summer, when sediments were progressively reduced. The denitrification capacity was always highest in the uppermost 1 cm of the sediment and declined with depth. The NO_3^- ammonification was usually higher with depth, but the maximum activity in late summer was observed within the upper 1 cm. The capacity for NO_3^- incorporation into organic material was investigated on two occasions in intact sediment cores and accounted for less than 5% of the total NO_3^- reduction. Denitrification accounted for between 13 and 51% of the total NO_3^- reduction, and NH_4^+ production accounted for between 4 and 21%, depending on initial rates during the time courses. Changes of the rates during the incubation were observed in the late summer, which reflected synthesis of denitrifying enzymes. This time lag was eliminated in experiments with mixed sediment because of preincubation with NO_3^- and alterations of the near-environmental conditions. The initial rates obtained in intact sediment cores therefore reflect the preexisting enzyme content of the sediment.

Three pathways of microbial NO_3^- reduction are generally recognized as being important in marine sediments, denitrification, NO_3^- ammonification (dissimilatory NO_3^- reduction to NH_4^+), and NO_3^- assimilation. Most information on the in situ activity and capacity for these processes is from widely different localities (8, 16, 30, 35); few studies have determined the three reduction routes simultaneously in the same sediment (21). Capacities are defined in this paper as the total preexisting content of NO_3^--reducing enzymes in the sediment, i.e., the maximum activity when NO_3^- is added to the sediment in nonlimiting concentrations.

Denitrification capacity has been reported to account for between 38 and 95% of the total NO_3^- reduction in marine sediments (16, 22). NO_3^- ammonification has accounted for from 2 to 52% (11, 22), and NO_3^- assimilation has accounted for from 4 to 33% (22). Assimilatory NO_3^- reduction to NH_4^+ should not be expected to be very important, since high NH_4^+ concentrations found in organic-rich coastal sediments should inhibit NO_3^- assimilation (4, 7).

It is well known that denitrifying capacity often is present in sediment layers below the NO_3^--containing surface zone (17, 30). This activity has been attributed to organisms having a fermentative metabolism or to organisms that use alternative electron acceptors (17). Furthermore, it has been observed that certain sulfate-reducing bacteria are able to reduce NO_3^- to NH_4^+ in a respiratory pathway (19, 27). In sediments, where the sulfate-reducing bacteria are localized either within or immediately below the NO_3^--containing zone, their role in NO_3^- reduction could be significant.

A number of physicochemical parameters may thus determine the distribution of denitrifying and NO_3^--ammonifying bacteria in estuarine sediment. MacFarlane and Herbert (24) enumerated both denitrifiers and NO_3^- ammonifiers by the most-probable-number method. During an annual cycle, NO_3^- ammonifiers were predominant, but there were almost no seasonal variations in the cell numbers of either group of bacteria. A different result was obtained in experiments with suspended-sediment samples that were amended with $^{15}NO_3^-$; the capacity for denitrification was higher than for NO_3^- ammonification, and both processes were at a maximum during the summer, indicating that cell number does not reflect the actual capacity of the population. High NO_3^- concentration favors denitrification relative to NO_3^- ammonification (21, 31), but the partitioning between two pathways may also be controlled by the oxidation level of the electron donor (25).

The present study investigated the significance and seasonal variation of the capacity for denitrification and NO_3^- ammonification in a relatively reduced sediment in which NO_3^- is depleted in summer. The study included detailed time courses for product formation both in intact cores and in mixed sediment. Since no specific technique for measuring NO_3^- ammonification at in situ levels of NO_3^- is readily applicable to this system, we studied the capacity and control of the process to examine its importance in this sediment.

MATERIALS AND METHODS

Location and sampling. The sampling site was in the shallow, freshwater-dominated part of the Norsminde Fjord estuary, Denmark. The sediment was a soft mud having an oxidized surface zone extending to 0.5 cm during summer and to 3 cm in depth during winter. This corresponded approximately to the depth of NO_3^- penetration. External input of NO_3^- from the tributary river exceeded the NO_3^- production from nitrification and gave a steep NO_3^- gradient in the sediment throughout the year. A maximum concentration of about 300 μM NO_3^- was recorded in the upper 0.5 cm in January, compared with a minimum concentration of about 10 μM in September. A more detailed description of the locality was given previously (14, 15).

Intact sediment cores were collected at monthly intervals from February 1985 to February 1987. The cores were

immediately brought to the laboratory and stored overnight in air-saturated water from the site.

Experiments with intact sediment cores. (i) Incubation. The partitioning of NO_3^- between denitrification and NO_3^- ammonification was investigated in intact cores from February 1985 to March 1986. In the final phase of the study, during September 1986 and February 1987, measurements of NO_3^- assimilation were included. The sediment was collected in Plexiglas (Rohm & Haas Co., Philadelphia, Pa.) cores (3.6 cm wide and 15 cm long) equipped with a vertical series of silicone-filled holes at 0.5-cm intervals. Within these tubes, the sediment cores could be adjusted to give an overlying water phase of 3 cm (30 ml) and a gas phase of 3 cm (30 ml). A combination of the C_2H_2-blockage technique to measure denitrification (36) and a ^{15}N isotope tracer technique to measure NO_3^- ammonification and NO_3^- assimilation was used. A 200-μl portion of a C_2H_2-saturated solution containing 12.5 mM $Na^{15}NO_3$ (99.8 atom%; B.O.C. Limited, London, England) and 12.5 mM NH_4Cl was injected into the sediment through each of the holes. The water phase received 1.2 ml of the solution. This gave a final concentration of about 700 μM NO_3^- in excess of ambient NO_3^-. The final specific activity of ^{15}N was between 50 and 99.8% in the cores, depending on the endogenous pool of NO_3^-. The actual specific activities for all depths were taken into account in the calculations.The cores were capped with gas-tight caps with small magnets mounted inside. Three milliliters of the gas phase were replaced by pure C_2H_2 gas, and the final C_2H_2 concentration was about 10% by volume (10 kPa of C_2H_2) in all phases.

Each incubation series comprised eight cores incubated at in situ temperature and with stirring of the water phase. Parallel cores were incubated for 0, 1, 2, and 4 h, respectively, except in September 1986 and February 1987, when the cores were sacrificed more frequently to obtain a more detailed time course for the incubation.

(ii) Extraction and analysis. Before a core was sacrificed, a gas sample (0.2 to 3.0 ml) was taken from the gas phase and transferred to an evacuated glass vial (Venoject; Terumo Europe N.V., Leuven, Belgium) and a water sample (10 ml) was collected in a 60-ml glass beaker. The sediment was cut into 0.5-cm fractions (upper 1 cm) or 1-cm fractions (1 to 4 cm depth). The samples were quickly transferred to wide-mouthed beakers containing 10 ml of 2 N KCl. All beakers were closed immediately, and N_2O was extracted by vigorous shaking for 2 min, after which gas samples were transferred to Venoject vials for later analysis of N_2O. The analyses were performed on a gas chromatograph (model 427; Packard) equipped with a ^{63}Ni electron capture detector, and operating conditions were as described by Jørgensen and Sørensen (15).

Extraction of NO_3^-, NO_2^-, and NH_4^+ into the KCl solution was continued for 10 min and was followed by centrifugation at 10,000 × *g* for 10 min; the supernatants were frozen for later analysis. The NO_3^-, NO_2^-, and NH_4^+ concentrations were determined by the colorimetric assays of Armstrong et al. (3) and Solorzano (34) by using a Chemlab Autoanalyzer. The atom% of ^{15}N on the NH_4^+ pool was determined by the microdiffusion procedure of Blackburn (5), using a Statron NOI 5 ^{15}N analyzer. About 0.5 cm^3 of the pellet from the centrifugation described above was used for determination of the ^{15}N content in the particulate nitrogen pool. This pellet was washed twice in 10 ml of 2 N KCl and three times in 10 ml of artificial seawater. The sediment was then dried at 55°C for 48 h and homogenized in a mortar. The ^{15}N content was measured with a Carlo Erba model NA 1500 Nitrogen Analyzer connected to a mass spectrometer (Isogas, Middlewich, England).

(iii) Calculations. The solubility coefficient for N_2O (26, 39) was used to calculate gas production in the sediment. Products of NO_3^- reduction recovered in the gas and water phases were assumed to originate from the upper 0.5 cm of sediment (2). Initial rates of N_2O and NH_4^+ accumulations, recorded during the first hour (or longer when production was linear) after NO_3^- application, were taken to represent the denitrification and NO_3^- ammonification capacities (maximum rates), respectively. Since C_2H_2 also inhibits the nitrification (13), the total NO_3^- reduction rate was estimated from the initial decrease in the NO_3^- concentration (14). The rate of incorporation of ^{15}N into the particulate nitrogen pool was taken to represent the rate of NO_3^- assimilation into organic matter.

All rates presented are the mean of two rates obtained from two parallel incubation series of four core samples each, and the standard deviation ranged between 2 and 42% for the NO_3^- reduction, between 3 and 52% for the denitrification, and between 5 and 25% for both the NH_4^+ production and ^{15}N incorporation in this sediment.

Rates per area (areal rates) were calculated by integration of the rates obtained at each depth from 0 to 4 cm.

Experiments with mixed sediment. (i) Preincubation. For experiments with mixed sediment, only the 0- to 2-cm fraction of the cores (5.6 cm wide and 15 cm long) was used. The sediment from eight cores (about 400 cm^3) was passed through a 2-mm sieve and was simultaneously diluted with O_2-free estuarine water which contained about 50 μM NO_3^-. The suspension was allowed to settle at 12°C overnight, after which the NO_3^- pool was completely exhausted.

(ii) Incubation. Fifteen-milliliter portions of the mixed sediment (water content of 54%, as in situ) were transferred to 20 24-ml serum vials by using a disposable syringe with a cut-off end. The flasks were immediately capped with butyl stoppers and purged with N_2 for 5 min. A 3-ml sample of C_2H_2 gas was then injected to the gas phase, and the flasks were vortexed for 2 min. This allowed about 1.5 ml of the C_2H_2 gas to dissolve, and the remaining overpressure was released. The final concentration of C_2H_2 was about 15% (vol/vol) (15 kPa of C_2H_2) in the pore water. Incubations were started by addition of 1 ml of O_2-free, artificial seawater containing 7.5 mM $Na^{15}NO_3$ (99.8 atom%) and 7.5 mM NH_4Cl. This gave final concentrations in the pore water of 700 μM for NO_3^- and 1,000 μM for NH_4^+. The flasks were vortexed for 1 min and incubated in a water bath maintained at 12°C.

Gas samples (50 μl) were taken at 15-min intervals from the headspaces of two duplicate flasks and transferred to Venoject vials for later analysis of N_2O.

The remaining 18 flasks were used for extraction of NO_3^-, NO_2^-, and NH_4^+ during the incubation and were subsequently sacrificed. The sediment was quantitatively transferred to 60-ml beakers by three 5-ml portions of 2 N KCl, and the extraction procedure was as described above.

The standard deviations of the mean values obtained for mixed sediment were less than 5% for all rates.

RESULTS

Seasonal and temporal distributions for denitrification and NO_3^- ammonification capacities are shown in Fig. 1. Maximum rates of denitrification were recorded from March to August, with a smaller peak of activity occurring from October to December. Except for the measurement in September, the activity was highest in the uppermost 0.5 cm of

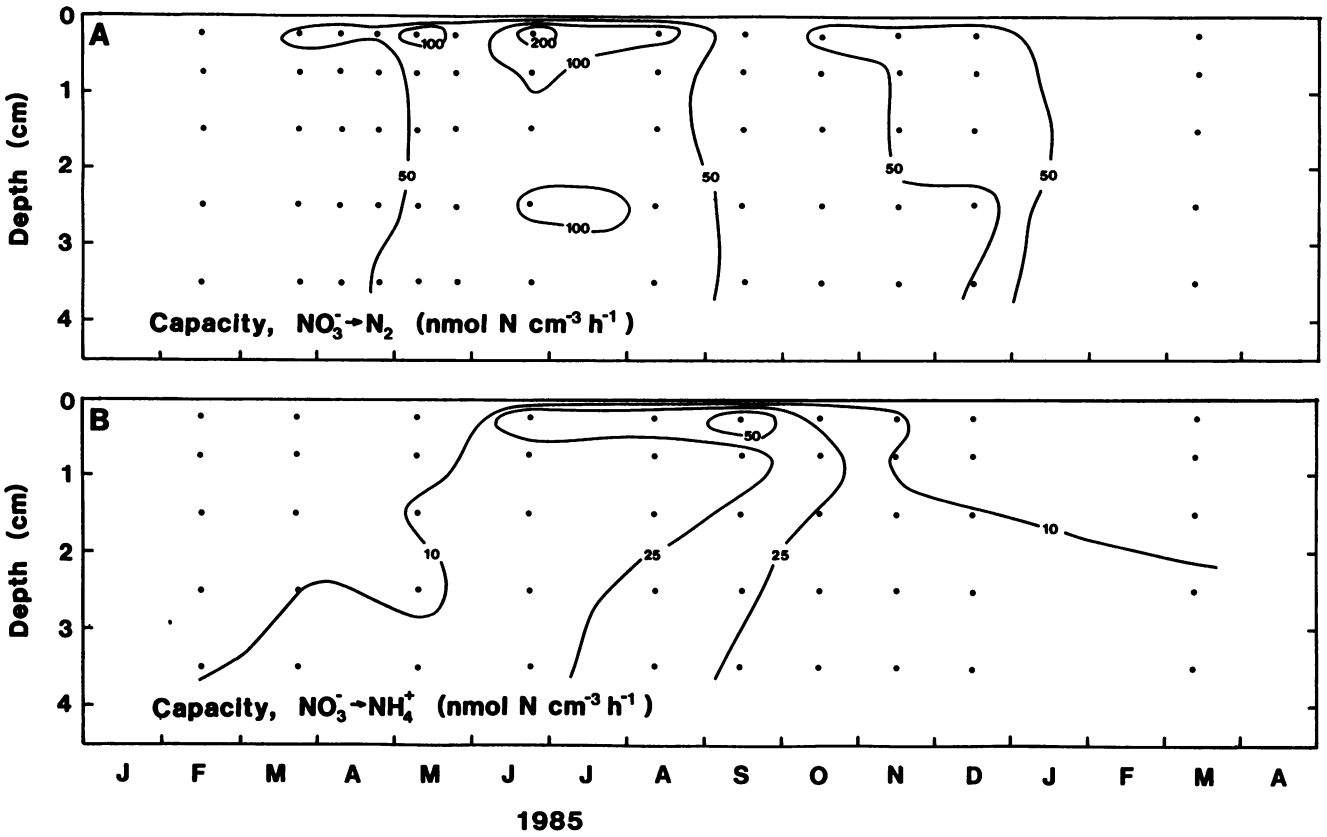

FIG. 1. Seasonal variation and depth distribution of capacity for denitrification (A) and NO_3^- ammonification (B) at in situ temperature in the sediment of Norsminde Fjord. Months are indicated on the x axis.

the sediment, but the layers below the natural denitrification zone (15) exhibited very high capacity as well. An absolute maximum rate of about 500 nmol of N cm^{-3} h^{-1} was measured in June. The capacity for NO_3^- ammonification was generally most significant in the deeper layers. However, activity was found closer to the surface during the summer; an absolute maximum rate of about 60 nmol of N cm^{-3} h^{-1} was detected in the upper 0.5 cm in September. The capacity for NO_3^- ammonification is thus high when the denitrification activity is at minimum during late summer. This coincided with a period of complete anoxia and relatively reduced conditions in the sediment.

The seasonal variation of in situ temperature and areal capacities for total NO_3^- reduction, denitrification, and NO_3^- ammonification are shown in Fig. 2. NO_3^- reduction was at maximum in June and was lowest in February, when the rates were about 250 mmol of N m^{-2} day^{-1} and 30 mmol of N m^{-2} day^{-1}, respectively. The annual pattern, with two maxima for denitrification capacity and with a predominance of NO_3^- ammonification in the intermittent period, is evident.

Detailed time courses for the incubations of intact sediment cores in September 1986 and February 1987 are shown in Fig. 3. The patterns for product formation were different on these two occasions. In September (Fig. 3A), the initial rate of $^{15}NH_4^+$ production (80 nmol of N cm^{-3} h^{-1}) exceeded that of N_2O production (30 nmol of N cm^{-3} h^{-1}). However, after about 1 h of incubation, denitrification increased and became the predominant process. At the same time, the NH_4^+ production decreased, although NO_3^- was still present. Increasing denitrification after a time lag was observed at all depths, although Fig. 3 only shows a cumulated result for the whole 0- to 4-cm surface zone. The same pattern of product formation was observed in both September 1984 and September 1985 (data not shown). The rate of ^{15}N incorporation into organic matter paralleled the NH_4^+ production and was about 11 nmol of N cm^{-3} h^{-1}. In February (Fig. 3B), both N_2O and $^{15}NH_4^+$ production was initially linear (96 and 20 nmol of N cm^{-3} h^{-1} respectively) and decreased only slightly with time. The overall rate of NO_3^- reduction also decreased with time. The rate of incorporation of ^{15}N into organic matter was detectable but very low.

Results from experiments with mixed sediment are shown in Fig. 4 for September 1986 and February 1987. Patterns of N_2O, $^{15}NH_4^+$, and organic ^{15}N production were similar on the two occasions; N_2O production was linear and exhibited no shifts, and NH_4^+ production and incorporation into organic matter were both slightly curvilinear and stopped after approximately 1 h of incubation. The rate of N_2O production was 109 nmol of N cm^{-3} h^{-1} in September and 265 nmol of N cm^{-3} h^{-1} in February, and the rates for NH_4^+ production were 12 and 40 nmol of N cm^{-3} h^{-1}, respectively, for the two months. The NO_3^- reduction was constant until the NO_3^- concentration was about 50 μM in the sediment.

To evaluate the recovery of the added NO_3^- in intact cores versus that in mixed sediment at different occasions, the initial rates for NO_3^- reduction and production of N_2O, $^{15}NH_4^+$, and organic ^{15}N (from Fig. 3 and 4) are compared in Table 1. In intact sediment, denitrification accounted for

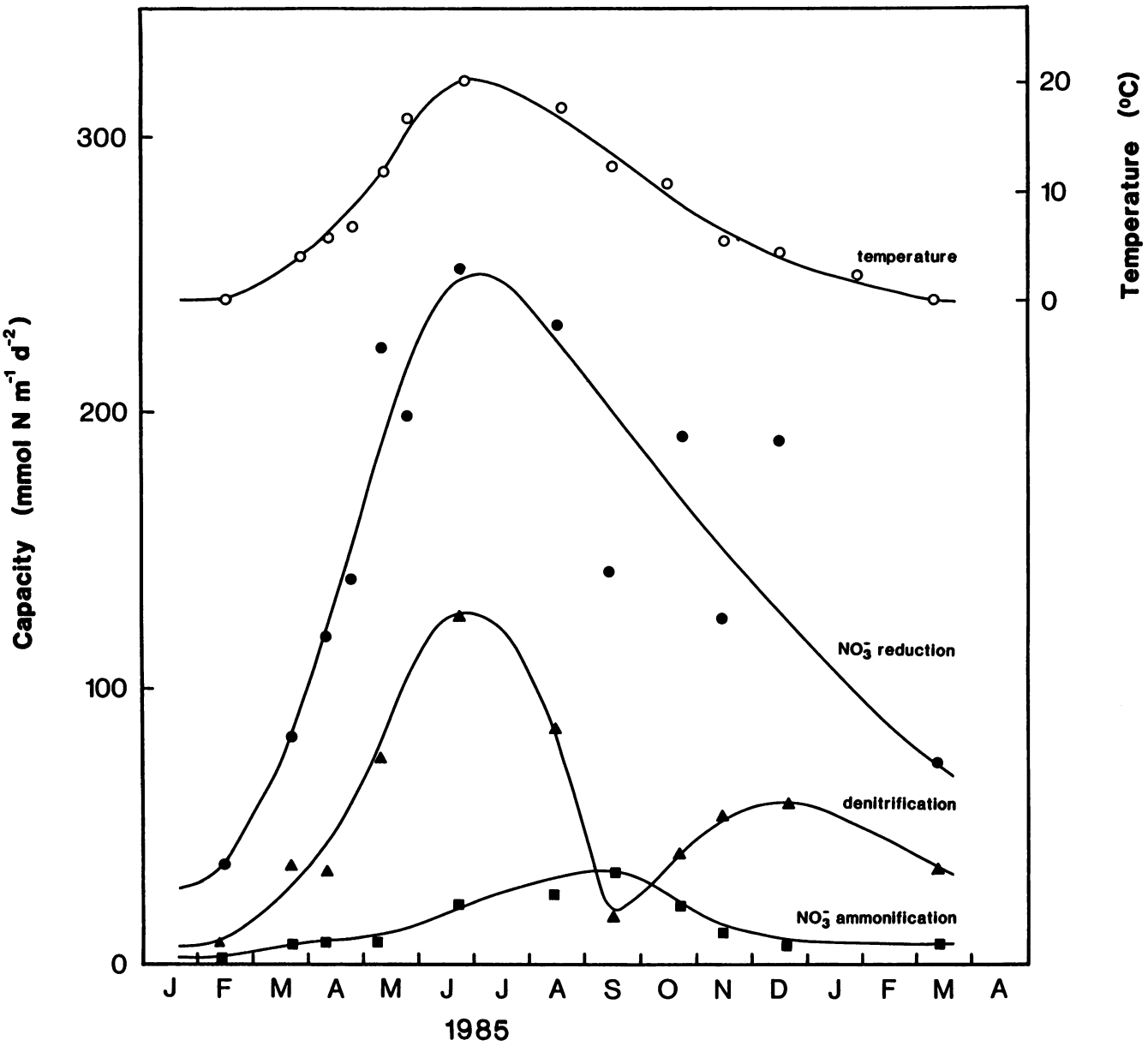

FIG. 2. Seasonal variation of the temperature and areal rates of capacity for total NO_3^- reduction, denitrification, and NO_3^- ammonification at in situ temperature in Norsminde Fjord. Months are indicated on the *x* axis.

between 11 and 27% and NO_3^- ammonification accounted for between 8 and 29% of total NO_3^- consumption. In mixed sediment, the recovery of NO_3^- as a gas was higher (between 30 and 80%), whereas the recovery of NO_3^- as NH_4^+ was lower (between 4 and 11%). In all cases the incorporation into organic matter was less than 4%. The NO_3^- disappearance accounted for was thus between 43 and 47% in intact cores and between 44 and 94% in mixed sediment.

DISCUSSION

NO_3^- reduction capacity in the intact cores was measured at in situ temperature; thus, the maximum in the summer (Fig. 2) may primarily reflect a regulation by temperature. The seasonal pattern showed that the capacity for denitrification exceeded that for NO_3^- ammonification throughout most of the year. An exception was the period in the late summer when the sediment was reduced all the way to the surface and a maximum of NO_3^- ammonification was found in the surface layer. Otherwise, the NO_3^- ammonification was generally restricted to the deeper layers of the sediment, in accordance with earlier results (11, 16, 35). The temporary decrease in denitrification capacity and the greater significance of NO_3^- ammonification in late summer were particularly interesting. Because of lower NO_3^- availability and increasing SO_4^{2-} reduction in the summer (14), the surface sediment is more reduced at this time of the year. Buresh and Patrick (8) and King and Nedwell (21) reported that low redox conditions and low NO_3^- concentrations stimulated the NO_3^- ammonification at the expense of the denitrification in estuarine sediment. Reduced conditions may favor

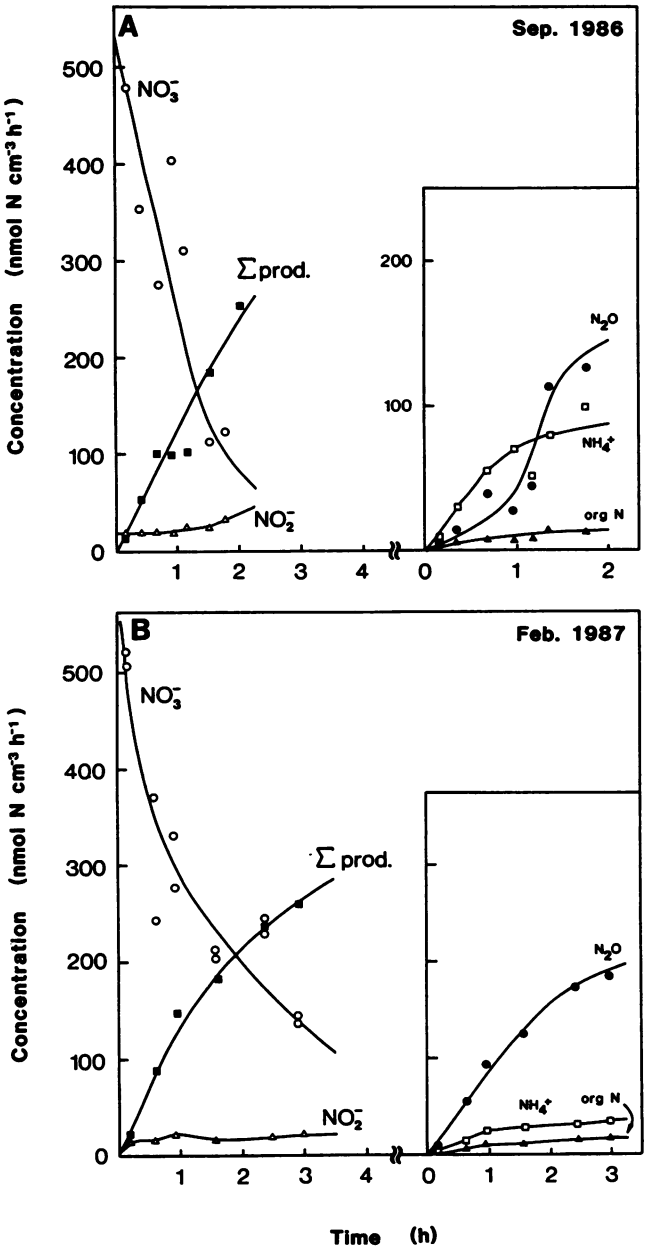

FIG. 3. Time course for the C_2H_2 incubation of intact sediment cores (0 to 4 cm) with 500 nmol of $^{15}NO_3^-$ cm^{-3} (700 μM) in September 1986 (A) and February 1987 (B) at in situ temperature. The sums of N_2O, $^{15}NH_4^+$, and organic ^{15}N (org N) as shown in the insets constitute the sums of products (Σ prod.).

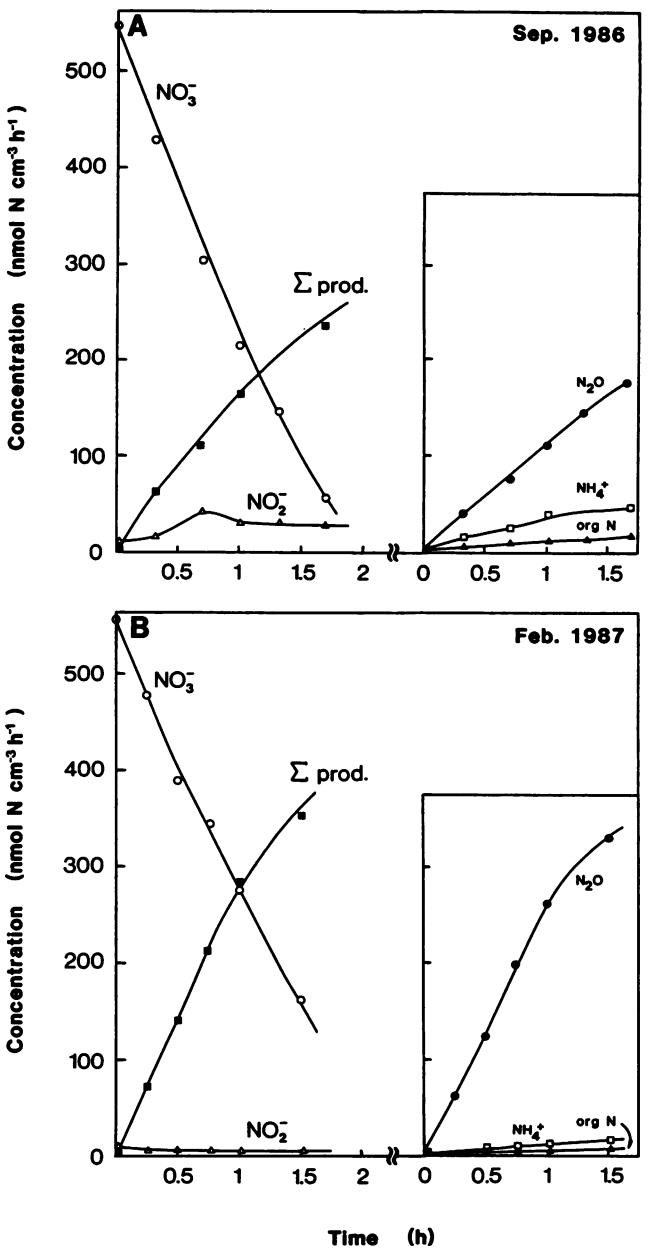

FIG. 4. Time courses for the anaerobic C_2H_2 incubation of mixed sediment (0- to 2-cm fraction) with 500 nmol of $^{15}NO_3^-$ cm^{-3} (700 μM) in September 1986 (A) and February 1987 (B) at 12°C. The sums of N_2O, $^{15}NH_4^+$, and organic ^{15}N (org N) as shown in the insets constitute the sums of products (Σ prod.).

NO_3^- ammonifiers because (i) denitrification is inhibited in the presence of free sulfide (1, 12, 29), (ii) NO_3^- ammonifiers have a more versatile metabolism and hence may grow under NO_3^--free conditions (9, 19, 20, 27), and (iii) NH_4^+ producers may possess constitutive enzymes for NO_3^- reduction (10, 28) which could be favorable where NO_3^- availability is highly variable.

When reduced sediment receives a high concentration of NO_3^-, the redox potential should increase because of the oxidizing capacity of NO_3^-. A stimulation of denitrification may thus be expected after the NO_3^- applications used here. This should be elucidated in the detailed time course of the incubations (Fig. 2). The time course from February (Fig. 3B and 4B), with ultimate production of both N_2O and NH_4^+, was representative for all occasions during the year except for the summer (September), when a shift in the activity was shown.

The immediate production of NH_4^+ after addition of NO_3^- suggested that the NO_3^--ammonifying bacteria had constitutive enzymes, since no NO_3^- was present in situ. By comparison, the initial time lag for gas production suggested that further capacity for denitrification could be induced by addition of NO_3^-. The initial phase of denitrification corresponds to the phase I described by Smith and Tiedje (33) for

TABLE 1. Rates of NO_3^- reduction and product formation in Norsminde Fjord sediments after amendment with $^{15}NO_3^-$ in intact sediment cores and mixed sediment

Sampling date	Material	Incubation temperature (°C)	NO_3^- reduction (nmol of N cm^{-3} h^{-1} [% of total]) resulting in:				
			Total	N_2O production	NH_4^+ production	Organic N production	NO_3^- not accounted for
September 1986	Intact sediment (0–4 cm)	13	280	30 (11)	80 (29)	11 (4)	159 (57)
February 1987	Intact sediment (0–4 cm)	4	265	96 (37)	20 (8)	9 (3)	159 (53)
September 1986	Mixed sediment (0–4 cm)	12	365	109 (30)	40 (11)	12 (3)	204 (56)
February 1987	Mixed sediment (0–2 cm)	12	300	265 (88)	12 (4)	6 (2)	17 (6)

soils. They argued that the first period of anaerobic incubation of soil samples reflected the preexisting denitrifying enzyme content, and they showed that the synthesis of denitrifying enzymes was fully derepressed (phase II) after a certain period. In this case, the sediment was anaerobic at the time of NO_3^- application and contained NO_3^- only at very low concentrations in the upper 1 cm, and the application of NO_3^- may have derepressed enzyme synthesis. This was further confirmed by the results from the experiments with mixed sediment (Fig. 4). Here, no time lag for N_2O production was observed on either occasion. In these experiments the enzymes were probably fully derepressed (phase II) because of preincubation with NO_3^-. The initial denitrification rate in September exceeded that of NO_3^- ammonification, in contrast to the results obtained with intact sediment. Thus, denitrification rates may be overestimated and NO_3^- ammonification rates may be underestimated in mixed sediments because of treatment of the sediment and preincubation with NO_3^-.

The NO_3^- ammonifiers seemed to decrease their activity in both intact and mixed sediment when the NO_3^- concentration decreased to about 200 nmol of N cm^{-3} (240 μM). An explanation for this may be that NO_3^- ammonifiers usually exhibit higher K_m values (100 to 500 μM NO_3^-) than denitrifiers (5 to 10 μM NO_3^-) and thus may not compete as well at lower NO_3^- levels (38).

Incorporation of nitrogen into the organic fraction accounted for less than 5% of total NO_3^- reduction (Table 1) in both experiments with intact sediment and experiments with mixed sediment. Furthermore, the incorporation was immediate, in contrast to the results of Smith et al. (32), who argued that for long-term incubations NO_3^- was first dissimilated to NH_4^+ and was then assimilated in the NH_4^+ form. The present work demonstrated a small but direct NO_3^- assimilation even at high NH_4^+ concentrations. Koike and Hattori (22) also found direct NO_3^- assimilation in short-term experiments, up to 33% of the total NO_3^- reduction. NO_3^- assimilation should generally be inhibited by NH_4^+ at both the level of uptake and the reduction to NH_4^+ in the cells (4). However, for dissimilatory purposes the NO_3^- uptake has been suggested to be a facilitated diffusion without an inhibitory effect by NH_4^+ (23). A possible explanation for the observed NO_3^- assimilation in the sediments may thus be an incorporation from an internal NH_4^+ pool in the cells, newly generated from an energy-yielding, dissimilatory NO_3^- reduction to NH_4^+.

Even when NO_3^- assimilation into particulate matter was included, the recovery of added NO_3^- was generally incomplete. In intact sediment the recovery was only between 43 and 50%, while that in the mixed sediment was between 50 and 95% (Table 1). The facts that the C_2H_2 blockage technique can be less than 100% effective at low NO_3^- concentrations (18, 30) and that low concentrations of sulfide can alleviate the C_2H_2 inhibition (37) may indicate that gas production was underestimated. It was, however, recently found that only up to 10% of $^{15}NO_3^-$ added to Norsminde Fjord sediment ended up as $^{15}N_2$ in the presence of C_2H_2 (unpublished data). The lack of efficacy of C_2H_2 is therefore not a major reason for the low recovery in this sediment. Other losses of ^{15}N may be important, e.g., fixation of $^{15}NH_4^+$ into the nonexchangeable NH_4^+ pool in silicate lattices in mineral particles (6), ^{15}NO production, and incorporation into dissolved organics (free amino acids, etc.) or extractable cell components. The simultaneous measurement of total NO_3^- reduction, denitrification, and NO_3^- ammonification presented here shows that estimation of one of the rates by subtraction of the other from the total NO_3^- reduction may overestimate the rate.

In conclusion, this work has contributed new information on the ecological significance and seasonal variation of the two dissimilatory pathways of NO_3^- reduction. The initial rates of denitrification and NO_3^- ammonification (phase I) provided the most useful information concerning in situ enzyme content; incubation and manipulation of the sediment altered the rates. The capacity for NO_3^- reduction to NH_4^+ was higher than for denitrification in the reduced sediment found in late summer. The in situ denitrification also has been shown to exhibit a minimum in the late summer (15), suggesting that in situ NO_3^- ammonification may be an important process at this time of the year.

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