Capacity for Denitrification and Reduction of Nitrate to Ammonia in a Coastal Marine Sediment

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The capacity for dissimilatory reduction of NO\textsubscript{3}\textsuperscript{−} to N\textsubscript{2} (N\textsubscript{2}O) and NH\textsubscript{4}\textsuperscript{+} was measured in \textsuperscript{15}NO\textsubscript{3}\textsuperscript{−}-amended marine sediment. Incubation with acetylene (7 × 10\textsuperscript{−3} atmospheres [normal]) caused accumulation of N\textsubscript{2}O in the sediment. The rate of N\textsubscript{2}O production equaled the rate of N\textsubscript{2} production in samples without acetylene. Complete inhibition of the reduction of N\textsubscript{2}O to N\textsubscript{2} suggests that the “acetylene blockage technique” is applicable to assays for denitrification in marine sediments. The capacity for reduction of NO\textsubscript{3}\textsuperscript{−} by denitrification decreased rapidly with depth in the sediment, whereas the capacity for reduction of NO\textsubscript{3}\textsuperscript{−} to NH\textsubscript{4}\textsuperscript{+} was significant also in deeper layers. The data suggested that the latter process may be equally as significant as denitrification in the turnover of NO\textsubscript{3}\textsuperscript{−} in marine sediments.

The reduction of NO\textsubscript{3}\textsuperscript{−} to N\textsubscript{2} and to NH\textsubscript{4}\textsuperscript{+} is mediated by bacteria which are able to perform one or both of these reductive pathways.

In anoxic environments, the respiratory reduction of NO\textsubscript{3}\textsuperscript{−} to N\textsubscript{2} is an alternative to O\textsubscript{2} respiration for the denitrifying bacteria. In rich organic sediments, e.g., those of many marine coastal areas, these conditions restrict the occurrence of denitrification to a zone immediately below the oxidized surface and reduced microniches within the surface zone.

The other pathway, the reduction of NO\textsubscript{3}\textsuperscript{−} to NH\textsubscript{4}\textsuperscript{+}, is a nutritional supplement for many bacteria if reduced nitrogen for assimilation is in short supply, but coastal marine sediments are most often rich in NH\textsubscript{4}\textsuperscript{+}, and the assimilatory demands are readily met.

This reduction may be entirely dissimilatory, however, where the reduced product is released, and the process may be linked to energy production (8). Thus, it is likely that the dissimilatory reduction of NO\textsubscript{3} to NH\textsubscript{4} actually does occur to a significant extent in NH\textsubscript{4}-rich sediments, but to the author’s knowledge, this NH\textsubscript{4}-producing pathway has never been quantified in marine sediments.

The capacity of a marine sediment for NO\textsubscript{3}− reduction by the two pathways was measured in the present study, in which sediment samples were incubated anaerobically with \textsuperscript{15}N-labeled NO\textsubscript{3}− in closed bottles, and the production of labeled N\textsubscript{2} (N\textsubscript{2}O) and NH\textsubscript{4}+ was followed.

The denitrification capacity was measured by the \textsuperscript{15}N\textsubscript{2} gas production in an assay where emission spectrophotometry was applied. Alternatively, denitrification was also estimated by the N\textsubscript{2}O production in other sediment samples where the “acetylene blockage technique” was applied. This technique was based on the findings of Fedorova et al. (5), who noted that the ultimate reduction of N\textsubscript{2}O to N\textsubscript{2} in the denitrification pathway could be blocked by C\textsubscript{2}H\textsubscript{2}, resulting in accumulation of N\textsubscript{2}O. The “acetylene blockage technique” was applied in culture studies by Balderston et al. (1) and Yoshinari and Knowles (21) and in soils by Yoshinari et al. (20). Their results suggested that it is a useful method for the measurement of denitrification activity, since N\textsubscript{2}O production could be easily assayed by gas chromatography (2, 9). The applicability of the acetylene blockage technique in marine sediments was tested in the present study, in which the technique was compared to a \textsuperscript{15}N\textsubscript{2} production assay.

The measured reduction capacities should provide information about the possible occurrence of the two reductive pathways in situ. The depth distribution of the reduction capacities in the sediment was recorded to get information about the types of bacteria involved.

MATERIALS AND METHODS

Sediment samples. Sediment samples were taken with a "Haps" corer (14) in April, 1977, at a location in the Limfjorden, Denmark, corresponding to station no. 5 of Jørgensen (12). The water depth was 10 m, and the bottom water temperature was 5 to 7°C. At this site, the salinity was 26%. Subcores 15 cm in length were taken from the Haps core with 3.5-cm-wide Plexiglas tubes and stored in the laboratory at in situ temperature in the dark. Experiments were initiated within the following 3 days.

Incubations. Immediately before the incubations
were made, the redox potential profile was recorded in a sediment core. A platinum electrode was pushed stepwise into the sediment from above, and the redox potential was read after 1 min. Sediment density and porosity were determined by weighing known volumes of sediment, which were then dried to constant weight at 110°C.

A whole core was mixed for experiment A, which was a control experiment in which the assays for \( ^{15}N_2 \) and \( N_2O \) production by denitrification were compared. The upper 12 cm of other cores was cut into four 3-cm segments for experiment B, in which \( N_2O \) and \( NH_4^+ \) production rates were followed. All segments were mixed thoroughly under \( N_2 \) in beakers before portions of 5 g were transferred into four series of 15-ml serum bottles. Each bottle was then purged extensively with \( O_2 \)-free \( N_2 \) and finally stopped with a greased (Ramsay vacuum grease) butyl rubber cap.

Pure \( C_2H_2 \) was injected in 100-µl quantities into each of the bottles which were assayed for \( N_2O \) production in the two experiments. The injected volume gave about 0.7% \( C_2H_2 \) in the gas phase of the bottles. Acetylene was omitted from the bottles for the \( ^{15}N_2 \) production assay in experiment A.

Finally, 100 µl of an \( Na^{14}NO_3 \) (96.3% \( ^{14}N \); VEB, Berlin) solution was injected, giving about 1.5 µmol of \( NO_3^- \)/cm³ in the bottles, and thoroughly mixed into the sediment on a Vortex mixer before the bottles were incubated in the dark at 5°C.

The unlabeled products of reduction of native \( NO_3^- \) were neglected, since native \( NO_3^- \) in the upper segments was less than 10% of the added \( ^{14}NO_3^- \). Thus, the \( NO_3^- \) pool and the pools of the intermediates \( NO_2^- \), \( NO_3^- \), and \( N_2O \) in denitrification were considered to be fully \( ^{15}N \)-labeled, and no attempt was made to discriminate between \( ^{14}N \) and \( ^{15}N \) in the assays for these compounds. Only for the production of \( N_2 \) and \( NH_4^+ \) was a discrimination necessary, since the native pools were high.

Gas analysis. A new bottle was used for each gas analysis to avoid multiple penetration of the rubber cap. The tightness of the caps was tested, and the loss of \( C_2H_2 \) from 15-ml, \( N_2 \)-purged bottles without sediment was only 2.5%/day.

In experiment A, the rate of denitrification was eventually measured by the rate of \( ^{15}N_2 \) production in that series of bottles where \( C_2H_2 \) was omitted. Using a Pressure-Lok gas-tight syringe, a 15-µl gas sample was taken from each bottle for \( ^{15}N \) analysis in a Statron NOI 4B 4 \(^{15}N \) analyzer. The needle of the locked syringe was inserted through a rubber septum (Hamilton, red) into a short side arm emerging from a three-way stopcock which also joined a vacuum line and the discharge tube in the analyzer. Evacuation of the system for 5 min removed the previous gas sample and excluded air contamination. The vacuum was better than 13.3 Pa when the vacuum line was closed off. Finally the syringe was unlocked, and the gas sample was injected and analyzed. Water vapor was removed by 0.5 g of "Drierite" (6 mesh) which was placed between two cotton plugs in the injection arm. The injected gas volume of 15 µl was found to give optimal intensity of the discharge. The minimal detectable \( ^{15}N \) enrichment in the gas samples was 0.2%. The measured \( ^{15}N \) abundance in pure \( N_2 \) from a cylinder was used as a standard of reference and subtracted from the values measured in the incubated bottles.

In the same experiment, this assay for \( ^{15}N_2 \) production was compared to the acetylene blockage technique which was used in a second series of bottles with \( ^{15}NO_3^- \)-amended sediment and 0.7% \( C_2H_2 \). The \( N_2O \) production rate was measured by gas chromatography. At intervals, 50-µl gas samples were taken from the bottles and assayed on a Packard Becker 417 gas chromatograph with a thermal conductivity detector operated at 140°C and a bridge current of 300 mA. The measuring side was a 2-m by 0.32-cm column of Porapak Q. He carrier flow was 20 ml/min. Full-scale deflection at 1 mV on the HP 7100 BM recorder corresponded to 20 nmol of \( N_2O \), and minimal detectable \( N_2O \) concentration in the gas phase of the bottles was 0.06%.

The acetylene blockage technique described was also applied in experiment B.

The concentration of \( ^{15}N_2 \) and \( N_2O \) in the bottles was expressed as \( \mumol \) of \( N/cm^3 \) of wet sediment, and the estimated production rates were expressed as \( \mumol \) of \( N/cm^3 \) per day. Correction was made for the solubility of \( N_2O \) in water.

Chemical assays. In experiment B, colorimetric assays for \( NO_3^- \), \( NO_2^- \), and total \( NH_4^+ \) were made at intervals. The sediment samples were killed with chloroform (200 µl/5 g of sediment). A 2-h extraction at 5°C with 0.5 M KCl (5 ml/5 g of sediment) was performed before the bottles were centrifuged at 2,000 x g for 10 min. A subsample of supernatant from each bottle was immediately removed and frozen until the chemical analyses were made.

Total \( NH_4^+ \) (soluble, interstitial \( NH_4^+ \) + adsorbed, exchangeable \( NH_4^+ \)) was determined by the phenol-hypochlorite method of Solórzano (17). The \( ^{15}N \) content of this pool was measured on the \( ^{15}N \) analyzer, using the technique of Fiedler and Proksch (6) for preparation of the discharge tubes and the microdiffusion technique of Conway (4) for preparation of the \( NH_4^+ -containing \) capillaries. Assays for \( N_2O \) were made by the method of Strickland and Parsons (18), and assays for \( NO_3^- \) were made by the modified brucine method of Kahn and Brezenksi (13).

All concentrations were expressed as \( \mumol \) of \( N/cm^3 \) of wet sediment, and \( ^{15}NH_4^+ \) production rates were expressed as \( \mumol \) of \( N/cm^3 \) per day.

RESULTS

Sediment characteristics. Some basic characteristics of the sediment are given in Table 1. The pronounced gradients for the redox potential and the \( NO_3^- \) concentration indicate that in situ \( O_2 \) and \( NO_3^- \) respiration were localized in the upper few centimeters of the sediment.

No method was available for the measurement of \( O_2 \) concentrations in the sediment, but it was anticipated from the extension of the brown-colored surface zone (precipitates of hydrous iron oxides) that \( O_2 \) penetrated about 2 cm into the sediment in situ.

The total \( NH_4^+ \) concentrations were high compared with the \( NO_3^- \) and \( NO_2^- \) concentra-
tions. Exchangeable NH$_4^+$ accounted for 50% of the total NH$_4^+$; this was a constant factor in the present sediment (T. H. Blackburn, unpublished results).

**Gas production assays.** The N$_2$ production rates in experiment A were based solely on the increments of the $^{15}$N content in the N$_2$ gas phase of the incubated bottles, since the native, unlabeled NO$_3^-$ pool was neglected. Any NO and N$_2$O that might have been produced was disregarded, since these gases only appeared transiently in trace amounts during an early stage of incubation. Isotopic fractionation in the denitrification process was also neglected in this context, because the reported fractionation factors are less than 1.023 (3).

Figure 1 shows the production of $^{15}$N$_2$ in the absence of C$_2$H$_2$ and the production of N$_2$O in the presence of 0.7% C$_2$H$_2$ in the two series of bottles in experiment A. The rates of $^{15}$N$_2$ and N$_2$O production were similar. Gas production ceased at the time when NO$_3^-$ was ultimately exhausted, and equal gas accumulations were obtained in the two series. It was concluded that significant $^{15}$N$_2$ production was absent in the bottles with C$_2$H$_2$ and that the reduction of N$_2$O to N$_2$ was completely blocked in the presence of 0.7% C$_2$H$_2$. The data showed that acetylene blockage technique was applicable to the present measurements of denitrification capacities in marine sediment.

The acetylene blockage technique was preferred in experiment B, since the gas chromatographic assay for N$_2$O production was more rapid and sensitive than the $^{15}$N$_2$ production assay.

**Denitrification.** The disappearance of NO$_3^-$ and the production of N$_2$O in experiment B is shown for the four sediment segments in Fig. 2. The rate of NO$_3^-$ disappearance decreased with depth in the sediment. Transient accumulation of NO$_3^-$ was observed in the two upper segments, whereas only trace amounts of NO could be detected in the bottles by the gas chromatographic assay. The production of N$_2$O began within 3 h after addition of NO$_3^-$ in the two surface segments, whereas increasing delays were observed in the two deeper segments. The N$_2$O production rates were apparently constant in all segments after the initial time lags when N$_2$O production was not detectable by the gas chromatographic assay, but sufficient points were not available to preclude an exponential increase. The presence of long time lags before N$_2$O production could be measured implied a risk of significant cell growth in the incubated bottles, and so the measured denitrification capacities in the two deeper segments were possibly overestimated.

The denitrification capacities in the segments were estimated as the maximal rates of N$_2$O production and are given in Table 2. The values ranged from 0.87 to 0.10 μmol of N/cm$^3$ per day in the sediment.

**Reduction of NO$_3^-$ to NH$_4^+$.** The production of $^{15}$NH$_4^+$ in the four segments is also shown in Fig. 2. In all segments, the production of $^{15}$NH$_4^+$ began immediately after addition of NO$_3^-$ . Since the production rates declined in the upper segments after the initial 5 to 10 h, the estimated capacities were based on the steady initial rates. The values are given in Table 2.

Decreasing values were found with depth in the sediment as in the case of denitrification, and the measured range was of similar magnitude, from 0.75 μmol of N/cm$^3$ per day in the top segment to 0.12 μmol of N/cm$^3$ per day in the deepest segment.

### Table 1. Sediment characteristics

<table>
<thead>
<tr>
<th>Sediment segment (cm)</th>
<th>Porosity (% [wt/wt])</th>
<th>Density (g/cm$^3$)</th>
<th>Eh (mV)</th>
<th>NO$_3^-$ (μmol of N/cm$^3$)</th>
<th>NO$_2^-$ (μmol of N/cm$^3$)</th>
<th>Total NH$_4^+$ (μmol of N/cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>76</td>
<td>1.10</td>
<td>+75</td>
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<td>1.15</td>
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<td>0.01</td>
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<td>1.75</td>
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<tr>
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<td>69</td>
<td>1.21</td>
<td>-250</td>
<td>-</td>
<td>-</td>
<td>1.65</td>
</tr>
<tr>
<td>9-12</td>
<td>65</td>
<td>1.20</td>
<td>-275</td>
<td>-</td>
<td>-</td>
<td>1.90</td>
</tr>
</tbody>
</table>

*$^a$ Soluble NH$_4^+$ + exchangeable NH$_4^+$.

*$^b$ Not detectable.

**Fig. 1.** Production of N$_2$O (●) from NO$_3^-$ respiration in the presence of 0.7% C$_2$H$_2$, and production of N$_2$ (■) from NO$_3^-$ respiration in the absence of C$_2$H$_2$.  

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Throughout the year, the vertical distribution of NO$_3^-$ changes by several centimeters in the sediment, but its presence is always restricted to the upper 5 to 6 cm (K. Henriksen, unpublished results). It is likely, however, that migrations of the bacteria and bioperturbation by burrowing animals may take denitrifying bacteria down to the deeper, NO$_3^-$-deficient layers. The bacteria would only be able to grow there, however, if they were also facultative fermenters. Possibly, bacteria which are capable of anaerobic fermentation and are also potential denitrifiers, e.g., some bacilli (7), are present in the deeper layers of these sediments.

**Denitrification capacity.** The potential for denitrification was, due to the exclusion of the alternative O$_2$ respiration, an indication of the abundance of the denitrifying bacteria. The high capacities which were measured in the upper two sediment segments confirmed that in situ denitrification was restricted to a few centimeters of surface sediment. The capacities which were measured in the two deeper segments were significant but possibly overestimated due to proliferating populations during the prolonged incubations. The denitrifying activity which did
occur in these layers was probably due to bacteria of a type which was fermenting in situ but was able to respire when supplied with NO$_3^-$.

Occurrence of other NO$_3^-$ reducers. The assimilatory reduction, in which NH$_4^+$ is incorporated into cell material, is usually repressed by high concentrations of NH$_4^+$. A few exceptions do exist, however; e.g., some clostridia where the enzyme synthesis is constitutive (15). If fermenters like the clostridia do reduce NO$_3^-$ to NH$_4^+$ in an NH$_4^+$-rich environment like the present sediment, the process may have an energetic rather than a nutritional value. The studies of Ishimoto and Egami (10) and Takahashi et al. (19) indicated that energy production was indeed linked to the reduction of NO$_3^-$ to NH$_4^+$ by some clostridia, and Hasan and Hall (8) showed that the presence of NO$_3^-$ enhanced ATP production in Clostridium perfringens. The process was clearly dissimilative, since NH$_4^+$ was released, and was also of fermentative character, since substrate-level phosphorylation was involved.

Constitutive enzyme synthesis in the NH$_4^+$-producing pathway was also demonstrated (16) for a denitrifying aerobe, Paracoccus denitrificans, as quoted by Payne (15).

Such capable fermenters and respirers were likely to cause the observed $^{15}$NH$_4^+$ production in the sediment under study.

Sediment capacity for reduction of NO$_3^-$ to NH$_4^+$. The measured capacities for this reductive pathway were of the same magnitude as those recorded for denitrification, and since the $^{15}$NH$_4^+$ production was also spontaneous in the deeper segments, it was evident that significant populations of capable bacteria were present in all layers.

The experiments suggested that the reduction of NO$_3^-$ to NH$_4^+$ may be quantitatively important in marine sediments.

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LITERATURE CITED


