APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1989, p. 2861–2870 Vol. 55, No. 11
0099-2240/89/112861-10$02.00/0

Nitrate Reduction in a Groundwater Microcosm Determined by ^{15}N Gas Chromatography-Mass Spectrometry

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Received 14 March 1989/Accepted 10 August 1989

Aerobic and anaerobic groundwater continuous-flow microcosms were designed to study nitrate reduction by the indigenous bacteria in intact saturated soil cores from a sandy aquifer with a concentration of 3.8 mg of NO_3^--N liter^{-1}. Traces of $^{15}NO_3^-$ were added to filter-sterilized groundwater by using a Darcy flux of 4 cm day^{-1}. Both assimilatory and dissimilatory reduction rates were estimated from analyses of $^{15}N_2$, $^{15}N_2O$, $^{15}NH_4^+$, and ^{15}N-labeled protein amino acids by capillary gas chromatography-mass spectrometry. N_2 and N_2O were separated on a megabore fused-silica column and quantified by electron impact-selected ion monitoring. NO_3^- and NH_4^+ were analyzed as pentafluorobenzoyl amides by multiple-ion monitoring and protein amino acids as their *N*-heptafluorobutyryl isobutyl ester derivatives by negative ion-chemical ionization. The numbers of bacteria and their [*methyl*-^{3}H]thymidine incorporation rates were simultaneously measured. Nitrate was completely reduced in the microcosms at a rate of about 250 ng g^{-1} day^{-1}. Of this nitrate, 80 to 90% was converted by aerobic denitrification to N_2, whereas only 35% was denitrified in the anaerobic microcosm, where more than 50% of NO_3^- was reduced to NH_4^+. Assimilatory reduction was recorded only in the aerobic microcosm, where N appeared in alanine in the cells. The nitrate reduction rates estimated for the aquifer material were low in comparison with rates in eutrophic lakes and coastal sediments but sufficiently high to remove nitrate from an uncontaminated aquifer of the kind examined in less than 1 month.

Despite the theoretically predicted instability of nitrate as a nitrogen species in most groundwater environments (31), nitrate is quite often found in concentrations exceeding normal aquifer background concentrations more than 50-fold. Contamination by nitrate is associated with both point sources (21, 29, 33, 49, 50) and regional fertilization and land irrigation practices (55, 63). The presence of high levels of nitrate and nitrite in deep saturated soils and the subsequent risks of contamination of natural and artificial water supplies have been of considerable concern, primarily because of health hazards such as the formation of carcinogenic nitrosamines and the possibility of methemoglobinemia (20). Infants are especially liable to methemoglobinemia owing to the low acidity of their gastic juices; this favors the reduction of nitrate to nitrite, which eventually enters the blood and combines with hemoglobin. As a precaution against health problems, the National Board of Health and Welfare in Sweden recommends that the nitrate-nitrogen concentration in drinking water should not exceed 6.8 mg liter^{-1}, and in the European Community directive the maximum admissible concentration is 11.6 mg liter^{-1}. Another concern is the potential impact of groundwater nitrate on surface waters, since groundwater influx is known to be a source of nitrate in lakes (7, 28) and coastal marine sediments (10, 56).

The fate of NO_3^- in soils is determined by a variety of chemical and biological processes, few of which are known in great detail. The biological processes are carried out by miscellaneous organisms, of which the respiratory denitrifiers, which reduce NO_3^- to N gases and generate ATP by electron transport phosphorylation, are the best known. Denitrification is a major process in the production of N_2O in grassland (53), especially under acidic soil conditions (3, 19, 38, 72). N_2O has become a major concern in recent years because of its detrimental effect on the stratospheric ozone layer (12, 18, 22, 44). Nitrifiers seem to be more important N_2O producers in fertilized agricultural soils (1, 23), whereas other sources, such as fungi, may be important in forest soils (51). True denitrifiers are less common in soils than nondenitrifying NO_3^- reducers (60), which respire NO_3^- to NO_2^- and NH_4^+ (47, 62) and eventually also to N_2O (4, 60).

Shallow aquifer sediments are normally oligotrophic environments with less than 0.1% organic carbon and less than 10 mg of C liter^{-1} in pore water. The numbers of bacteria in subsurface soils have been estimated to be 100- to 1,000-fold smaller than in agricultural soils, and only 1 to 10% are metabolically active (17, 27, 43). Groundwater bacteria have lower physiological activities than bacteria in sewage sludge and surface waters (26), and their growth rates are significantly lower than those of bacteria in marine or freshwater sediments or in surface soils (67).

Denitrification is known to occur in groundwater environments (31, 46, 58, 61, 70, 71), whereas other nitrate-reducing processes are virtually unknown. Most of the denitrifying activity is associated with attached bacteria and limited by nitrate when in situ nitrate concentrations are less than 10 mg liter^{-1}.

The purpose of this work was to test three hypotheses associated with nitrate reduction in oligotrophic aquifer material. (i) Assimilatory NO_3^- reduction was assumed to be negligible owing to the presence of repressing NH_4^+ (47). If the concentration of NH_4^+ in the aquifer material becomes low, e.g., owing to limited leaching through the unsaturated soil, competition with nitrifiers for NH_4^+ will increase the likelihood of assimilatory NO_3^- reduction in aerobic groundwater. (ii) Dissimilatory NO_3^- reduction was not expected in the saturated soil unless oxygen, as a superior electron acceptor, was reduced to a minimum. Hence, NO_3^- concentrations should reduce only slowly and chemically convert to

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N gases at redox potentials of <700 mV (15). As a consequence of this and the assumptions in hypothesis (i), we expected NO_3^- reductions to be negligible in aerobic aquifer material, with a residence time for water in the order of some months. (iii) We expected dissimilatory NO_3^- reduction to be the major respiratory process in absence of O_2 as an electron acceptor, based on the relatively high energy yield for the electron transport to that oxidant in comparison with, e.g., SO_4^{2-} and CO_2 (47). By introducing permanently moderate reducing conditions ($E_h > -300$ mV) in the aquifer material, reduction to both N gases and NH_4^+ end products should be theoretically possible. Whichever process becomes the most competitive may depend on, e.g., the relative density of the organisms and their resource requirements.

The hypotheses were addressed by studying dynamic groundwater microcosms with the indigenous soil matrix and microorganisms, supplied with filter-sterilized groundwater and $^{15}NO_3^-$. Although a microcosm is not identical to the real system studied, it offers the advantage of laboratory scale manipulations of variables and processes, upon which the representative description of the true system is based. A microcosm is also a cost-efficient physical model for the studies of the fate of ^{15}N-labeled compounds, for which two methods traditionally are available, isotope mass spectrometry and optical emission spectrometry. For both methods, conversion of nitrogen-containing compounds to nitrogen gas has been a prerequisite; this has limited the applications to samples that contain relatively large amounts of nitrogen and a high percentage of ^{15}N. With the development and interfacing of the quadropole mass spectrometer with capillary gas chromatography, both nitrogen gases and their precursors can be analyzed for their isotope ratios at ambient concentrations without prior sample concentration.

MATERIALS AND METHODS

Sampling site. The saturated soil was sampled in mid-April 1987 at a sandy field at Vomb, 30 km east of Lund, southern Sweden. The aquifer supports the cities of Lund and Malmö with drinking water, which is produced by artificial infiltration of lake water from the nearby Lake Vombsjön.

A continuous-flight auger was used to drill holes to a depth of about 7 m, about 1 m below the groundwater table. The auger was replaced by an autoclaved core barrel carrying a 0.9-liter stainless steel cylinder (20.4 cm by 7.4 cm [outer diameter]). The cylinder was aseptically filled by pushing the barrel sufficiently deep into the saturated soil and then sealing it with threaded stainless steel caps at both ends. A Whatman GF/A filter was placed on the inside of the top cap to reduce channeling at the water inlet and on the inside of the bottom cap to prevent soil particles from escaping and clogging the effluent tube, which had a shutoff valve.

The columns were placed in a constant-temperature room (11°C), where the experiments were conducted within 2 h after sampling. Groundwater was collected from a well, J 5, close to the drilling site and stored at 4°C prior to use. The carbon concentration of the aquifer material in soil was 0.2%, the dissolved organic carbon (DOC) concentration in the groundwater was 6 mg liter^{-1}, and the NO_3^--N concentration was 2.5 mg liter^{-1}.

Experimental design. The columns were resaturated to recharge pore water lost during sampling. An autoclaved aspirator-type bottle with a hose connection outlet near the bottom and filled with filter-sterilized groundwater was connected to the effluent valve of a column by an autoclaved Teflon tubing. A pressure of approximately 0.5 kPa was applied by the siphon technique until water appeared at the open inlet valve, within about 2 days. The pore volume of the saturated soil was 0.45 m^3 m^{-3}, and the average wet weight of the soil was 0.725 kg column^{-1}. The column inlet was then connected with Teflon tubing to a glass carboy containing filter-sterilized groundwater with added $Na^{15}NO_3$ (99%; Amersham) at 3.5% of the groundwater concentration. Two columns were connected to a 10-liter carboy with oxygenated water, and another two were connected to a carboy with deoxygenated water (Fig. 1). The carboy was sealed by using a glass stopper with three valves. One valve was used to supply air through a Gelman Acrodisc filter (pore size, 0.2 μm) to prevent contamination by airborne bacteria. The second valve was connected to a Pyrex glass tube (outer diameter, 2 mm; Corning Glass Works) in the carboy and used to withdraw samples and add helium (see below). The third valve connected another glass tube in the carboy to Teflon tubing supporting two columns via a T-joint of glass.

Oxygen in the air was removed before entering the deoxygenated carboy in an Erlenmeyer (E)-flask containing a solution of saturated iron(II)sulfide, prepared by adding iron(III)citrate to a solution of sodium sulfide. Air entered the E-flask through 1-m Teflon tubing to prevent the solution from overflowing during air pressure changes. The E-flask was connected to a safety bottle to prevent the solution from entering the carboy during air pressure changes. A 0.2-μm sterile filter was placed on the Teflon tubing between the safety bottle and a T-joint, with one end connected to the carboy stopper valve and the other end connected by a T-joint to a check valve and an electric vacuum pump.

With the tubing between the carboy and the oxygen trap closed, vacuum was applied to the carboy by using the pump. Then helium was bubbled through the water via Teflon tubing with an air filter while the water was stirred with a magnetic stirrer. Vacuum and helium bubbling were applied alternately until most of the oxygen was removed from the water. The vacuum was used because oxygen in the water phase was removed faster when the oxygen in the gas phase was eliminated. The oxygen concentration in the water was measured by the method of Winkler (28a); the lowest measured value was 0.2 mg liter^{-1} (1.8% saturation), and the highest was 0.7 mg liter^{-1} (6.3% saturation).

The effluent valves were connected with Teflon tubing to an Ismatec MV-GE peristaltic pump. Silicone pump tubings (ENE 07; outer diameter, 2 mm) were used in the pump. The average Darcy velocity at the field site was 4 cm day^{-1} or less, and the pump was set to give 4 cm day^{-1} in the columns. Each column produced about 0.08 liter day^{-1}, and the mean residence time of water in the column was 5 days. Oxygen concentrations in the effluent water stabilized after 2 weeks of pumping, and the microcosms were considered to be in equilibrium with respect to hydraulics, chemistry, and biological activity; the sampling program was then started. All sampling and maintenance were performed under sterile conditions.

Filter sterilization. The groundwater was filtered in three consecutive steps. In the first and second steps, a Whatman GF/A fiber glass filter (pore size, 1.2 μm) and a Gelman GA-6 filter (pore size, 0.45 μm), respectively, were mounted in a Millipore 100-ml stainless pressure holder connected to a 10-liter stainless steel pressure carboy, and a pressure of about 200 kPa applied. In the last step, water filtered through the GA-6 filter was siphoned into the autoclaved glass carboy through an autoclaved Gelman GA-8 filter (pore size,

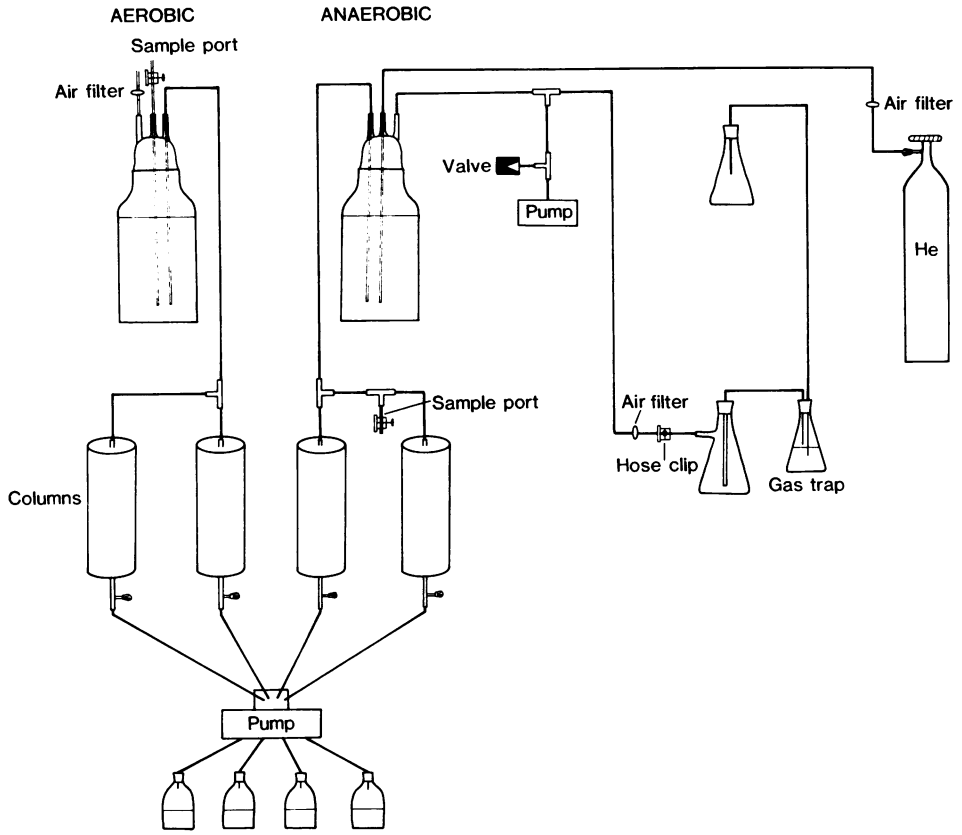


FIG. 1. Schematic drawing of the groundwater microcosms. See the text for an explanation.

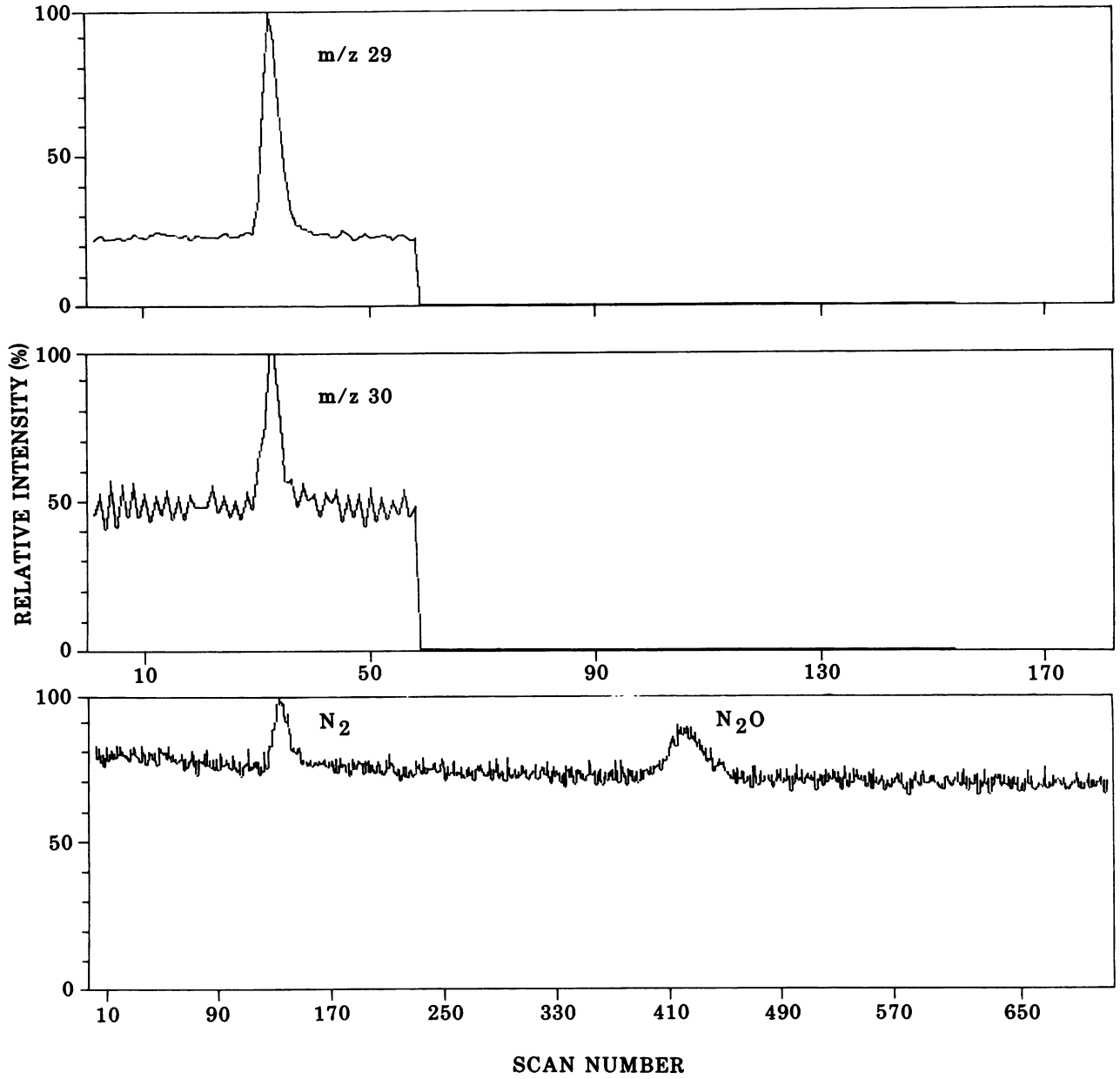
0.2 μm) mounted in the autoclaved Millipore filter holder. Filtration of 10 liters of groundwater lasted 3 to 4 h and was performed at 4°C. The reduced hydrostatic pressure was used to minimize the probability of a breakthrough of small bacterial cells through the filter.

Sampling. Effluent water was sampled in 100-ml autoclaved serum bottles. The bottles were capped with rubber septa, and water entered via a cannula (20 mm by 0.4 mm [outer diameter]) attached to the end of the Teflon tubing. Water was collected every day for 2 weeks for analysis of nitrogen gases, nitrate, ammonium, and amino acids. Water was collected for another week in 20-ml serum bottles to determine the number of bacteria and their activity. The oxygen concentration was repeatedly measured during the sampling program in the filter-sterilized water and in the effluent. After the system had reached equilibrium, a test was made of the error in the nitrogen gas analyses due to biological activity in the serum bottles during sampling. Two serum bottles were empty prior to sampling, and 9 ml of 0.2% sodium azide solution was added to another two to inhibit bacterial metabolism. The water sampled for 1 day was analyzed for N_2 and N_2O, and no differences were found between the treatments.

Analysis of $^{15}N_2$ and $^{15}N_2O$. The serum bottles were stored at 4°C until analyzed. They were then shaken for 5 min in an electric wrist-action shaker, and 1 ml of the headspace was analyzed by gas chromatography (GC)-mass spectrometry. The extraction efficiency of the headspace analysis was estimated by saturating 75 ml of deionized water in a 100-ml serum bottle with N_2 or N_2O. The water volume was diluted 1,000-fold, and 75 ml of the diluted solution was shaken for 5 min in a 100-ml serum bottle on the wrist-action shaker. A 1-ml sample of the headspace was analyzed on a Varian 3700 gas chromatograph equipped with a thermal conductivity detector and a Porapak Q column. Peak areas were quantified with a Hewlett-Packard 3390A digital integrator and standardized against gas mixtures made by serial dilution. The gas phase was then discharged and replaced by the same volume of helium. The bottle was shaken for another 5 min to equilibrate the gas and water phases, and 1 ml of the gas phase was analyzed by GC. This procedure was repeated until no more N_2 or N_2O could be detected. It was found that about 35% of dissolved N_2 and 46% of dissolved N_2O was extracted to the gas phase by the first extraction.

GC-mass spectrometry was performed by using a Carlo Erba 4160 GC in combination with a Ribermag R 10-10c quadropole mass spectrometer. The column was constructed from a megabore fused-silica column (2 m by 0.5 mm [inner diameter]) that was packed at the laboratory with HayeSep Q, 140/170 mesh, by a combination of vacuum and nitrogen gas pressure. The polymer was prewashed in acetone and conditioned overnight at 250°C. Both column ends were sealed with Valco low dead-volume filter to retain column packing. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The oven, injector, and detector temperatures were 40, 125, and 130°C, respectively. Electron impact and selected ion-monitoring mass spectra were obtained by using a 70-eV ionizing energy and 150°C ion source temperature (Fig. 2).

The $^{15}N/^{14}N$ ratio was expressed as $(^{15}N/^{14}N)_{sample} - (^{15}N/^{14}N)_{standard}$. The percentage of ^{15}N in N_2 was calculated from $^{30}N_2/(^{29}N_2 + ^{28}N_2)$, and the percentage of ^{15}N in N_2O was calculated from $^{46}N_2O/(^{45}N_2O + ^{44}N_2O)$. The contribution of the mass of the unlabeled fragment ion to the (M + 1)



ion due to the natural abundance of the stable isotope was corrected by a factor 0.0037 times the number of N atoms in the molecule and a factor of 0.0004 for O in N_2O.

Pure gas standards were received in 5.7-liter Alfax containers with a maximum pressure of 10.2×10^5 Pa and containing 104 μl of N_2O per liter in N_2 and 107 ± 6 μl of N_2 per liter in He.

Analysis of $^{15}NH_4^+$ and $^{15}NO_3^-$. NH_4^+ was analyzed by a modification of the method suggested by Fujihara et al. (25). NO_3^- was analyzed as NH_4^+ after reduction by Dewarda reagent in alkaline solution.

A 2-ml water sample was placed in a Pyrex glass tube (150 by 20 mm) for purification; 1 ml of a saturated Na_2CO_3 solution was added, and the tube was capped with a rugged glass staff dipped into 100 μl of 0.1 M H_2SO_4. The tube was slanted and left for 6 h at 35°C. The glass staff was transferred to another tube containing 2 ml of 5% $NaHCO_3$ solution and 15 μl of 98% pentafluorobenzoyl chloride (Aldrich). NH_4^+ adsorbed on the acid-coated staff was derivatized to pentafluorobenzoyl amide by shaking the mixture for 5 min. The mixture was transferred to another 150- by 20-mm tube with a Pasteur pipette, and the tube was shaken for another 5 min with 1 ml of ethyl acetate to extract pentafluorobenzoyl amide. The aqueous phase was then discarded, and the ethyl acetate phase was washed with 2 ml of 6% H_3PO_4 for 30 s. The aqueous phase was discarded, and the organic phase was taken for analysis by GC-mass spectrometry.

The Carlo Erba gas chromatograph was equipped with a 25-m fused-silica capillary column (inner diameter, 0.2 mm) statically coated with SE 54 (5) and connected directly to the ion source. The injector temperature was 220°C, and the

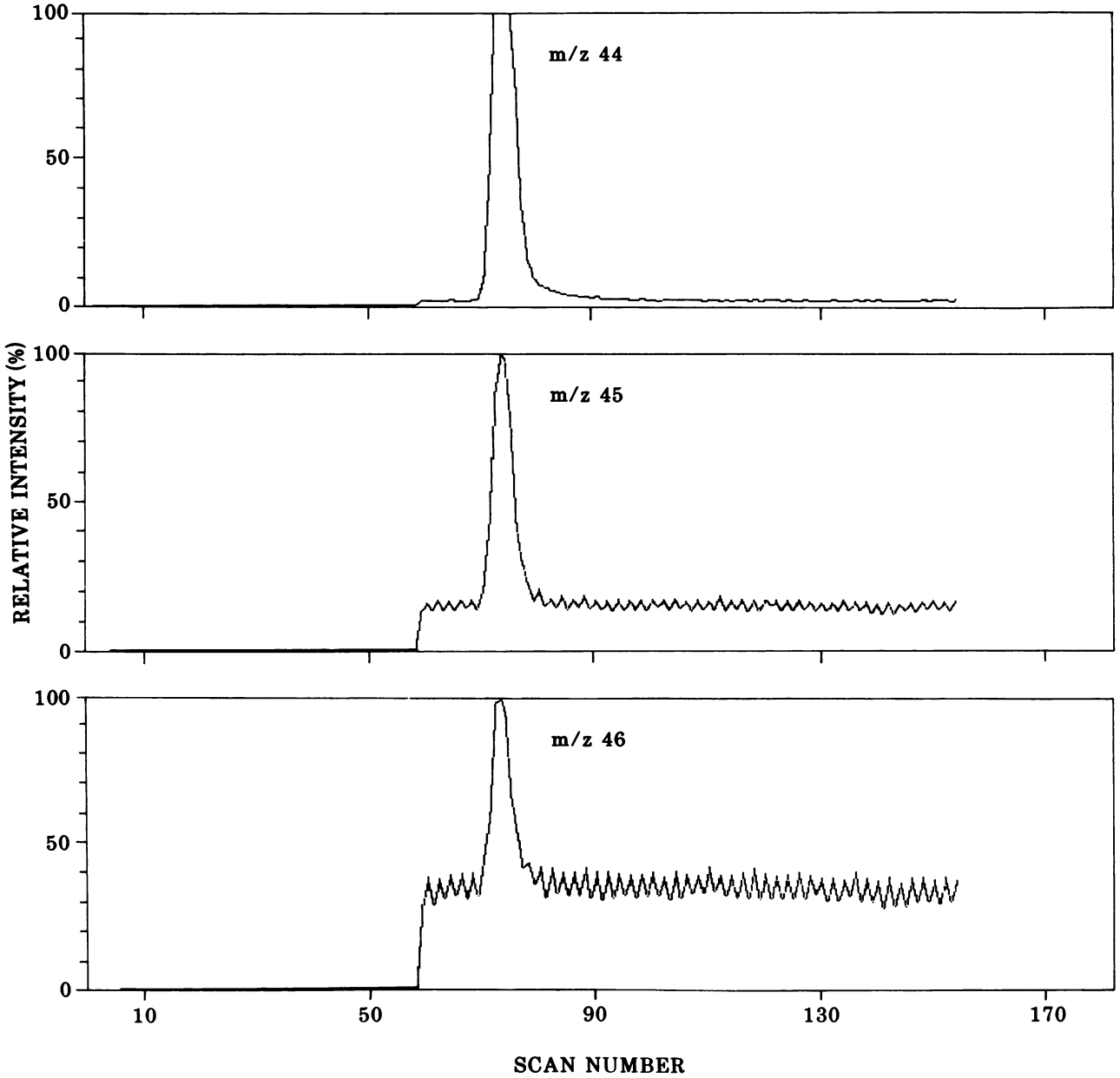


FIG. 2. Total ion chromatogram of a mixed N_2 and N_2O standard in water (bottom left) and mass fragmentograms of N_2 (*m/z* 29 and 30) and N_2O (*m/z* 44, 45, and 46). Injected gas volumes contained 0.12 μg of each component.

detector temperature was 210°C. The oven was programmed to heat the sample from 70 to 148°C at 20°C min^{-1}. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The pentafluorobenzoyl amide was determined by multiple-ion monitoring with a 70-eV ionizing energy and with reference to a standard curve of NO_3^- and NH_4^+ with various percentages labeled. The ^{15}N abundance ratio was determined from the molecular ions at *m/z* 211 ($C_6F_5CONH_2$) and 212, and the pentafluorobenzoyl amide was quantified from *m/z* 195 (C_6F_5CO).

Analysis of ^{15}N-labeled protein amino acids. Samples from two consecutive days were pooled and centrifuged at 20,000 × *g*. The pellet was suspended in 1 ml of 6 M HCl, 20 μg of internal standard (norleucine) was added, and the sample was hydrolyzed at 110°C for 24 h. The hydrolysate was diluted with distilled water, lyophilized in a Leybold-Heraeus Lyovac GT2 freeze-drier, and dissolved in 0.5 ml of 25% acetic acid containing 5 mg of $SnCl_2$ 100 ml^{-1}.

The hydrolysate was cation exchanged and derivatized with isobutanol–3 M HCl and heptafluorobutyric anhydride by the method of Bengtsson and Odham (2) with slight modifications. The derivatized amino acids were quantitatively analyzed with a Varian 3700 GC equipped with a flame ionization detector and an all-glass split-splitless injection system. The injector temperature was 200°C, and the detector temperature was 250°C. Injections were made splitless with the oven at 80°C for 2 min. The temperature was increased linearly at 6°C min^{-1} to 230°C.

The isotope ratio was determined with the Carlo Erba-Ribermag GC-mass spectrometry system. The GC was

equipped with a 25-m fused-silica capillary column (inner diameter, 0.2 mm) statically coated with SE-54. Helium was used as the carrier gas at 1 ml min^{-1}. Methane (9.33 Pa; >99.95% purity) for negative-ion chemical ionization was ionized at 94 eV with the ion source temperature at 125°C. The injector temperature was 250°C, and the detector temperature was 300°C. The oven temperature was programmed from 80 to 230°C at 6°C min^{-1} with 2 min at the initial temperature.

Number of bacteria. The number of cells in the effluent water samples was counted by direct microscopy in a Helber counting chamber; 60 squares were counted for each sample.

A sample of 2 g of drained and gently washed (filter-sterilized groundwater) saturated soil was suspended in 20 ml of sterilized 0.2% Calgon–0.1% peptone solution and shaken for 30 min at 160 rpm. Acridine orange solution (50 μl of a 0.1% solution) was added after serial dilution of the suspension. The mixture was left for 2 min at room temperature and then slowly filtered through a black 0.22-μm filter (Millipore-MF dyed with Ebony black). The number of fluorescent cells was then counted in a Zeiss 2 Fl microscope equipped for phase-contrast observations.

Thymidine incorporation. [*methyl*-^{3}H]thymidine incorporation was used to estimate the bacterial activity by a modification of the method suggested by Fuhrman and Azam (24). Samples were incubated at 11°C with 50 nM (250 μCi $liter^{-1}$; 84.1 Ci $mmol^{-1}$) [^{3}H]thymidine (Du Pont, NEN Research Products). At the end of the incubation, the soluble pools of the cells were extracted with 10% trichloroacetic acid. The trichloroacetic acid-insoluble material was filtered on a 0.22-μm Millipore GS filter. The filters were rinsed with 5% trichloroacetic acid and dissolved in ethyl acetate, and the radioactivity was assayed by liquid scintillation spectrometry (Beckman LS 1800 spectrometer). Blank values from controls treated with 0.2% NaN_3 were subtracted.

RESULTS

Numbers of bacteria and their activity. The aerobic aquifer material contained 5×10^6 cells g of core $material^{-1}$ at the start and end of the experiment. Anaerobiosis reduced the numbers to 2×10^6 cells g^{-1}. A total of 6×10^6 cells ml^{-1} were counted in effluent samples from the anaerobic columns; this was half the counts from the aerobic columns (Fig. 3). Nearly 50% of the bacteria in the original aquifer material vanished during the oxygen removal and were most probably obligate aerobes. Hence, it seems unlikely that the original aquifer material, except in local inhomogeneities, stays anaerobic for very long periods. In contrast, the [^{3}H]thymidine incorporation rate in the anaerobic microcosm was more than 4 times higher than in the aerobic microcosm ($P < 0.05$ by the Mann-Whitney test [Fig. 3]).

Oxygen reduction. The oxygen concentration in the aerobic columns was reduced from an average of 9.9 mg $liter^{-1}$ in the carboy to 1.3 ± 0.08 (standard error) in the effluent, which is equivalent to a reduction of 0.87 mg of O_2 kg of $soil^{-1}$ day^{-1}. That corresponds to an oxidation of 0.77 mg of organic matter kg of $soil^{-1}$ day^{-1}, assuming that 1.22 mg of O_2 oxidizes 1 mg of organic matter (48). DOC in the filter-sterilized groundwater was 4.5 mg $liter^{-1}$ and that in the effluent water was 1.8 mg $liter^{-1}$. Assuming that all of the DOC loss was due to microbial metabolism, DOC would contribute 0.216 mg of C day^{-1} as an electron donor. With an average soil mass of 0.725 kg, a carbon concentration of 0.2%, and organic carbon as the sole electron donor, DOC metabolism represents about 40% of the total carbon reduced in the columns. Although several of the assumptions for the estimation probably are violated (part of the DOC was certainly sorbed to soil particles, the in situ [0.45-μm filter] DOC concentration was 5 to 6 mg $liter^{-1}$ rather than 4.5 mg $liter^{-1}$, etc.), there is no doubt that the aquifer system requires periods of higher import of electron donors and reduced metabolism to maintain equilibrium. Put another way, one may ask how rapid and regular losses of carbon occur in aquifer material.

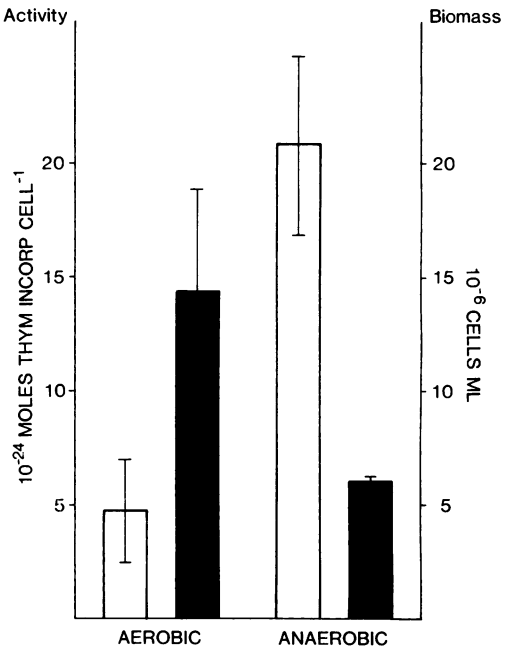


FIG. 3. Average biomass (mean and standard error; 10^{-6} cells ml^{-1}) and activity (10^{-24} mol of thymidine $cell^{-1}$) of bacteria in effluent water from columns. Symbols: □, activity; ■, biomass.

The oxygen concentration in the anaerobic system remained at 0.6 ± 0.07 (standard error) mg $liter^{-1}$ from the carboy to the effluent. Since the analytical method would distinguish between samples containing 0.6 and 0.0 mg of O_2 $liter^{-1}$, we are confident that the bacteria ignored O_2 at this low concentration and switched to other electron acceptors to enhance their DNA or protein synthesis.

Nitrate reduction. $^{15}NO_3^-$ was not detectable in the effluent of any of the columns. With a flow rate of 0.08 liter day^{-1}, each column reduced 46 μg of NO_3^--^{15}N per week, that is, 64 μg of NO_3^--^{15}N kg^{-1} $week^{-1}$. The potential NO_3 reduction capacity in the aquifer could then be estimated as 64/0.035 = 1.8 mg kg^{-1} $week^{-1}$, assuming a constant reduction rate.

On average, more than twice as much $^{15}N_2$ was produced in the aerobic microcosm as in the anaerobic microcosm, although the differences were insignificant owing to a huge variation in the aerobic system (Fig. 4). The daily $^{15}N_2$ production was calculated as 78 μg $liter^{-1}$ in the aerobic microcosm and 31 μg $liter^{-1}$ in the anaerobic microcosm, corresponding to 57 and 23 μg of $^{15}N_2$ kg^{-1} $week^{-1}$, respectively. $^{15}N_2O$ was not detected in any sample.

$^{15}NH_4^+$ production occurred in the reverse order. The anaerobic and aerobic microcosms produced 63 and 28 μg of $^{15}NH_4^+$ $liter^{-1}$ day^{-1}, respectively ($P < 0.05$ [Fig. 5]). These quantities corresponded to 35 and 16 μg kg^{-1} $week^{-1}$ under anaerobic and aerobic conditions, respectively.

Assimilatory nitrate reduction was observed only in the

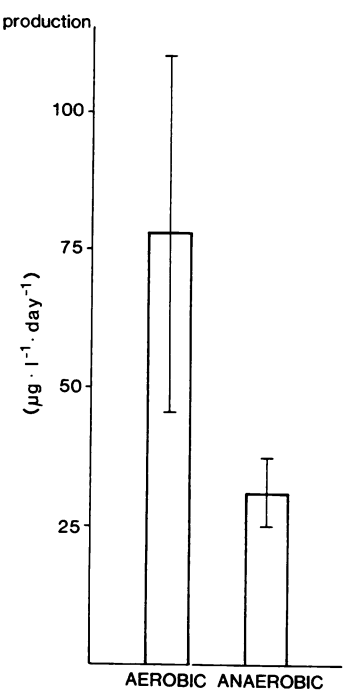


FIG. 4. $^{15}N_2$ (mean and standard error) in effluent water from columns.

aerobic microcosm, and only one amino acid, alanine, was labeled in the protein hydrolysate. The average concentration was 88 ng of [^{15}N]alanine liter^{-1} day^{-1}, corresponding to 9 ng of [^{15}N]alanine kg^{-1} week^{-1}.

Mass balance of $^{15}NO_3$ reduction. Of the $^{15}NO_3^-$ applied to the aerobic microcosm, 80 to 90% was reduced to $^{15}N_2$ as an end product, compared with about 35% in the anaerobic microcosm (Fig. 6). More than 50% of $^{15}NO_3^-$ in the anaerobic microcosm underwent dissimilatory reduction to $^{15}NH_4^+$. The assimilatory NO_3^- reduction in the aerobic microcosm corresponded to 0.01‰ of the NO_3^- reduced.

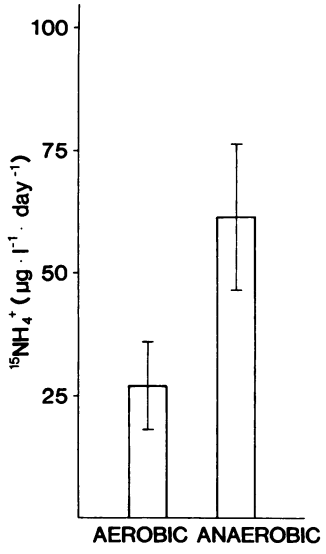


FIG. 5. $^{15}NH_4^+$ (mean and standard error) in effluent water from columns.

The ^{15}N budget was unbalanced, with opposite bias in the two microcosms. The output exceeded the input by 13.6% in the aerobic microcosm and fell below the input by 10.2% in the anaerobic microcosm.

DISCUSSION

The substantial reduction of NO_3^- to N_2 in the aerobic aquifer material was not expected from our hypotheses, basically because denitrification has been accepted as an anaerobic alternative to O_2-linked respiration and because O_2 is a superior electron acceptor; the mean energy yields for the transfer of 1 molar equivalent of electrons from an organic compound to O_2 and NO_3^- are 26.5 kcal (110.9 kJ) and approximately 18 kcal (75 kJ), respectively (47), although the difference in efficiency depends on the organism and electron donor used. A slightly lower relative energy yield for NO_3^- reduction has actually been reported (16, 39). O_2 has long been accepted as a repressor of denitrification reductases (36, 47, 54), but the mechanisms by which O_2 controls them are not fully understood and may vary among species. Some bacteria have constitutive reductases, i.e., enzymes synthesized under both aerobic and anaerobic conditions (40, 52), whereas the synthesis rather than the activity seems to be repressed in others (57). Other reports (41, 45) confirm that aerobic denitrification occurs, even at high O_2 concentrations, and that, in addition, N_2O production should be favored by the presence of O_2, since nitrous oxide reductases are the most oxygen-sensitive enzymes. It may be speculated that such bacteria have been selected for simultaneously utilizing O_2 and NO_3^- as terminal electron acceptors by the transient environment in which they live, where periods of aerobiosis would otherwise reduce their growth rate.

A combination of a high density of such aerobic denitrifiers in the aquifer material and the significant reduction of dissolved O_2 may be responsible for the N_2 production in the microcosm; 11 mg of NO_3^- liter^{-1} could not compete with 9.9 mg of O_2 liter^{-1} in the water supply for electron transfer but becomes theoretically equal in efficiency when the level of O_2 is reduced to about 4 mg liter^{-1}. Hence, at some distance from the surface, conditions in the microcosm may even be energetically favorable for NO_3^--linked respiration. Oxygen was used to convert organic carbon to CO_2 at a rate of 0.94 mg of CO_2 kg^{-1} day^{-1} (conversion factor, 1.22 [48]), a relatively high rate, also reflected in the 60% loss of DOC in the microcosm. This indicates that a significant proportion of the organic carbon in the microcosm was available for metabolism; this fact supports the observed denitrification rate, since denitrification rates and available organic carbon are known to be well correlated, at least when the NO_3^--N concentrations are high (6, 38, 64, 65).

The absence of $^{15}N_2O$ production in the microcosms was probably due to a combination of a pH effect and relatively low NO_3^- concentration (19, 59). N_2O production tends to cease at pH 7.0 (38) and at NO_3^--N concentrations of about 10 mg liter^{-1} and be favored when high concentrations of NO_2^- accumulate or when low concentrations of NO_3^- are combined with a limiting supply of fermentable substrates. It is possible that some N_2O was accumulated in the upper half of the aerobic columns owing to inhibition of N_2O reductases by high concentrations of O_2 but subsequently reduced when O_2 trapped electrons.

The predominance of NH_4^+ as the product of NO_3^- reduction in the anaerobic microcosm is in agreement with earlier observations of stable anaerobic environments (34,

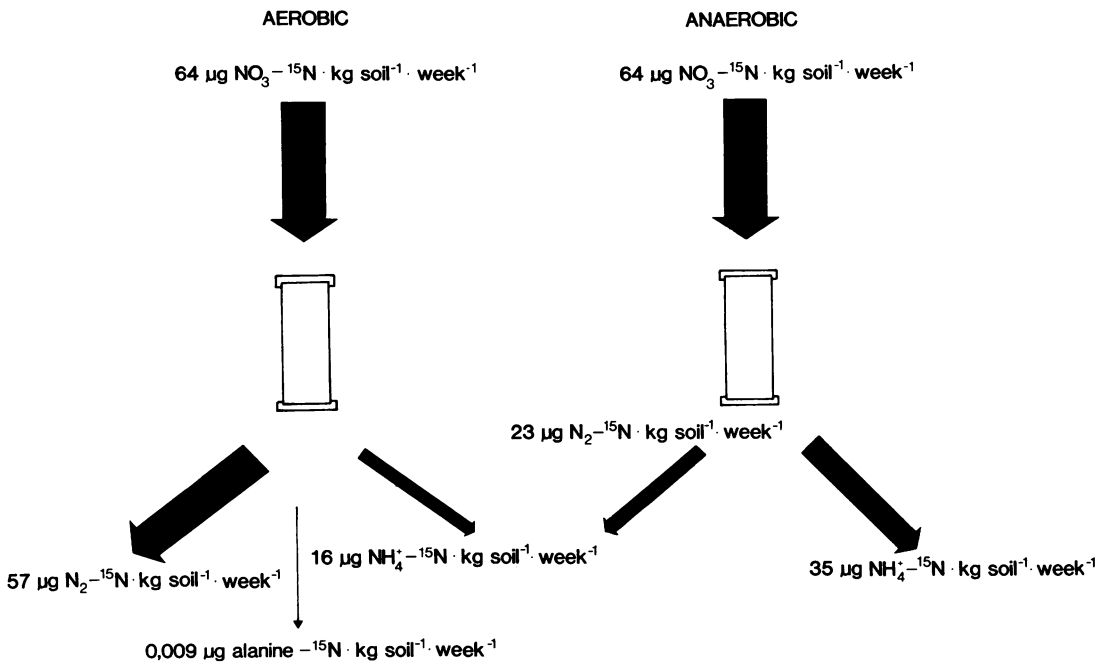


FIG. 6. Mass balance of NO_3^--^{15}N (in micrograms per kilogram per week) in the microcosms.

37, 42, 62), where the limited density of electron acceptors should make the $8e^-$ per N reduction to NH_4^+ more favorable than the $5e^-$ per N reduction to N_2. Smith and Duff (61) found no evidence for NO_3^- reduction to NH_4^+ in the sewage water-contaminated aquifer material they studied, although anoxic conditions prevailed in some samples, and suggested that this was due to carbon limitation. Delwiche (13) calculated the free energy associated with NO_3^- reduction and found that N_2 production would be the most efficient reaction in electron donor-limited environments and NH_4^+ production would be the least, whereas NH_4^+ reduction would be most advantageous for bacteria when NO_3^- is limiting and electron donors are abundant. As discussed by Tiedje et al. (68), denitrification may be favored when the carbon-to-electron-acceptor ratio is low, whereas reduction to NH_4^+ becomes more important as the ratio increases. This is further supported by observations of the conversion of NO_3^- to NH_4^+ in anoxic soils amended with mono- and disaccharides (8, 61), although such amendments may also stimulate denitrification in certain soils (9). The potential for NO_3^- reduction to NH_4^+ seems to be present in most aerobic soils (11), and nondenitrifying bacteria are more abundant than denitrifiers in the few cases examined (47, 62), but the factors controlling their activity are still unknown. Possible differences in NO_3^- reduction within denitrifying and nondenitrifying groups of species may explain the variability among replicates (Fig. 4 and 5) and the partial lack of N mass balance (Fig. 6); this is a field for future research.

Even less is known about assimilatory NO_3^- reduction and the diversity and dynamics of the organisms. The process is strongly regulated by NH_4^+ (47), but the NH_4^+ concentration in soils at which the nitrate assimilation is inhibited is unknown. The NH_4^+ concentration in the anaerobic microcosm, 9 µg liter^{-1}, was apparently sufficiently high to inhibit nitrate assimilation, whereas the lower concentration in the effluent of the aerobic microcosm, 4 µg liter^{-1}, would not totally outcompete assimilatory reduction. Since no ^{15}N appeared in the bacteria from the anaerobic microcosm with its higher NH_4^+ concentration, there is a high probability that ^{15}N in protein in the aerobic microcosm originated from $^{15}NO_3^-$. The possibility that ^{15}N was synthesized primarily for cell-wall-specific alanine is currently being examined.

The magnitude of NO_3^- reduction in the field is more difficult to evaluate owing to spatially and temporally variable inputs of nitrate and mixing of waters of different origins. Very slow in situ denitrification in aquifers has been reported (31, 71), but rates closer to our estimates from the microcosms, 0.22 and 0.58 mg of N liter^{-1} day^{-1}, have also been found. Trudell et al. (70) measured denitrification rates in the order of 0.19 to 3.12 mg liter^{-1} day^{-1} upon injection of nitrate into a 3-m-deep sandy aquifer. They also found a linear negative correlation between the denitrification rate and NO_3^--N concentrations between 8 and 15 mg liter^{-1} and a curvilinear relationship at lower concentrations. However, this relationship may vary from site to site by a factor of 10 or more (32, 66). Their maximum rates, which are about 1 order of magnitude higher than our averages, were observed at concentrations of about 2 to 4 mg liter^{-1}. Also, Morris et al. (46) found considerable potential denitrification rates for deep-aquifer material amended with very high concentrations of NO_3^- and suggested that contamination of groundwater at the site they sampled should not be allowed. Denitrification rates in an aquifer may decrease with the distance from a sewage effluent contamination source (61) and be nitrate rather than carbon limited (58).

The NO_3^- reduction rates in the groundwater microcosms and low productive aquatic environments are similar. An oxygenated pond sediment with 0.9 mg of NO_3^- liter^{-1} denitrified a maximum of 0.3 mg of N liter^{-1} day^{-1} (14), and the denitrification rates varied between 1.34 and 1.75 mg of N liter^{-1} day^{-1} in two eutrophic lakes with high NO_3^- concentrations, 37 to 47 mg liter^{-1} (69). Our estimates of about 250 ng of N reduced g^{-1} day^{-1} also agree with the lower values compiled by Hattori (30) for NO_3^- reduction in coastal and estuarine sediments, some of which have maxi-

mum reduction rates that are at least 30 times higher than ours. All of these rates are very much lower than the rates obtained in wastewater treatment, 12 g liter^{-1} or more (35).

If we assume that sufficient concentrations of carbon are available as an electron donor and that the O_2 concentration is low, such that the NO_3^- reduction rate is at least 0.22 mg of N liter^{-1} day^{-1}, infiltration would eliminate 6.8 mg of NO_3^--N liter^{-1}, the upper health limit recommended for drinking water by the National Board of Health and Welfare in Sweden, in 30 days. If concentrations of NO_3^- became higher, the removal time will be more than doubled.

ACKNOWLEDGMENTS

The work was supported by a grant from the National Swedish Environment Protection Board.

Staff members of the Malmö City drilling rigg and the Vomb water works are gratefully acknowledged for field assistance and advice.

LITERATURE CITED

1. **Aulakh, M. S., D. A. Rennie, and E. A. Paul.** 1984. Gaseous N losses from soils under zero-till as compared with conventional-till management systems. J. Environ. Qual. **13**:130–136.
2. **Bengtsson, G., and G. Odham.** 1979. A micromethod for the analysis of free amino acids by gas chromatography and its application to biological systems. Anal. Biochem. **92**:426–443.
3. **Blackmer, A. M., and J. M. Bremner.** 1978. Inhibitory effect of nitrate on reduction of N_2O to N_2 by soil microorganisms. Soil. Biol. Biochem. **10**:187–191.
4. **Bleakley, B. H., and J. M. Tiedje.** 1982. Nitrous oxide production by organisms other than nitrifiers or denitrifiers. Appl. Environ. Microbiol. **44**:1342–1348.
5. **Blomberg, L., J. Buijten, K. Markides, and T. Wännman.** 1982. Peroxide initiated *in situ* curing of silicone gums for capillary column gas chromatography. J. Chromatogr. **239**:51–60.
6. **Bowman, R. A., and D. D. Focht.** 1974. The influence of glucose and nitrate concentrations upon denitrification rates in a sandy soil. Soil Biol. Biochem. **6**:297–301.
7. **Brock, T. D., D. R. Lee, D. Janes, and D. Winek.** 1982. Groundwater seepage as a nutrient source to a drainage lake, Lake Mendota, Wisconsin. Water Res. **16**:1255–1263.
8. **Buresh, R. J., and W. H. Patrick, Jr.** 1978. Nitrate reduction to ammonium in anaerobic soil. Soil Sci. Soc. Am. J. **42**:913–918.
9. **Burford, J. R., and J. M. Bremner.** 1975. Relationships between the denitrification capacities of soils and total water-soluble and readily decomposable soil organic matter. Soil Biol. Biochem. **7**:389–394.
10. **Capone, D. G., and M. F. Bautista.** 1985. A groundwater source of nitrate in nearshore marine sediments. Nature (London) **313**:214–215.
11. **Caskey, W. H., and J. M. Tiedje.** 1979. Evidence for clostridia as agents of dissimilatory reduction of nitrate to ammonium in soils. Soil Sci. Soc. Am. J. **43**:931–936.
12. **Crutzen, P. J.** 1976. Upper limits on atmospheric ozone reductions following increased application of fixed nitrogen to soil. Geophys. Res. Lett. **3**:169–172.
13. **Delwiche, C. C.** 1978. Biological production and utilization of N_2O. Pure Appl. Geophys. **116**:414–422.
14. **Dodds, W. K., and R. D. Jones.** 1987. Potential rates of nitrification and denitrification in an oligotrophic freshwater sediment system. Microb. Ecol. **14**:91–100.
15. **Drever, J. I.** 1982. The geochemistry of natural waters. Prentice-Hall, Inc., Englewood Cliffs, N.J.
16. **Elliott, R. G., and C. M. Gilmour.** 1971. Growth of *Pseudomonas stutzeri* with nitrate and oxygen as terminal electron acceptors. Soil Biol. Biochem. **3**:331–335.
17. **Federle, T. W., D. C. Dobbins, J. R. Thornton-Manning, and D. D. Jones.** 1986. Microbial biomass, activity, and community structure in subsurface soils. Ground Water **24**:365–374.
18. **Firestone, M. K., R. B. Firestone, and J. M. Tiedje.** 1979. The influence of nitrate, nitrite, and oxygen on the composition of the gaseous products of denitrification in soil. Soil Sci. Soc. Am. J. **43**:1140–1144.
19. **Firestone, M. K., R. B. Firestone, and J. M. Tiedje.** 1980. Nitrous oxide from soil denitrification: factors controlling its biological production. Science **208**:749–751.
20. **Forman, D., S. Al-Dabbagh, and R. Dolt.** 1985. Nitrates, nitrites and gastric cancer in Great Britain. Nature (London) **313**: 620–625.
21. **Foster, S. S. D., A. C. Cripps, and A. Smith-Carington.** 1982. Nitrate leaching to groundwater. Philos. Trans. R. Soc. London Ser. A **296**:477–489.
22. **Freney, J. R., O. T. Denmead, and J. R. Simpson.** 1978. Soil as a source or sink for atmospheric nitrous oxide. Nature (London) **273**:530–532.
23. **Freney, J. R., O. T. Denmead, and J. R. Simpson.** 1979. Nitrous oxide emission from soils at low moisture contents. Soil Biol. Biochem. **11**:167–173.
24. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. **66**:109–120.
25. **Fujihara, S., T. Nakashima, and Y. Kurogochi.** 1986. Determination of $^{15}NH_3^+$ by gas chromatography-mass spectrometry. Application to the measurement of putrescine oxidation by human plasma. J. Chromatogr. **383**:271–280.
26. **Gehlen, M., H. J. Trampisch, and W. Dott.** 1985. Physiological characterization of heterotrophic bacterial communities from selected aquatic environments. Microb. Ecol. **11**:205–219.
27. **Ghiorse, W. C., and D. L. Balkwill.** 1983. Enumeration and morphological characterization of bacteria indigenous to subsurface environments. Dev. Ind. Microbiol. **24**:213–224.
28. **Gibbs, M. M.** 1979. Groundwater input to Lake Taupo, New Zealand: nitrogen and phosphorus inputs from Taupo township. N.Z. J. Sci. **22**:235–241.

28a. **Golterman, H. L.** 1971. Methods for chemical analysis of fresh waters. IBP handbook no. 8. Blackwell Scientific Publications, Oxford.

29. **Gormly, J. R., and R. F. Spalding.** 1979. Sources and concentrations of nitrate-nitrogen in groundwater of the Central Platte Region, Nebraska. Ground Water **17**:291–301.
30. **Hattori, A.** 1983. Denitrification and dissimilatory nitrate reduction, p. 191–232. *In* E. J. Carpenter and D. G. Capone (ed.), Nitrogen in the marine environment. Academic Press, Inc., New York.
31. **Howard, K. W. F.** 1985. Denitrification in a major limestone aquifer. J. Hydrol. **76**:265–280.
32. **Kaspar, H. F., R. A. Asher, and I. C. Boyer.** 1985. Microbial nitrogen transformations in sediments and inorganic nitrogen fluxes across the sediment/water interface on the South Island west coast, New Zealand. Estuarine Coastal Shelf Sci. **21**: 245–255.
33. **Katz, B. G., J. B. Lindner, and S. E. Ragone.** 1980. A comparison of nitrogen in shallow groundwater from sewered and unsewered areas, Nassau County, New York, from 1952 through 1976. Ground Water **18**:607–616.
34. **Keeney, D. R., R. L. Chen, and D. A. Graetz.** 1971. Importance of denitrification and nitrate reduction in sediments to the nitrogen budget in lakes. Nature (London) **233**:66–67.
35. **Klapwijk, A., J. C. M. van der Hoeven, and G. Lettinga.** 1981. Biological denitrification in an upflow sludge blanket reactor. Water Res. **15**:1–6.
36. **Knowles, R.** 1981. Denitrification. Ecol. Bull. **33**:315–329.
37. **Koike, I., and A. Hattori.** 1978. Denitrification and ammonia formation in anaerobic coastal sediments. Appl. Environ. Microbiol. **35**:278–282.
38. **Koskinen, W. C., and D. R. Keeney.** 1982. Effect of pH on the rate of gaseous products of denitrification in a silt loam soil. Soil Sci. Soc. Am. J. **46**:1165–1167.
39. **Kristjansson, J. K., B. Walter, and T. C. Hollocher.** 1978. Respiration-dependent proton translocation and transport of nitrate and nitrite in *Paracoccus denitrificans* and other denitrifying bacteria. Biochemistry **23**:5014–5019.
40. **Krul, J. M., and R. Veeningen.** 1977. The synthesis of the

dissimilatory nitrate reductase under aerobic conditions in a number of denitrifying bacteria, isolated from activated sludge and drinking water. Water Res. **11**:39–43.

41. **Lloyd, D., L. Boddy, and K. J. P. Davies.** 1987. Persistence of bacterial denitrification capacity under aerobic conditions: the rule rather than the exception. FEMS Microb. Ecol. **45**:185–190.
42. **MacRae, D. C., R. R. Ancajas, and S. Salandanan.** 1968. The fate of nitrate nitrogen in some tropical soils following submergence. Soil Sci. **105**:327–334.
43. **Marxsen, J.** 1988. Investigations into the number of respiring bacteria in groundwater from sandy and gravelly deposits. Microb. Ecol. **16**:65–72.
44. **McElroy, M. B., S. C. Wofsy, and Y. L. Yung.** 1977. The nitrogen cycle: pertubations due to man and their impact on atmospheric N_2O and O_3. Philos. Trans. R. Soc. London Ser. B **277**:159–181.
45. **Meiberg, J. B. M., P. M. Bruinenberg, and W. Harder.** 1980. Effect of dissolved oxygen tension on the metabolism of methylated amines in *Hyphomicrobium X* in the absence and presence of nitrate: evidence for "aerobic" denitrification. J. Gen. Microbiol. **120**:453–463.
46. **Morris, J. T., G. J. Whiting, and F. H. Chapelle.** 1988. Potential denitrification rates in deep sediments from the southeastern coastal plain. Environ. Sci. Technol. **22**:832–836.
47. **Payne, W. J.** 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. **37**:409–452.
48. **Petrusewicz, K., and A. Macfadyen.** 1970. Productivity of terrestrial animals. Principles and methods. IBP handbook no. 13. Blackwell Scientific Publications, Oxford.
49. **Porter, K. S.** 1980. An evaluation of sources of nitrogen as causes of groundwater contamination in Nassau County, Long Island. Ground Water **18**:617–625.
50. **Robertson, F. N.** 1979. Evaluation of nitrate in the groundwater in the Delaware Coastal Plain. Ground Water **17**:328–337.
51. **Robertson, G. P., and J. M. Tiedje.** 1987. Nitrous oxide sources in aerobic soils: nitrification, denitrification and other biological processes. Soil Biol. Biochem. **19**:187–193.
52. **Robertson, L. A., and J. G. Kuenen.** 1984. Aerobic denitrification: a controversy revived. Arch. Microbiol. **139**:351–354.
53. **Ryden, J. C.** 1981. N_2O exchange between a grassland soil and the atmosphere. Nature (London) **292**:235–237.
54. **Sacks, L. E., and H. A. Barker.** 1949. The influence of oxygen on the nitrate and nitrite reduction. J. Bacteriol. **58**:11–22.
55. **Saffigna, P. G., and D. R. Keeney.** 1977. Nitrate and chloride in groundwater under irrigated agriculture in central Wisconsin. Ground Water **15**:170–177.
56. **Sewell, P. L.** 1982. Urban groundwater as a possible nutrient source for an estuarine benthic algal bloom. Estuarine Coastal Shelf Sci. **15**:569–576.
57. **Simpkin, T. J., and W. C. Boyle.** 1988. The lack of repression by oxygen of the denitrifying enzymes in activated sludge. Water Res. **22**:201–206.
58. **Slater, J. M., and D. G. Capone.** 1987. Denitrification in aquifer soil and nearshore marine sediments influenced by groundwater nitrate. Appl. Environ. Microbiol. **53**:1292–1297.
59. **Smith, M. S.** 1982. Dissimilatory reduction of NO_2^- to NH_4^+ and N_2O by a soil *Citrobacter* sp. Appl. Environ. Microbiol. **43**:854–860.
60. **Smith, M. S., and K. Zimmerman.** 1981. Nitrous oxide production by nondenitrifying soil nitrate reducers. Soil Sci. Soc. Am. J. **45**:865–871.
61. **Smith, R. L., and J. H. Duff.** 1988. Denitrification in a sand and gravel aquifer. Appl. Environ. Microbiol. **54**:1071–1078.
62. **Sørenson, J.** 1978. Capacity for denitrification and reduction of nitrate to ammonia in a coastal marine sediment. Appl. Environ. Microbiol. **35**:301–305.
63. **Spalding, R. F., J. R. Gormly, B. H. Curtiss, and M. E. Exner.** 1978. Nonpoint nitrate contamination of groundwater: a field study. J. Contam. Hydrol. **1**:143–161.
64. **Stanford, G., J. O. Legg, S. Dzienia, and E. C. Simpson, Jr.** 1975. Denitrification and associated nitrogen transformations in soils. Soil Sci. **120**:147–152.
65. **Stanford, G., R. A. van der Pol, and S. Dzienia.** 1975. Denitrification rates in relation to total and extractable soil carbon. Soil Sci. Soc. Am. Proc. **39**:284–289.
66. **Starr, J. L., and J.-V. Parlange.** 1975. Non-linear denitrification kinetics with continuous flow in soil columns. Soil Sci. Soc. Am. Proc. **39**:875–880.
67. **Thorn, P. M., and R. M. Ventullo.** 1988. Measurement of bacterial growth rates in subsurface sediments using the incorporation of tritiated thymidine into DNA. Microb. Ecol. **16**:3–16.
68. **Tiedje, J. M., A. J. Sexstone, D. D. Myrold, and J. A. Robinson.** 1982. Denitrification: ecological niches, competition and survival. Antonie van Leeuwenhoek J. Microbiol. **48**:569–583.
69. **Tirén, T., J. Thorin, and H. Nömmik.** 1976. Denitrification measurements in lakes. Acta Agric. Scand. **26**:175–184.
70. **Trudell, M. R., R. W. Gillham, and J. A. Cherry.** 1986. An in-situ study of the occurrence and rate of denitrification in a shallow unconfined sand aquifer. J. Hydrol. **83**:251–268.
71. **Vogel, J. C., A. S. Talma, and T. H. E. Heaton.** 1981. Gaseous nitrogen as evidence for denitrification in groundwater. J. Hydrol. **50**:191–200.
72. **Weier, K. L., and J. W. Gilliam.** 1986. Effect of acidity on denitrification and nitrous oxide evolution from coastal plain soils. Soil Sci. Soc. Am. J. **50**:1202–1205.