APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1997, p. 931–937 Vol. 63, No. 3
0099-2240/97/$04.00+0

Effects of Nitrate Availability and the Presence of *Glyceria maxima* on the Composition and Activity of the Dissimilatory Nitrate-Reducing Bacterial Community†

JOHANNA W. NIJBURG,[1]* MARCO J. L. COOLEN,[1] SASKIA GERARDS,[1]
PAULIEN J. A. KLEIN GUNNEWIEK,[1] AND HENDRIKUS J. LAANBROEK[2]

Department of Plant-Microorganism Interactions, Centre for Terrestrial Ecology,[1] and Department of Microbial Ecology, Centre for Limnology,[2] Netherlands Institute of Ecology, 6666 ZG Heteren, The Netherlands

Received 10 June 1996/Accepted 6 December 1996

The effects of nitrate availability and the presence of *Glyceria maxima* on the composition and activity of the dissimilatory nitrate-reducing bacterial community were studied in the laboratory. Four different concentrations of NO_3^-, 0, 533, 1434, and 2,905 μg of NO_3^--N g of dry sediment^{-1}, were added to pots containing freshwater sediment, and the pots were then incubated for a period of 69 days. Upon harvest, NH_4^+ was not detectable in sediment that received 0 or 533 μg of NO_3^--N g of dry sediment^{-1}. Nitrate concentrations in these pots ranged from 0 to 8 μg of NO_3^--N g of dry sediment^{-1} at harvest. In pots that received 1,434 or 2,905 μg of NO_3^--N g of dry sediment^{-1}, final concentrations varied between 10 and 48 μg of NH_4^+-N g of dry sediment^{-1} and between 200 and 1,600 μg of NO_3^--N g of dry sediment^{-1}, respectively. Higher input levels of NO_3^- resulted in increased numbers of potential nitrate-reducing bacteria and higher potential nitrate-reducing activity in the rhizosphere. In sediment samples from the rhizosphere, the contribution of denitrification to the potential nitrate-reducing capacity varied from 8% under NO_3^--limiting conditions to 58% when NO_3^- was in ample supply. In bulk sediment with excess NO_3^-, this percentage was 44%. The nitrate-reducing community consisted almost entirely of NO_2^--accumulating or NH_4^+-producing gram-positive species when NO_3^- was not added to the sediment. The addition of NO_3^- resulted in an increase of denitrifying *Pseudomonas* and *Moraxella* strains. The factor controlling the composition of the nitrate-reducing community when NO_3^- is limited is the presence of *G. maxima*. In sediment with excess NO_3^-, nitrate availability determines the composition of the nitrate-reducing community.

There are two dissimilatory nitrate-reducing pathways that can be distinguished: denitrification and dissimilatory nitrate reduction to ammonia (DNRA). Due to the production of nitric oxide (NO) and nitrous oxide (N_2O), which are environmentally harmful gasses, denitrification has received considerable attention over the last 15 to 20 years (7, 33, 34). Denitrification is the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and, in general, dinitrogen gas (N_2). Denitrification leads to nitrogen losses from soils and sediments. In contrast, DNRA, the reduction of NO_3^- to NO_2^- and ammonia (NH_4^+), leads to nitrogen conservation. DNRA is of minor significance in terrestrial soils (35) but plays a greater role in water-saturated systems, such as freshwater and estuarine sediments (9, 13–15, 17, 33). The two dissimilatory nitrate-reducing processes are performed by two different groups of microorganisms. Most denitrifiers are obligatory oxidative organisms (19, 34), whereas bacteria that dissimilate NO_3^- to NH_4^+ have a fermentative metabolism in most cases (33). The availability of suitable electron acceptors (i.e., organic carbon) and the amount of available electron donor (i.e., NO_3^-) determine to a large extent which nitrate-reducing process will occur. The ratio between available donor and acceptor is also important (34). In anoxic waterlogged sediments without NO_3^-, only the bacteria that dissimilate NO_3^- to NH_4^+ will be able to grow due to their fermentative abilities.

Aerenchymatous plants create an oxic-anoxic interface in the root zone by releasing oxygen into this zone. The release of oxygen by the roots may stimulate nitrification (1, 3, 10, 25) and subsequently denitrification after diffusion of NO_3^- into the reduced zone of the sediment. A correlation between nitrification and denitrification has been observed in the rhizosphere of aerenchymatous plants (3, 24). Furthermore, partially submerged aerenchymatous plants also affect the nitrate-reducing microbiota due to uptake of nitrate (7, 12) and excretion of organic compounds into the rhizosphere (23, 26).

Glyceria maxima (Hartm.) Holmb. (reed sweet grass), an aerenchymatous plant common in The Netherlands, releases considerable amounts of oxygen into the root zone (5). In the rhizosphere of *G. maxima*, large numbers of obligatory aerobic nitrifying bacteria have been detected, indicating that oxygen must be available to these bacteria (4). The potential nitrate-reducing bacteria have been studied in the presence and absence of *G. maxima* in terms of both species composition and nitrate metabolism (21). From this study, the hypothesis that *G. maxima* has a great influence on the composition of the nitrate-reducing community when NO_3^- is limiting was put forward. When sufficient NO_3^- is available, the effect of *G. maxima* is minimal. However, there was no direct correlation between NO_3^- availability and the quantitative increase in the total number and activity of the potential nitrate-reducing bacteria. Since nitrate-reducing bacteria are common members of the total organotrophic bacterial community in sediments, their presence in the rhizosphere and bulk sediment does not necessarily mean that their proliferation has been due to ni-

* Corresponding author. Mailing address: Centre for Terrestrial Ecology, P.O. Box 40, 6666 ZG Heteren, The Netherlands. Phone: (31)-(0)26 4791306. Fax: (31)-(0)26 4723227. E-mail: Nijburg@cto.nioo.knaw.nl.

† Publication number 2200, Centre for Terrestrial Ecology, Netherlands Institute of Ecology.

trate reduction. Other factors might be responsible for their growth in the rhizosphere and bulk sediment.

In this study, quantitative measurements concerning the species composition and the numbers and activity of the nitrate-reducing community in the presence of *G. maxima* and with the addition of different amounts of NO_3^- were performed. The aim of the study was to determine if the presence of *G. maxima* or the availability of NO_3^- controls the composition and activity of the dissimilatory nitrate-reducing community.

MATERIALS AND METHODS

Sampling location and procedure. Bulk sediment (the upper 0 to 15 cm) was collected in December 1991 from Lake Drontermeer (52°58′N, 5°50′E) close to *G. maxima* vegetation. The sediment was transported to the laboratory in airtight buckets at 4°C. The sediment was thoroughly mixed in the laboratory, and the initial properties were determined. Each of 24 500-ml pots was filled with 850 g of moist sediment. The water content of the sediment was 35% ± 3.7% (wt/vol). To study the effect of different NO_3^- inputs on the dissimilatory nitrate-reducing community in the presence of *G. maxima*, the pots were incubated under four different conditions. Three nonsterile seedlings were planted in each pot. Water was added to the pots three times a week to compensate for evapotranspiration by the plants. During incubation there was ~2 cm of water above the sediment. The water was enriched with different concentrations of KNO_3: 0, 10, 50, or 100 mM. The solutions were added by using syringes with long needles to distribute the NO_3^- solution as evenly as possible throughout the sediment. The pots were placed in a plant growth chamber (Vötsch HPS-1500; Heraeus, Wijk bij Duurstede, The Netherlands) with a light-dark regime of 16 h-8 h (light intensity, ~200 $\mu mol \cdot m^{-2} \cdot s^{-1}$), a temperature of 20 to 15°C, and air humidity of 80%. To monitor the NO_3^-, NO_2^-, and NH_4^+ concentrations during the incubation period, rhizon sediment solution samples (6 cm long; nominal pore size, 0.1 μm; Eijkelkamp, Giesbeek, The Netherlands) were inserted into the pots below the water-sediment interface at two depths (top layer, 5.5 cm; bottom layer, 10.5 cm). Interstitial water samples were taken three times a week from the top and bottom layers to monitor changes in inorganic nitrogen concentrations. It became clear that the young seedlings in pots receiving 100 mM KNO_3 were not able to grow, probably due to the high salt level. Nevertheless, the addition of NO_3^- to these pots was continued. From these pots only bulk sediment could be harvested, whereas from all other pots only rhizosphere sediment was harvested after 69 days of incubation.

Sediment determinations. Throughout the incubation period, concentrations of NO_3^-, NO_2^-, and NH_4^+ were determined in the interstitial water samples. The samples were analyzed with a Traacs 800 autoanalyzer (Technicon Instruments Corp., Tarrytown N.Y.) with a detection level of 10 μM for all three compounds. After harvest, total N was measured spectrophotometrically in digests obtained by treating a sediment sample with a mixture of H_2SO_4-Se and salicylic acid (22). The pH(H_2O) of the sediments was determined by shaking 5 g of moist sediment with 10 ml of water for 2 h. The pH(KCl) and the inorganic nitrogen concentrations were determined by shaking 5 g of moist sediment with 10 ml of 1 M KCl for 2 h. After being shaken, samples were centrifuged at 15,000 × *g* in a Biofuge A bench centrifuge (Heraeus Christ, Dijkstra Verenigde, Almere, The Netherlands) for 10 min and the supernatants were analyzed as described above. The total organic carbon of the sediment was determined according to the Mebius procedure (20).

Plant determinations. The total dry weight of the shoots and roots was determined for each pot by drying the plants for 2 weeks at 70°C. Total nitrogen was determined as described above (22).

Determination of the total number of potential nitrate-reducing bacteria. The total number of potential nitrate-reducing bacteria was determined before the incubation and after harvest by plating dilution series onto agar (1%, wt/vol) plates containing nutrient broth (NB) medium enriched with 10 mM KNO_3 (33). Depending on the amount of sediment available, 1.26 to 4.45 g of moist sediment was suspended in 9 ml of phosphate buffer (per liter, 1.34 g of Na_2HPO_4, 0.35 g of NaH_2PO_4, and 8.5 g of NaCl; pH 7.3 to 7.5) for the dilution series. For each pot and sediment type, the total number of bacteria was determined by five independent dilution series. The tubes (10^{-3} through 10^{-8} dilutions) were placed in an ultrasonic bath two times for 15 s each. For every tube, 0.1 ml of suspension was spread onto each of five NB plates. The plates were incubated anoxically for 2 weeks at 20°C. The total number of potential nitrate-reducing bacteria was determined on 100% NB and on 10% NB to examine the influence of different carbon concentrations in the selective medium on the total number of detectable potential nitrate-reducing bacteria.

Determination of potential nitrate-reducing activity. The medium used to determine the potential nitrate-reducing activity contained 4.5 mM KNO_3, 10 mM D-glucose, and 22.0 mM KH_2PO_4 at pH 7.2. The measurements were performed in 1-liter serum flasks each containing 500 ml of medium. Four activity determinations were conducted for rhizosphere sediment samples; bulk sediment activities were performed in duplicate. Depending on the amount of sediment available, 11.21 to 77.40 g of moist sediment was added to each of the flasks. The flasks were flushed for 10 min with N_2, and 10 kPa of acetylene (C_2H_2) was added subsequently to inhibit the reduction of N_2O to N_2. The flasks were incubated at 20 ± 3°C for 24 h with shaking (100 rpm). During the incubation period of 8 h, gas and water samples were taken every hour after C_2H_2 addition and one sample was removed after 24 h to determine the concentrations of N_2O, NO_3^-, NO_2^-, and NH_4^+. The nitrogen concentrations in the water samples were determined as described above. The N_2O concentration was measured with a gas chromatograph (6000 VEGA series 2; Carlo-Erba Instruments, Milan, Italy) equipped with a hot wire detector and a Porapack Q column. N_2 was used as the carrier gas (flow rate, 30 ml per min). The column, injector, and detector temperatures were 80, 120, and 119°C, respectively. The peak area was computed by an integrator (Shimadzu model CR3A; Interscience, Breda, The Netherlands), with a detection limit of approximately 1 μg of N_2O ml^{-1}.

Composition of the nitrate-reducing community. The composition of the nitrate-reducing community was determined before the experiment and after harvesting the pots. The plates used for calculating the total number of potential nitrate-reducing bacteria were used to determine the composition of dominant bacteria of the nitrate-reducing community. Twenty-five colonies from the most diluted positive plates of each treatment (675 colonies total) were randomly picked and isolated as pure cultures on tryptic soy broth (TSB) plates with 1% (wt/vol) agar. The isolated pure cultures were initially divided into different groups by four tests: the Gram test (32), the oxidase test with 1% tetramethyl-*p*-phenylenediamine-HCl, the catalase test with 10% H_2O_2, and the oxidation-fermentation test with glucose as substrate (8). The gram-positive strains were examined microscopically for endospores. The strains were also tested for endospores by pasteurization of old liquid cultures for 15 min at 80°C. Cultures which were able to grow after pasteurization were considered to be *Bacillus* strains. The gram-negative strains were further identified by the API-20NE and API-20E tests (API System, S.A., Montalieu-Vercieu, France) to at least the genus level.

Nitrate metabolism of the nitrate-reducing community. To determine the metabolism of the dominant bacteria of the nitrate-reducing community, the isolated strains were tested in TSB (100%) with and without KNO_3 (10 mM) in 100-ml serum bottles. The bottles were flushed with N_2 gas for 10 min, and subsequently each was injected with 10 kPa of C_2H_2. The bottles were incubated for 2 weeks at 20°C. At the end of the incubation period, the media were tested for N_2O, NO_3^-, NO_2^-, and NH_4^+ concentrations. Based on the major end products formed, the strains were divided into four groups: group I, N_2O producers, reducing >80% of the added NO_3^- to N_2O; group II, NO_2^- accumulators, reducing >50% of the added NO_3^- to NO_2^- and N_2O; group III, NH_4^+ producers, reducing about 50% of the added NO_3^- to NH_4^+, with no NO_3^-, NO_2^-, or N_2O being detectable; and group IV, no nitrate reducers, with only a fraction of the added NO_3^- having disappeared and no NO_2^-, NH_4^+, or N_2O being present. During the isolation and purification procedures, some strains were nonculturable under our laboratory conditions.

Data analysis. Sediment characteristics were analyzed by one-way analysis of variance (ANOVA). Differences between the means were tested for significance by using the least significant difference (LSD) procedure. The means of the data from five plates of the five independent dilution series were also analyzed by one-way ANOVA to determine the total number of potential nitrate-reducing bacteria. The potential nitrate-reducing activity was determined by computing the best line by linear regression after 24 h of incubation. The *r* values were tested for significance at 95 and 99% probability levels.

RESULTS

Interstitial nitrogen concentrations during incubation. The changes in concentrations of the different interstitial N compounds in pots to which different amounts of NO_3^- have been added are given in Fig. 1. NO_2^- was not detectable during the entire course of the incubation period. The NH_4^+ concentration was lowest in pots which were not supplemented with NO_3^- and in pots receiving water enriched with 10 mM KNO_3. In the top layers of pots receiving water enriched with 50 mM KNO_3, NH_4^+ concentrations remained almost constant over the course of the incubation. The NO_3^- concentration in these top layers increased from 9.9 μg of NO_3^--N g of dry sediment^{-1} at the outset of the experiment to about 420 μg of NO_3^--N g of dry sediment^{-1} at harvest. The nitrogen patterns in pots that received water with 100 mM KNO_3 were almost the same as those in the pots that received water with 50 mM KNO_3, except that NH_4^+ production occurred in the top layers near the beginning of the incubation. In the bottom layers, the NO_3^- concentration increased until the end of the experiment.

Sediment and *G. maxima* characteristics. The total amounts of NO_3^- added to the pots during the incubation period are given in Table 1. The ratio of the total amount of NO_3^- added

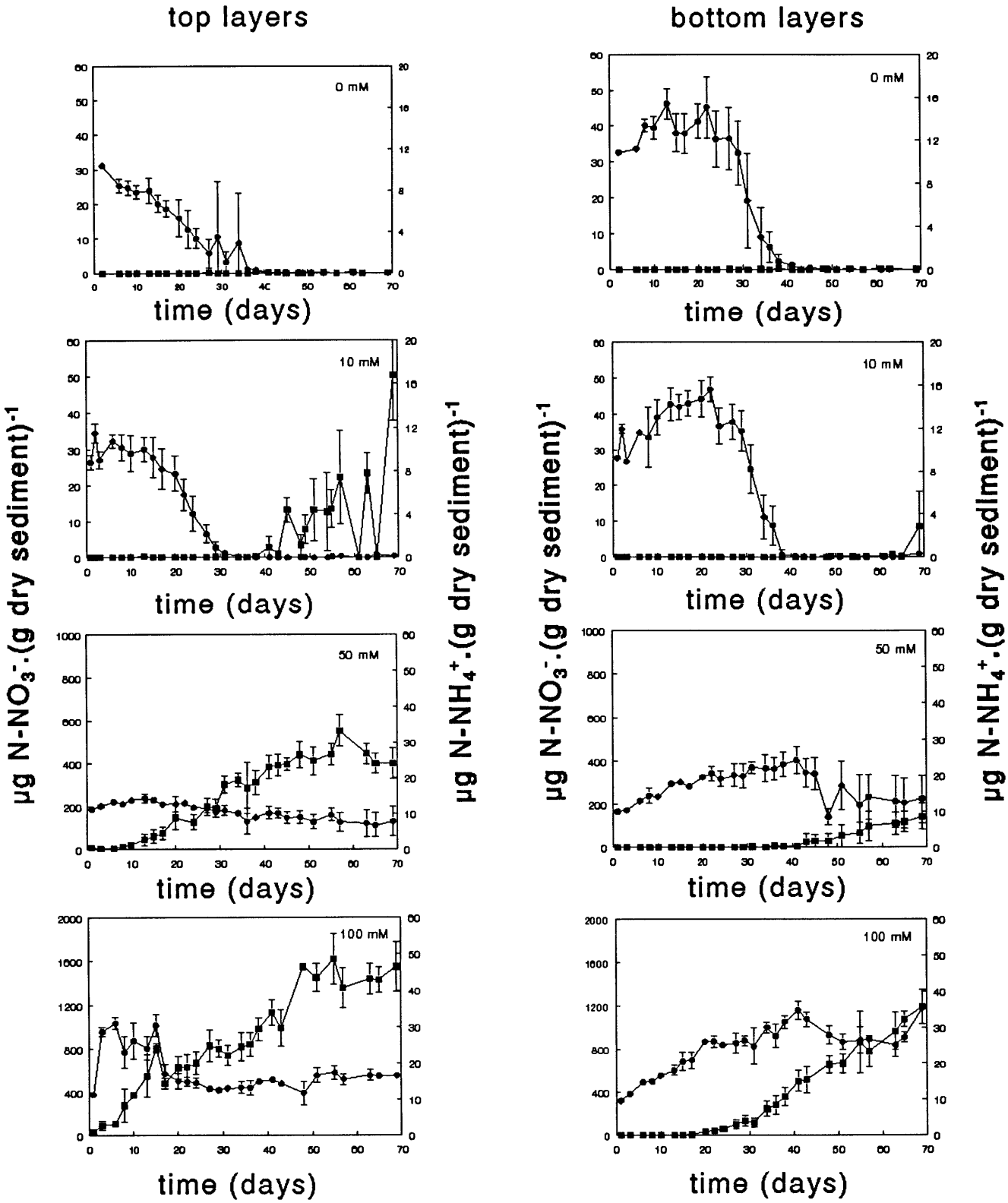

FIG. 1. Interstitial NO_3^- (squares) and NH_4^+ (circles) concentrations (in micrograms of N per gram of dry sediment) during the incubation period in pots receiving different amounts of NO_3^- in the presence of *G. maxima*. Top layers are 5.5 cm below the water/sediment interface; bottom layers are 10.5 cm below the interface. NO_3^- concentrations of water supplies are given in the different panels (0, 10, 50, and 100 mM). Bars represent standard deviations.

TABLE 1. Total nitrate added during incubation, nitrogen concentrations in the sediment and in *G. maxima* after harvest, and percentages of total N taken up by *G. maxima* and in presumptive gaseous products produced during incubation[a]

Concn of nitrate solution added (mM)	Total nitrogen added	Total nitrogen in[b]:				Fate of missing nitrogen (%)	
		Sediment		*G. maxima*		Uptake by plant	Presumably lost in gaseous products
		Rhizosphere	Bulk	Shoot	Root		
0	0	972b		11,508a	7,266a	91	9
10	533	823a		18,746b	11,506b	18	82
50	1,434	914b		44,926c	29,540c	2	98
100	2,905		1,498c			0	84

[a] Unless otherwise stated, concentrations are given in micrograms of N per gram of dry sediment or plant material.
[b] The means of the total nitrogen concentrations in the sediment and in the shoots and roots of *G. maxima* were statistically analyzed by one-way ANOVA followed by LSD. Significant differences ($P < 0.05$) within columns are indicated by different letters. The initial total nitrogen concentration was 1,038 μg of N of dry sediment^{-1}.

was different from the ratio of the initial NO_3^- additions (0:1:2.6:5.4 and 0:1:5:10, respectively). This was due to differences in plant biomass and subsequent evapotranspiration. The total fresh biomass of plants that received no NO_3^- was 25.0 ± 5 g. *G. maxima* plants receiving 533 or 1,434 μg of NO_3^--N g of dry sediment^{-1} had a total fresh biomass of 32.0 ± 9 g or 2.89 ± 1.5 g, respectively. There was no significant difference in the shoot/root ratio between plants that received no NO_3^- and plants provided with 533 μg of NO_3^--N g of dry sediment^{-1}, with values of 1.1 and 0.99, respectively, based on dry weight. In contrast, the roots from plants that received 1,434 μg of NO_3^--N g of dry sediment^{-1} were relatively short, resulting in a shoot/root ratio of 2.15. The seedlings that received 2,905 μg of NO_3^--N g of dry sediment^{-1} were not able to grow and died near the beginning of the experiment, as described earlier. Table 1 shows that despite the addition of NO_3^- to a total of 1,434 μg of NO_3^--N g of dry sediment^{-1}, the total nitrogen concentrations in the sediment at the end of the experiment were still significantly lower than the initial concentration of 1,038 μg of NO_3^--N g of dry sediment^{-1}. Only with the highest NO_3^- addition was the total nitrogen concentration significantly higher than the initial concentration. For calculating the percentage of nitrogen uptake by the plant and the percentage of presumably gaseous products, the assumption was made that in the young seedlings the nitrogen concentration was zero. The decrease in total sediment nitrogen (organic and inorganic nitrogen and added NO_3^-) per pot is the total amount of nitrogen that was either reduced by the nitrate-reducing community or taken up by the roots. The percentage of presumable nitrate reduction to gaseous products increased when more NO_3^- was added, except at 100 mM. In Table 2, the mineral nitrogen concentrations, pH(H_2O), pH(KCl), and organic carbon content of the sediment samples are given. The initial concentration of NO_3^- was almost zero. Only with addition of 1,434 or 2,905 μg of NO_3^--N g of dry sediment^{-1} did the NO_3^- concentration significantly increase. Compared to the initial NH_4^+ concentration of 19.7 μg of NH_4^+-N g of dry sediment^{-1}, the NH_4^+ concentrations decreased significantly during incubation, regardless of the NO_3^- addition. The pH(H_2O) and pH(KCl) increased with increasing NO_3^- concentration, especially for the two highest NO_3^- concentrations added. The organic carbon percentage significantly decreased in the rhizosphere and in the bulk sediment when NO_3^- was added.

Total number of potential nitrate-reducing bacteria. The results (Table 3) showed that addition of NO_3^- resulted in a significant increase in the total number of CFU. The total number of CFU determined on 10% NB was not significantly different from that determined on 100% NB (data not shown). The initial number of potential nitrate-reducing CFU was 2.52×10^6 g dry sediment^{-1}. The total number of CFU in pots receiving no NO_3^- was not significantly different from the number at the beginning of the incubation. For the dominant isolated strains, the percentages of strains nonculturable on TSB and non-nitrate-reducing strains are also given in Table 3. The total number of culturable nitrate-reducing strains can be calculated assuming that the colonies chosen for study are representative of all CFU detected. The total number of potential nitrate-reducing strains in the rhizosphere significantly increased with increasing NO_3^- addition.

Potential nitrate-reducing activity. The potential nitrate-reducing activities in the rhizosphere of *G. maxima* and bulk sediment incubated with different concentrations of NO_3^- are given in Table 4. The nitrogen conversions were calculated by linear regression for a 24-h incubation period. The NO_3^--N decrease and the N_2O-N increase were significant for all incubations, whereas the NH_4^+-N increase was not significant. NO_2^- was detectable in low concentrations at all sampling times during all incubations (data not shown). In the rhizosphere with NO_3^- addition, the nitrate reduction rate was 2.4

TABLE 2. Sediment parameters before starting the experiment ($t = 0$) and after harvest[a]

Total NO_3^--N added (μg dry sediment^{-1})	Sediment type	Concn of N as:		pH		Organic C (%)
		NO_3^-	NH_4^+	H_2O	KCl	
0 ($t = 0$)	Nonrhizosphere	0.0a	19.7d	7.5c	7.3ab	1.6c
0	Rhizosphere	0.1a	2.8a	7.2a	7.2a	1.4bc
533	Rhizosphere	19.0a	2.1a	7.4b	7.4b	1.3b
1,434	Rhizosphere	209.0b	4.0b	7.7d	7.7c	1.0a
2,905	Nonrhizosphere	996.0c	14.1c	7.8d	7.9d	1.0a

[a] Concentrations are in micrograms of N per gram of dry sediment. Significant differences ($P < 0.05$) between the means within columns, as determined by one-way ANOVA followed by LSD, are indicated by different letters.

TABLE 3. Effect of addition of different amounts of nitrate on total number of CFU and potential nitrate-reducing strains in the rhizosphere of *G. maxima* and in bulk sediment[a]

Total nitrogen added (μg g dry sediment^{-1})	Type of sediment	Total CFU[b]	Total no. of strains isolated	% Nonculturable strains	% Non-nitrate-reducing strains	No. of potential nitrate-reducing strains[b]
0	Rhizosphere	3.2×10^6a	200	36	39	0.8×10^6a
533	Rhizosphere	4.6×10^7b	250	29	34	1.7×10^7b
1,434	Rhizosphere	3.3×10^8c	125	14	15	2.3×10^8c
2,905	Bulk	11.7×10^8d	100	42	11	5.5×10^8d

[a] For the dominant strains isolated ($N = 675$), the percentages of non-TSB-culturable strains and non-nitrate-reducing strains also are presented, resulting in the total number of potential nitrate-reducing strains per gram of dry sediment.

[b] The means of five independent dilution series were analyzed by one-way ANOVA followed by LSD. Significant differences ($P < 0.05$) within columns are indicated by different letters. The initial number of potential nitrate-reducing CFU was 2.52×10^6 g of dry sediment^{-1}.

times higher than in that without NO_3^- addition. There was no electron donor limitation during the potential nitrate-reducing activity measurements, as glucose was still detectable in the medium after incubation (data not shown). Since the regression of the NH_4^+ production was not significant in all incubations, results of the DNRA were not presented.

Composition of the nitrate-reducing community. Although the percentage of strains not able to reduce nitrate was rather high in our experiment (Table 3), the genus composition did not change when these non-nitrate-reducing strains were excluded from the total number of culturable strains. The results presented do represent the dominant culturable potential nitrate-reducing bacteria. At the beginning of the experiment, the dominant nitrate-reducing community consisted of denitrifying *Pseudomonas fluorescens* strains. In Fig. 2, the genus composition of the dominant culturable bacteria of the nitrate-reducing community is given. Without NO_3^- addition, the nitrate-reducing community in the rhizosphere consisted almost completely (84%) of gram-positive species—58% *Bacillus* species, 8% rods, and 18% cocci—with only a small percentage gram-negative *Aeromonas*, *Moraxella*, and *Pseudomonas* species. With the addition of 533 μg of NO_3^--N g of dry sediment^{-1}, the composition of the nitrate-reducing community changed only slightly. When 1,434 μg of NO_3^--N g of dry sediment^{-1} was added, only 5% of the isolated strains were gram positive. *Moraxella* species showed the most striking increase, from 0% when 533 μg of NO_3^--N g of dry sediment^{-1} was added during incubation to 44% with addition of 1,434 μg of NO_3^--N g of dry sediment^{-1}. In the bulk sediment, the community consisted of 96% *Pseudomonas* and *Moraxella* species. The dominant *Pseudomonas* strains were further identified as *P. chlororaphis*, *P. fluorescens*, and *P. alcaligenes.*

Nitrate metabolism of the nitrate-reducing community. In Fig. 3, the nitrate metabolism of the dominant culturable bacteria of the nitrate-reducing community is given in relation to different amounts of added NO_3^-. When no NO_3^- was added, the nitrate-reducing community consisted of 90% NO_2^- accumulators and NH_4^+ producers. With addition of 533 μg of NO_3^--N g of dry sediment^{-1}, the changes were minimal. When 1,434 μg of NO_3^--N g of dry sediment^{-1} was added, the denitrifying strains became dominant (65%) and the percent-

TABLE 4. Potential nitrate-reducing activities in the rhizosphere of *G. maxima* and bulk sediment after incubation with different amounts of added nitrate[a]

Nitrate added (μg)	Type of sediment	Rate of[a]:	
		Nitrate reduction	N_2O production[b]
0	Rhizosphere	16.7**	0.7** (8)
533	Rhizosphere	39.7**	3.5** (18)
1,434	Rhizosphere	39.8*	11.6** (58)
2,905	Bulk	20.6**	4.5** (44)

[a] The activities were calculated by linear regression. The values were significant at the $P < 0.01$ (**) or the $P < 0.05$ (*) level.

[b] The values in parentheses are the percentages of nitrate reduced to N_2O. Rates are given in micrograms of N per gram of dry sediment per hour.

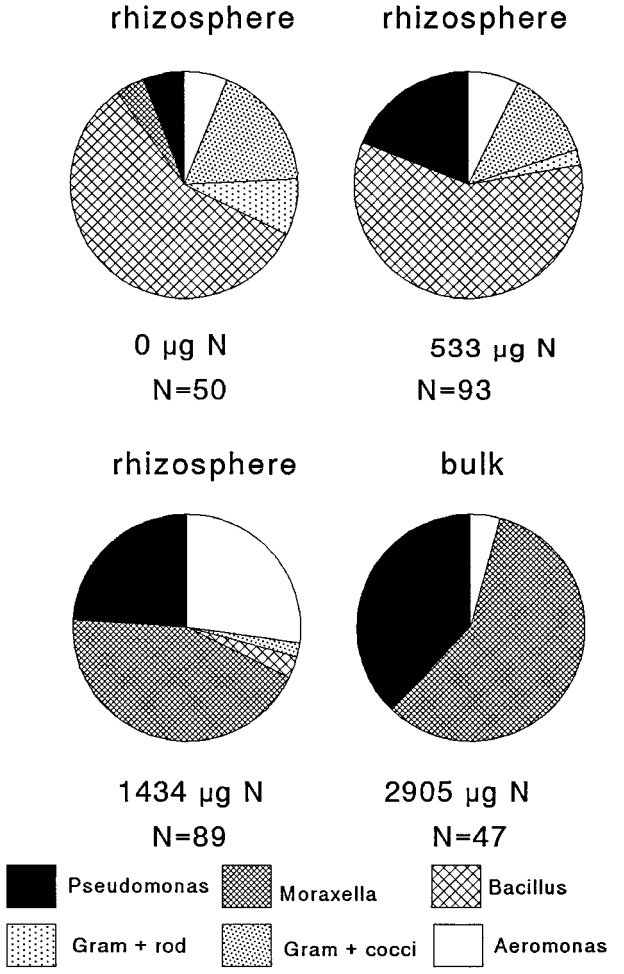

FIG. 2. Genus composition of the culturable dominant bacteria of the nitrate-reducing community in the rhizosphere and bulk sediment of *G. maxima* in relation to addition of different concentrations of nitrate (in micrograms of NO_3^--N per gram of dry sediment). N, number of culturable nitrate-reducing strains (total strains tested, 675).

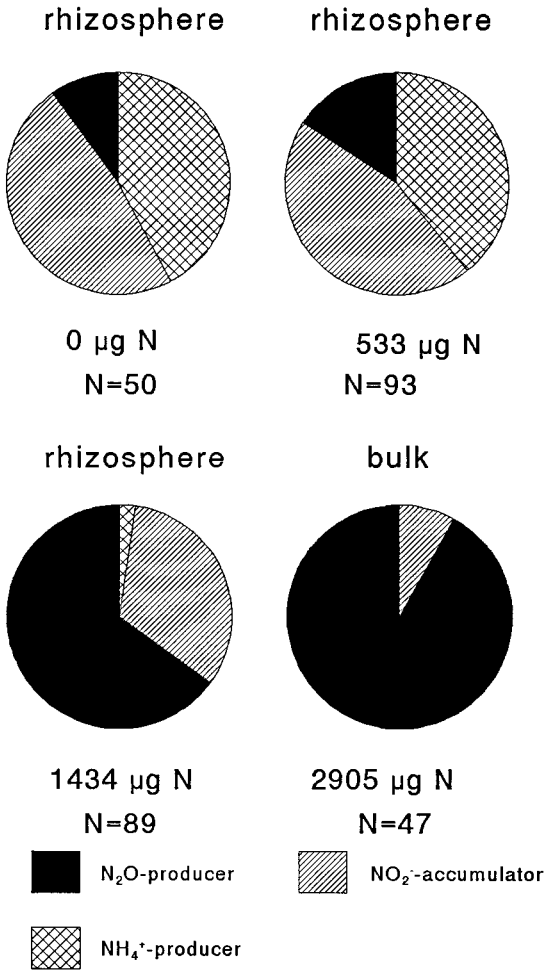

FIG. 3. Nitrate metabolism of the culturable dominant bacteria of the nitrate-reducing community in the rhizosphere and bulk sediment of *G. maxima* in relation to addition of different concentrations of nitrate (in micrograms of NO_3^--N per gram of dry sediment). N, number of culturable nitrate-reducing strains (total strains tested, 675).

age of NH_4^+-producing strains decreased to 2%. In the bulk sediment, the nitrate-reducing community consisted almost completely of denitrifying strains.

DISCUSSION

Interstitial nitrogen concentrations. The NH_4^+ patterns in pots to which NO_3^- was not added can be explained by the uptake of NH_4^+ by *G. maxima*. In the beginning of the experiment, the roots of *G. maxima* were most likely abundant only in the upper layers of the pot, explaining the immediate decrease of NH_4^+ in this zone during the first 30 days (Fig. 1). With increasing length of incubation, the roots penetrated deeper into the sediment and the NH_4^+ concentration decreased in the bottom layers as well. The increase of NO_3^- in the top layers in pots receiving water enriched with 10 mM KNO_3 may indicate that the NO_3^--consuming processes were less important in the top layers than in the bottom layers. This can be explained by the higher root biomass, which leads to increased uptake of nitrogen, or by the more anoxic environment in the bottom layers, resulting in a higher nitrate-reducing rate. NH_4^+ was produced in the bottom layers of pots receiving water enriched with 50 mM KNO_3 during the first 40 days. This can be explained by the dissimilatory reduction of NO_3^- to NH_4^+ or mineralization. The uptake of nitrogen by *G. maxima* is negligible, since the roots did not reach the bottoms of these pots. In the bottom layers of pots receiving water enriched with 100 mM KNO_3, NH_4^+ was produced from the outset of incubation. This may have been caused by mineralization which was not compensated for by nitrogen uptake by *G. maxima*, as the plants in these pots died.

The dissimilatory nitrate-reducing bacterial community. The percentage of isolated strains that were nonculturable under our laboratory conditions was on the same order as those for strains isolated from various soils and sediments (2, 11). When no NO_3^- was added to the rhizosphere sediment, only 9% of the nitrogen was presumably denitrified (Table 1). The denitrification activity accounted for 8% of the total potential nitrate-reducing activity (Table 4), and 90% of the community consisted of NO_2^--accumulating or NH_4^+-producing gram-positive strains (Fig. 2 and 3). In estuarine sediments, denitrification accounted for 2.5 to 13% of the total potential nitrate-reducing activity under nitrate-limiting conditions as determined by [^{15}N]nitrate addition (14). When NO_3^- was added to the pots, the total number of potential nitrate-reducing bacteria (Table 3), the potential nitrate-reducing activity, and the percentage of N_2O derived from NO_3^- reduction all increased (Table 4). In sediment receiving 533 µg of N-NO_3^- g of dry sediment^{-1}, the potential nitrate-reducing rate could have been overestimated due to NO_3^- immobilization in the absence of NH_4^+. Chloramphenicol was not added during potential nitrate-reducing activity measurements, but sediments from pots receiving the two largest amounts of NO_3^- contained NH_4^+, which is known to inhibit NO_3^- assimilation (13). In the rhizosphere, with addition of increasing amounts of NO_3^-, denitrification accounted for a greater percentage of the total nitrate reduction, increasing from 8 to 58%. This elevated percentage, as well as the increase in the total number of potential nitrate-reducing bacteria in the rhizosphere, may be explained by the release of oxygen and exudates by *G. maxima* (1, 3, 7, 23, 24, 29). The denitrification activity in the rhizosphere can be stimulated only when sufficient N oxides are present in the sediment (16, 17, 29). In estuarine sediments, nitrate reduction was higher at high NO_3^- concentrations than at low NO_3^- concentrations (14, 15). The increasing numbers of potential nitrate-reducing bacteria in our experiments could indicate that NO_3^- was the limiting factor for NO_3^- reduction when no or little NO_3^- was added. The roots probably successfully compete with the nitrate-reducing community for NO_3^-. Increased NO_3^- reduction to N_2O, as observed with addition of increasing amounts of NO_3^-, could be due to denitrifiers having a competitive advantage over fermentative, dissimilatory bacteria which reduce nitrate to NH_4^+ in the utilization of NO_3^-. *Pseudomonas* strains increased in numbers, at the expense of the gram-positive, NO_2^--accumulating rods and cocci (Fig. 2). However, it is also possible that NO_3^- was first reduced to NO_2^- by fermentative bacteria and the NO_2^- was further reduced to N_2O by denitrifying bacteria. Fermentative bacteria of the genus *Aeromonas* were still members of the nitrate-reducing community after addition of NO_3^- to high levels. In bulk sediment with the greatest amount of NO_3^- added, the total number of potential nitrate-reducing bacteria did not further increase (Table 3) whereas the percentage of N_2O produced by the reduction of NO_3^- decreased in the potential nitrate-reducing assays (Table 4). Explanations for the incomplete nitrogen balance observed during the measurements of potential denitrifying activity could be the insufficient inhibition of N_2O reduction by acetylene (27, 28) or the sensitivity of nitrite reductase to oxygen (31, 36).

Research concerning the composition of the nitrate-reducing community in different soils (11, 30) and estuarine sediments (9, 18) showed that each environment apparently has its own specific nitrate-reducing community. In the rhizosphere and nonrhizosphere of the aerenchymatous plant *Typha angustifolia*, many of the *Enteriobacteriaceae* as well as *Aeromonas* and *Vibrio* species were present (6). There was no distinct effect of the presence of *T. angustifolia* on the nitrate-reducing community. In the rhizosphere of *G. maxima*, gram-positive bacteria were dominant when NO_3^- was limited (21). Our results showed that in the rhizosphere sediment, *Bacillus* strains were dominant when NO_3^- was limited. It is very likely that in the presence of an aerenchymatous plant the composition of the nitrate-reducing community is determined by the aerenchymatous plant rather than the sediment type. Under conditions of excess NO_3^-, denitrifying strains were dominant in the rhizosphere sediment. Our results confirm the hypothesis of Nijburg and Laanbroek (21) that *G. maxima* has a large effect on the composition of the nitrate-reducing bacteria when NO_3^- is limited. The presence of *G. maxima* is the main factor controlling the composition of the nitrate-reducing community. To clarify whether oxygen concentration or carbon availability or both parameters can be important for the composition of the nitrate-reducing community in the rhizosphere of an aerenchymatous plant, further research is necessary.

Under conditions of excess NO_3^-, there is a direct correlation between numbers and activity and the composition of the nitrate-reducing community in the rhizosphere of *G. maxima*. This does not necessarily mean that the total number of gram-positive strains decreases. The addition of NO_3^- could have resulted in an increase of denitrifying gram-negative *Pseudomonas* or *Moraxella* strains, due to the fact that they are better competitors for NO_3^- than are gram-positive strains. Nevertheless, with excess NO_3^-, the main factor controlling the nitrate-reducing community in the rhizosphere of *G. maxima* is NO_3^- availability.

ACKNOWLEDGMENTS

We thank Wiecher Smant for determining the total nitrogen concentrations of *G. maxima*, Roel Wagenaar for the total nitrogen and organic carbon determinations of the sediment, and Wietse de Boer, Jan Woldendorp, Paul Bodelier, and George Kowalchuk for valuable comments on the manuscript.

REFERENCES

1. **Armstrong, W., J. Armstrong, and P. M. Beckett.** 1990. Measurement and modelling of oxygen release from roots of *Phragmites australis*. Adv. Water Pollut. Control **11:**41–52.
2. **Blösl, M., and R. Conrad.** 1992. Influence of an increased pH on the composition of the nitrate-reducing microbial populations in an anaerobically incubated acidic forest soil. Syst. Appl. Microbiol. **15:**624–627.
3. **Bodelier, P. L. E., J. A. Libochant, C. W. P. M. Blom, and H. J. Laanbroek.** 1996. Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. Appl. Environ. Microbiol. **62:**4100–4107.
4. **Both, G. J.** 1990. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
5. **Brändle, R. A.** 1991. Flooding resistance of rhizomatous amphibious plants, p. 35–46. *In* M. B. Jackson, D. D. Davies, and H. Lambers (ed.), Plant life under oxygen derivation. SBP Academic Publishing, The Hague, The Netherlands.
6. **Brunel, B., J. D. Janse, H. J. Laanbroek, and J. W. Woldendorp.** 1992. Effect of transient oxic conditions on the composition of the nitrate-reducing community from the rhizosphere of *Typha angustifolia*. Microb. Ecol. **24:**51–61.
7. **Christensen, P. D., and J. Sørensen.** 1986. Temporal variation of denitrification activity in plant-covered, littoral sediment from Lake Hampen, Denmark. Appl. Environ. Microbiol. **51:**1174–1179.
8. **Collins, S. C. H., and P. M. Lyne.** 1964. Microbial methods, p. 149–173. Butterworths, London, United Kingdom.
9. **Dunn, G. M., J. N. Wardell, R. A. Herbert, and C. M. Brown.** 1980. Enrichment, enumeration and characterization of nitrate-reducing bacteria present in sediments of the River Tay Estuary. Proc. R. Soc. Edinburgh Sect. B (biol.) **78:**47–56.
10. **Engelaar, W., J. C. Symens, H. J. Laanbroek, and C. W. P. M. Blom.** 1995. Preservation of nitrifying capacity and nitrate availability in waterlogged soils by radial oxygen loss from roots of wetland plants. Biol. Fertil. Soils **20:**243–248.
11. **Gamble, T. N., M. R. Betlach, and J. M. Tiedje.** 1977. Numerically dominant denitrifying bacteria from world soils. Appl. Environ. Microbiol. **33:**926–939.
12. **Hanson, G. C., P. M. Groffman, and A. J. Gold.** 1991. Denitrification in riparian wetlands receiving high and low groundwater nitrate inputs. J. Environ. Qual. **23:**917–922.
13. **Kaspar, H. F., and J. M. Tiedje.** 1981. Denitrification and dissimilatory nitrate reduction to ammonium in digested sludge. Can. J. Microbiol. **27:** 878–885.
14. **King, D., and D. B. Nedwell.** 1985. The influence of nitrate concentration upon the end-products of nitrate dissimilation by bacteria in anaerobic salt marsh sediment. FEMS Microbiol. Ecol. **31:**23–28.
15. **King, D., and D. B. Nedwell.** 1987. The adaptation of nitrate-reducing bacterial communities in estuarine sediments in response to overlying nitrate load. FEMS Microbiol. Ecol. **45:**15–20.
16. **Knowles, R.** 1982. Denitrification. Microbiol. Rev. **46:**43–70.
17. **Koike, I., and A. Hattori.** 1978. Denitrification and ammonia formation in anaerobic coastal sediments. Appl. Environ. Microbiol. **35:**278–282.
18. **MacFarlane, G. T., and R. A. Herbert.** 1982. Nitrate dissimilation by *Vibrio* spp. isolated from estuarine sediments. J. Gen. Microbiol. **128:**2463–2468.
19. **Martin, K., L. L. Parsons, R. E. Murray, and M. S. Smith.** 1988. Dynamics of soil denitrifier populations: relationships between enzyme activity, most-probable-number counts, and actual N gas loss. Appl. Environ. Microbiol. **54:**2711–2716.
20. **Nelson, D. W., and L. E. Summer.** 1982. Total carbon, organic carbon and organic matter, p. 542–577. *In* A. L. Page, R. H. Miller, D. R. Keeney, R. Miller, Jr., D. E. Baker, and J. D. Rhoades (ed.), Methods of soil analysis: chemical and microbiological properties, 2nd ed. Soil Science Society of America, Madison, Wis.
21. **Nijburg, J. W., and H. J. Laanbroek.** 1997. The influence of *Glyceria maxima* and nitrate input on the composition and nitrate metabolism of the dissimilatory nitrate-reducing bacterial community. FEMS Microb. Ecol. **22:**57–63.
22. **Novazamsky, I., V. J. G. Houba, R. van Eck, and W. van Vark.** 1983. A novel digestion technique for multi-element plant analysis. Commun. Soil Sci. Plant Anal. **14:**239–249.
23. **Prade, K., and G. Trolldenier.** 1990. Denitrification in the rhizosphere of plants with inherently different aerenchyma formation: wheat (*Triticum aestivum*) and rice (*Oryza sativa*). Biol. Fertil. Soils **9:**215–219.
24. **Reddy, K. R., W. H. Patrick, Jr., and C. W. Lindau.** 1989. Nitrification-denitrification at the plant root-sediment interface in wetlands. Limnol. Oceanogr. **34:**1004–1013.
25. **Sand-Jensen, K., C. Prahl, and H. Stokholm.** 1981. Oxygen release from roots of submerged aquatic macrophytes. Oikos **38:**349–354.
26. **Scaglia, J., R. Lensi, and A. Chalamet.** 1985. Relationship between photosynthesis and denitrification in planted soil. Plant Soil **84:**37–43.
27. **Simarmata, T., G. Benckiser, and J. C. G. Ottow.** 1993. Effect of an increasing carbon:nitrate-N ratio on the reliability of acetylene in blocking the N_2O-reductase activity of denitrifying bacteria in soil. Biol. Fertil. Soils **15:**107–112.
28. **Slater, J. M., and D. G. Capone.** 1989. Nitrate requirement for acetylene inhibition of nitrous oxide reduction in marine sediments. Microb. Ecol. **17:**143–157.
29. **Smith, M. S., and J. M. Tiedje.** 1979. The effect of roots on soil denitrification. Soil Sci. Soc. Am. J. **43:**951–955.
30. **Smith, M. S., and K. Zimmerman.** 1981. Nitrous oxide production by non-denitrifying soil nitrate reducers. Soil. Sci. Soc. Am. J. **45:**865–871.
31. **Stouthamer, A. H.** 1991. Metabolic regulation including anaerobic metabolism in *Paracoccus denitrificans*. J. Bioenerg. Biomembr. **23:**163–185.
32. **Suslow, T. V., M. N. Schroth, and M. Isaka.** 1982. Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology **72:**917–918.
33. **Tiedje, J. M.** 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179–245. *In* A. J. B. Zehnder (ed.), Biology of anaerobic micro-organisms. Wilder, New York, N.Y.
34. **Tiedje, J. M., A. J. Sexstone, D. D. Myrold, and J. A. Robinson.** 1982. Denitrification: ecological niches, competition and survival. Antonie Leeuwenhoek **48:**569–583.
35. **Woldendorp, J. W.** 1963. Ph.D. thesis. University of Wageningen, Wageningen, The Netherlands.
36. **Zumft, W. G., A. Viebrock, and H. Körner.** 1988. Biochemical and physiological aspects of denitrification, p. 245–279. *In* J. A. Cole and S. J. Ferguson (ed.), The nitrogen and sulphur cycles: 42nd Symposium of the Society for General Microbiology, held at the University of Southampton, January 1988. Cambridge University Press, Cambridge, United Kingdom.