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Competition for Ammonium between Nitrifying and Heterotrophic Bacteria in Continuously Percolated Soil Columns

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Although the absence of nitrate formation in grassland soils rich in organic matter has often been reported, low numbers of nitrifying bacteria are still found in these soils. To obtain more insight into these observations, we studied the competition for limiting amounts of ammonium between the chemolithotrophic ammonium-oxidizing species *Nitrosomonas europaea* and the heterotrophic species *Arthrobacter globiformis* in the presence of *Nitrobacter winogradskyi* with soil columns containing calcareous sandy soil. The soil columns were percolated continuously at a dilution rate of 0.007 h^{-1}, based on liquid volumes, with medium containing 5 mM ammonium and different amounts of glucose ranging from 0 to 12 mM. *A. globiformis* was the most competitive organism for limiting amounts of ammonium. The numbers of *N. europaea* and *N. winogradskyi* cells were lower at higher glucose concentrations, and the potential ammonium-oxidizing activities in the uppermost 3 cm of the soil columns were nonexistent when at least 10 mM glucose was present in the reservoir, although 10^7 nitrifying cells per g of dry soil were still present. This result demonstrated that there was no correlation between the numbers of nitrifying bacteria and their activities. The numbers and activities of *N. winogradskyi* cells decreased less than those of *N. europaea* cells in all layers of the soil columns, probably because of heterotrophic growth of the nitrite-oxidizing bacteria on organic substrates excreted by the heterotrophic bacteria or because of nitrate reduction at reduced oxygen concentrations by the nitrite-oxidizing bacteria. Our conclusion was that the nitrifying bacteria were less competitive than the heterotrophic bacteria for ammonium in soil columns but that they survived as viable inactive cells. Inactive nitrifying bacteria may also be found in the rhizosphere of grassland plants, which is rich in organic carbon. They are possibly reactivated during periods of net mineralization.

For grassland soils rich in carbon, the absence or low rates of nitrate production have often been reported (20, 37, 38). In addition, in these soils the numbers and activities of nitrifying bacteria have been found low compared with those in soils with other vegetations (9, 31, 32). A possible explanation for these observations was put forth by Verhagen and Laanbroek (50). They showed that in experiments with mixed cultures of *Nitrosomonas europaea*, *Nitrobacter winogradskyi*, and *Arthrobacter globiformis* in dual energy-limited chemostats, the nitrification process was inhibited by NH_4^+ assimilation by the more competitive heterotrophic bacteria. The fate of ammonium was shown to be dependent on the C/N ratio of the medium used. At low C/N ratios, the heterotrophic bacteria were carbon limited, leaving a surplus of ammonium available for nitrification. When the heterotrophic bacteria became nitrogen limited at high C/N ratios, the nitrification process was inhibited and nitrate concentrations fell to zero.

In a system with n limiting substrates, n different bacterial populations can at best coexist in steady-state situations (19, 48, 52), unless cell wall attachment, production of an autoinhibitor, or differences in cell motility properties exist in the system (2, 17, 29). In the chemostat experiments mentioned above (50), the ammonium-oxidizing and heterotrophic bacteria coexisted since there were two limiting substrates at low C/N ratios in the medium, whereas at high C/N ratios in the medium, a condition that caused both populations to be nitrogen limited, the nitrifying bacteria were expected to be washed out, as they were assumed to be the weakest competitors. However, nitrifying bacteria were still found in the cultures at high C/N ratios in the medium. The presence of the nitrifying bacteria was explained by growth on the walls of the culture vessels. Attached ammonium- and nitrite-oxidizing cells were reported to be more active than free-living ones (3, 18, 26). Therefore, attached nitrifying bacteria survived in the chemostats in the presence of heterotrophic cells and probably used small quantities of the ammonium supplied to the system. The small amounts of nitrate formed were taken up by the nitrogen-limited heterotrophic bacteria and were assimilated. Therefore, it would be interesting to perform similar competition experiments with soil columns, in which large portions of the nitrifying population are probably adsorbed to soil particles. Because adsorbed nitrifying cells are more competitive than free-living cells, more nitrifying bacteria will likely survive the suppression of the nitrifying process by heterotrophic bacteria in these experiments than in the chemostat experiments mentioned above (50), in which there were drastic decreases in the numbers of nitrifying bacteria.

The aim of this study was to investigate the competition for limiting amounts of ammonium between *N. europaea* and *A. globiformis* in the presence of *N. winogradskyi* with soil columns continuously percolated with medium with different C/N ratios at a dilution rate of 0.007 h^{-1}, based on liquid volumes.

MATERIALS AND METHODS

Soil sampling. Soil was collected from the top layer (0 to 30 cm) of a calcareous grassland near Brummen, The Netherlands (52°05'N, 06°09'E). The soil was sieved (2-mm-pore-

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size sieve) to remove roots and stones and was well mixed. It was a sandy soil containing 21.3% coarse sand, 69.3% fine sand, 4.1% silt, and 5.3% clay. The pHs of H_2O and KCl extracts and the calcium carbonate and organic matter concentrations in the sieved, mixed soil were 7.8, 7.4, 1.6, and 4.2%, respectively. The soil was stored before use at 4°C for 2 weeks.

Microorganisms and culture conditions. *A. globiformis* was used as the heterotrophic bacterium in this study. It was isolated from the rhizosphere of Ribwort plantain (*Plantago lanceolata*) that was growing in a pot containing the soil described above. The isolation and culturing of the heterotrophic bacterium have been described (50). *N. europaea* ATCC 19718 and *N. winogradskyi* ATCC 25391 were used as the nitrifying bacteria in the soil column experiments. They probably are the dominant nitrifying bacteria in the grassland soil mentioned above because of a positive reaction with antibodies specific for them in the most diluted positive tubes in a most-probable-number (MPN) enumeration. The culturing of a mixed nitrifying population before inoculation into the soil columns was similar to that described by Verhagen and Laanbroek (50).

Competition experiments. Competition experiments were performed in the presence of *N. winogradskyi* to prevent possible toxic effects of nitrite on the competing organisms. The experiments were performed with 30-cm-long acrylate tubes with an internal diameter of 4 cm. At the bottom of the tube, a hydrophilic nylon filter with a pore size of 0.2 μm was held in place by hard plastic gauze and a stopper with a small opening. This opening was connected to a bottle used to sample the percolate. A water column of 1 m maintained a soil moisture content of 24%, which was 60% of the water-holding capacity of this soil. The upper end of the tube was closed by a rubber stopper, which was perforated with a glass tube for dripping sterile medium with or without glucose continuously on top of the soil column. A small steel tube passing through the rubber stopper was connected to a conical flask and used for inoculation with bacteria. It also served as an air exhaust during the experiments. The 190-ml gas phase above the soil column was refreshed continuously by pumping filter-sterilized air into the gas phase at a rate of 25 ml/min.

Each tube was filled with 250 g of the above-described soil, which was subsequently percolated with demineralized water for 1 to 2 days until it was compacted and a soil column of 15 cm was formed. After sterilization by gamma irradiation (4.5 megarads), the soil column was percolated with sterile demineralized water for 2 weeks to remove possible toxic compounds formed by the radiation. It was then percolated with mineral medium containing (per liter) the following: $(NH_4)_2SO_4$, 0.33 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 0.5 g; and trace element solution, 1 ml. The composition of the trace element solution has been described (50). The pH of the medium was adjusted with 0.1 N NaOH; after sterilization, the final pH was 7.5. When ammonium was found in the percolated samples the soil column was inoculated with a mixture of 25 ml of a 4-week-old mixed batch culture of *N. europaea* and *N. winogradskyi*, containing 5×10^6 and 3×10^6 cells per ml, respectively, and 5 ml of a 3-day-old glucose-free culture of *A. globiformis*, precultured on 1 mM glucose and containing 3×10^7 cells per ml. Percolation with mineral medium containing 5 mM ammonium was continued until 5 mM nitrate was found in the percolated samples. Then, new vessels containing the mineral medium described above but supplemented with 0.40, 0.79, 1.19, 1.59, 1.98, or 2.38 g of glucose $\cdot H_2O$ per liter were connected. Two columns were used for each glucose concentration, and four columns received no glucose. Percolated samples were taken three times per week to determine concentrations of ammonium, nitrite, nitrate, and glucose and pH. After 10 weeks of percolation, the soil columns were harvested. Each column was divided into three parts; layer A (0 to 3 cm), layer B (3 to 6 cm), and layer R (6 to 15 cm), which were analyzed separately.

MPN enumerations. For MPN enumerations (42), suspensions of 5.0 g of moist soil and 45 ml of sterile phosphate buffer, containing 139 mg of K_2HPO_4 and 27 mg of KH_2PO_4 per liter (pH 7.0), were shaken at 100 rpm for 4 h. Subsamples of the suspensions were diluted in sterile microtiter plates containing the appropriate medium for the ammonium- or nitrite-oxidizing bacteria. Twelve replicates were made per dilution. The dilution procedure and the composition of the media have been described (50). MPNs of bacteria were obtained from statistical tables that were generated by a computer program.

FA enumerations. For fluorescent-antibody (FA) enumerations (51), a sample of moist soil from a column (1.00 g) was mixed with 0.5 g of glass beads, 2.8 g of chelating resin (sodium form; dry mesh, 50/100; Sigma, St. Louis, Mo.), and 6.0 ml of 0.1% (wt/vol) cholic acid (sodium salt; Sigma). The mixture was shaken for 2 h at 100 rpm and then allowed to stand for 1 min. The supernatant was successively filtered through nylon filters with pore sizes of 20 and 5 μm. With a syringe, 0.5 ml of Percoll (Pharmacia, Uppsala, Sweden) was placed under 1.00 ml of the obtained filtrate (30). After centrifugation at $10,000 \times g$ for 10 min in a Biofuge A table centrifuge, the upper 1.0 ml was filtered through a black polycarbonate membrane filter (pore size, 0.2 μm; Nuclepore Corp., Pleasanton, Calif.) (21). The procedure for staining the bacteria on the filter with antiserum prepared from blood from an immunized rabbit has been described (50).

Determination of potential nitrifying activities. The medium used for the determination of the potential ammonium-oxidizing activity contained (per liter) the following (45): $(NH_4)_2SO_4$, 0.33 g; K_2HPO_4, 0.14 g; KH_2PO_4, 27 mg; and $NaClO_3$, 1.06 g. That used for the determination of the potential nitrite-oxidizing activity contained (per liter) the following (45): $NaNO_2$, 6.9, 13.8, 20.7, or 34.5 mg; K_2HPO_4, 0.14 g; and KH_2PO_4, 27 mg. The pHs of both media were 7.5. The maximum ammonium-oxidizing activity was reached at an ammonium concentration of 2 mM (12). Higher concentrations of ammonium did not affect oxidation rates (12). In this study, medium containing 5 mM ammonium was used. However, it is not known at which nitrite concentration maximum nitrite-oxidizing activities are reached, and substrate inhibition of nitrite-oxidizing bacteria may occur at higher concentrations. Therefore, potential nitrite-oxidizing activities were determined with four different nitrite concentrations. The rates of nitrite formation and consumption were calculated with linear regression. For determination of the potential nitrite-oxidizing activities, the rates were plotted against the mean nitrite concentrations and the V_{max} was calculated with the direct linear method, by use of a computer program (12).

Samples (5.0 g) of a well-mixed layer were supplemented with 25 mg of $CaCO_3$ and 12.5 ml of one of the media described above. For the determination of the potential ammonium-oxidizing activity, nitrite oxidation was inhibited by 10 mM chlorate present in the medium (4). For the determination of the potential nitrite-oxidizing activity, 25 μl

of 1% (wt/vol) nitrapyrin (Dow Chemical Co., Midland, Mich.) in 96% ethanol was added to inhibit ammonium oxidation (6, 34). The mixtures were incubated at 25°C and shaken at 150 rpm. The formation of nitrite in the potential ammonium oxidation determinations and the consumption of nitrite in the potential nitrite oxidation determinations were monitored for 6 h. Samples used for the determination of nitrite were taken every hour. After centrifugation of a sample at 15,000 × *g* in a Biofuge A table centrifuge for 5 min, 0.50 ml of the sample was mixed with 0.50 ml of a 2 M KCl solution. Samples were stored at 4°C and analyzed within 1 day.

Determinations of mineral nitrogen concentrations, pH, and organic matter concentrations. Mineral nitrogen concentrations were determined by shaking 2.5 g of moist soil with 25 ml of a 1 M KCl solution. After 4 h, a sample was taken and centrifuged at 15,000 × *g* in a Biofuge A table centrifuge for 5 min. The supernatant was stored at 4°C and analyzed within 1 day. The pH of an H_2O extract was determined by shaking 2.5 g of moist soil with 12.5 ml of demineralized water for 2 h. Organic matter concentrations were determined by the analysis of weight losses after heating of about 5 g of dry soil at 550°C for 4 h.

Analytical methods. Concentrations of ammonium, nitrite, and nitrate were determined by use of a Traacs 800 autoanalyzer (Technicon Instruments Corp., Tarrytown, N.Y.) with a detection level of 0.01 mM N for all three compounds. Glucose concentrations were determined with a glucose test (Boehringer Mannheim Diagnostica, Mannheim, Germany) based on the photometric determination of glucose with glucose oxidase and peroxidase. The detection level for glucose was 0.05 mM. Dissolved oxygen concentrations were measured by use of an oxygen monitor (Strathkelvin Instruments, Glasgow, United Kingdom) fitted with a microelectrode.

RESULTS

At the end of the experiments, dissolved oxygen concentrations were measured in every 0.5 mm of the uppermost 4 cm and at a depth of 7 cm of a soil column percolated with medium containing 10 mM glucose. Each column was divided into three parts at harvesting after 10 weeks of percolation: layer A, 0 to 3 cm; layer B, 3 to 6 cm; and layer R, 6 to 15 cm. Numbers of nitrifying bacteria were determined by MPN counts. In addition, the potential ammonium- and nitrite-oxidizing activities were determined. Numbers of heterotrophic bacteria were determined in layer A (0 to 3 cm) by FA counts. Mineral nitrogen concentrations and pH were determined in 1 M KCl and water extracts, respectively. Organic matter concentrations were also determined. Samples of the medium reservoirs were taken to determine ammonium and glucose concentrations and pH.

Mineral nitrogen concentrations. In soil columns percolated with mineral medium for 10 weeks, 5 mM ammonium was almost completely converted to 5 mM nitrate by the nitrifying bacteria (Fig. 1). However, nitrate concentrations were lower in the presence of high glucose concentrations and were zero at 10 and 12 mM glucose. Ammonium and nitrite concentrations in the percolated samples were below the detection levels for all soil columns.

Ammonium concentrations in soil extracts of layer A were higher than those in soil extracts of layers B and R (Fig. 2A). In the upper layer, they increased between 2 and 6 mM glucose but were constant above 6 mM glucose. Ammonium concentrations in layers B and R were zero at 12 mM glucose. Nitrate concentrations in all three layers decreased above 2 mM glucose (Fig. 2B). Nitrate concentrations in layers A and R were equal and were zero above 8 mM glucose. Nitrate concentrations in layer B were higher than those in layers A and R and were zero above 10 mM glucose.

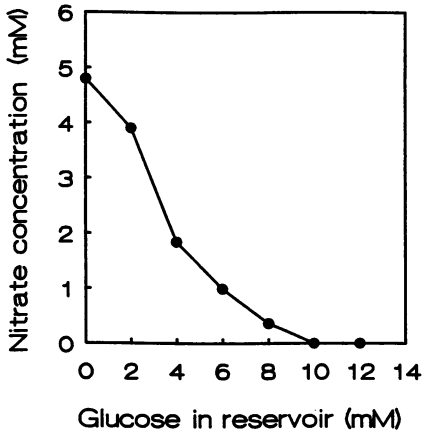

FIG. 1. Concentrations of nitrate in the percolated material from soil columns after percolation for 10 weeks with medium containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C. Data are the means for duplicate soil columns.

Glucose concentrations. Glucose concentrations in the percolated samples were below the detection level during percolation at all glucose concentrations supplied.

Numbers and potential activities of *N. europaea* cells. The numbers of ammonium-oxidizing bacteria decreased with increasing glucose concentrations in all three layers (Fig. 3A). At 12 mM glucose, they amounted to 15, 14, and 27% of those at 0 mM glucose in layers A, B, and R, respectively. For all soil columns, the numbers of *N. europaea* cells in layers A and B were equal, whereas the numbers in layer R were three to four times lower than those in the upper layers.

The potential ammonium-oxidizing activities of *N. europaea* decreased with increasing glucose concentrations (Fig. 3B). The potential activities were lowest in layer A at all glucose concentrations supplied. Above 10 mM glucose, no ammonium-oxidizing activity was found in this layer, although the bacteria were present in high numbers (Fig. 3A). The activities were highest in layer B up to 6 mM glucose, whereas above 6 mM glucose, they were similar to those in layer R. The activities in layer B decreased above 2 mM glucose until they became constant above 8 mM glucose.

Numbers and potential activities of *N. winogradskyi* cells. In all soil columns, the numbers of bacteria in layers B and R were equal, except for those at 12 mM glucose (Fig. 4A). The numbers in layer A were two to four times higher than those in the underlying layers. In layers A and B, the numbers of nitrite-oxidizing bacteria were constant up to 10 mM glucose, but at 12 mM glucose, an increase in the numbers was observed. At 10 mM glucose, the numbers in layers A and B amounted to 28 and 55% of those at 0 mM glucose, respectively. The numbers of *N. winogradskyi* cells in layer R were more or less constant at all glucose concentrations supplied. At 10 mM glucose, the numbers amounted to 71% of those at 0 mM glucose.

The potential nitrite-oxidizing activities of *N. winogradskyi* in soil columns percolated with mineral medium were almost equal in layers A and B (Fig. 4B). The potential

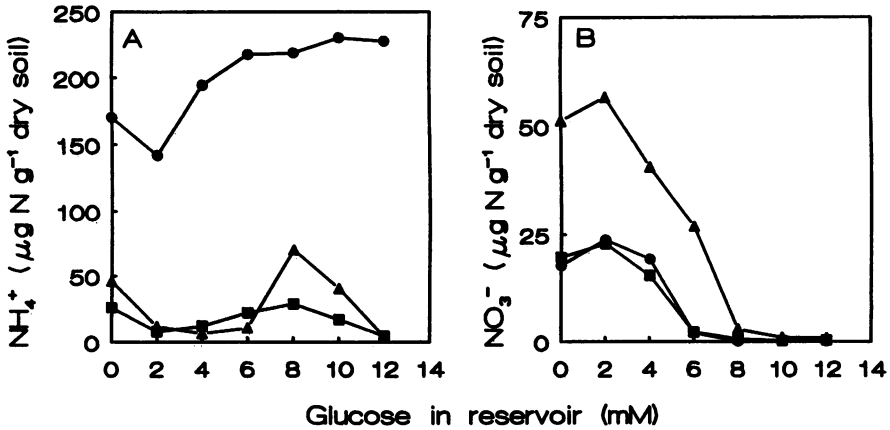

FIG. 2. Concentrations of ammonium (A) and nitrate (B) in 1 M KCl extracts (1:5) of the three layers of soil columns percolated for 10 weeks with medium containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C. Symbols: ●, layer A (0 to 3 cm); ▲, layer B (3 to 6 cm); ■, layer R (6 to 15 cm). Data are the means for duplicate soil columns.

activities in layer R were four to five times lower than those in the upper layers. The potential activities in layer A decreased between 0 and 6 mM glucose, whereas above 6 mM glucose, a sharp increase in activity to 1,400 μmol h^{-1} g of dry $soil^{-1}$ at 12 mM glucose was measured. In layer B, the potential activities were constant up to 8 mM glucose, but at higher glucose concentrations, a sharp increase in activity to 725 μmol h^{-1} g of dry $soil^{-1}$ at 12 mM glucose was measured. The potential activities in layer R were almost equal at all glucose concentrations supplied.

Ratios between the numbers of *N. europaea* and *N. winogradskyi* cells. In layer A, almost equal numbers of ammonium- and nitrite-oxidizing bacteria were present in the soil columns (Table 1). In this layer, all ratios between the numbers of *N. europaea* and *N. winogradskyi* cells were about 1, except for those at 12 mM glucose, at which a low ratio was found because of the relatively sharp decrease and increase in the numbers of ammonium- and nitrite-oxidizing bacteria, respectively (Fig. 4A). The ratios in layer B were higher than those in layer A because of the lower numbers of *N. winogradskyi* cells in layer B than in layer A (Fig. 4A), whereas the numbers of *N. europaea* cells were equal in both layers (Fig. 3A). The ratios in layer B amounted to 2 to 4 at all glucose concentrations, except for 12 mM glucose, at which a low ratio was found for the same reason as in layer A. In layer R, the ratios decreased from 1.2 to 0.5 with increasing glucose concentrations because of a steady decrease in the numbers of *N. europaea* cells.

Ratios between the potential activities of *N. europaea* and *N. winogradskyi*. Generally, the potential activities of the nitrite-oxidizing bacteria in layers A and B were higher than those of the ammonium-oxidizing bacteria in layers A and B at all glucose concentrations up to 6 and 8 mM, respectively (Table 2). No ratios for the potential activities of the nitrifying bacteria were determined at higher glucose concentrations because of the observed sharp increase in the potential nitrite-oxidizing activities (Fig. 4B). In layer R, the potential activities of the ammonium-oxidizing bacteria were higher than those of the nitrite-oxidizing bacteria up to 6 mM glucose, whereas at glucose concentrations higher than 6 mM, the potential activities of *N. winogradskyi* were higher than those of *N. europaea*.

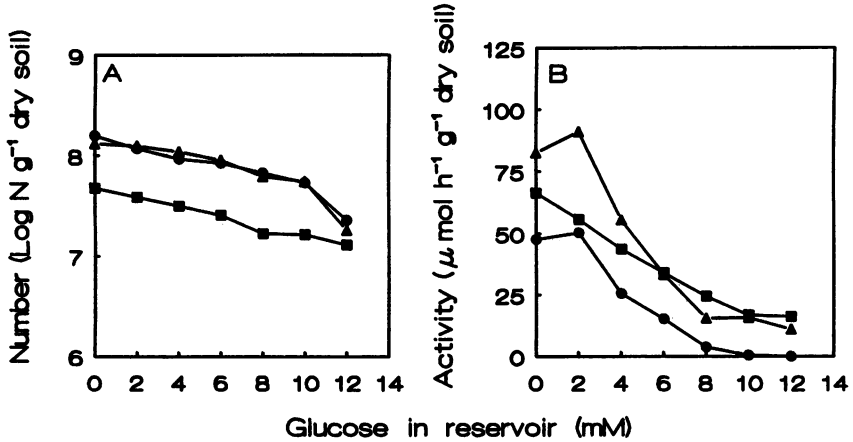

FIG. 3. Numbers (A) and potential ammonium-oxidizing activities (B) of *N. europaea* cells, grown in the presence of *N. winogradskyi* and *A. globiformis*, in the three layers of soil columns percolated for 10 weeks with medium containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C. Symbols are as defined in the legend to Fig. 2. Cells were enumerated by the MPN technique. Data are the means for duplicate soil columns. N in panel A represents number.

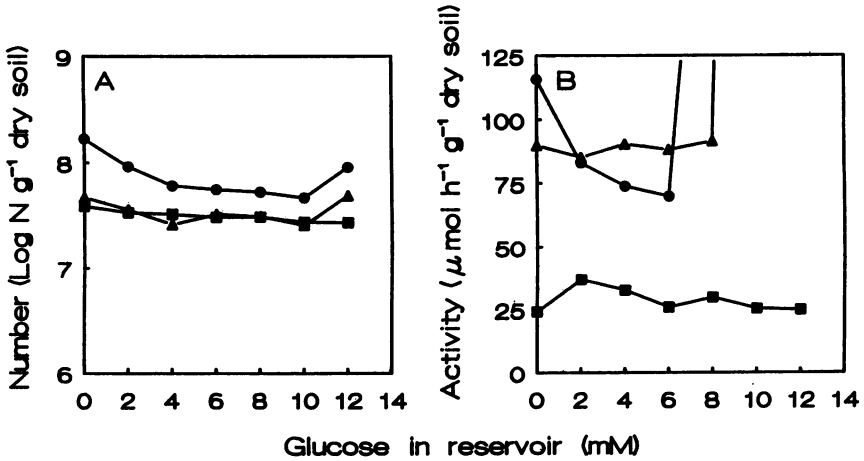

FIG. 4. Numbers (A) and potential nitrite-oxidizing activities (B) of *N. winogradskyi* cells, grown in the presence of *N. europaea* and *A. globiformis*, in the three layers of soil columns percolated for 10 weeks with medium containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C. Symbols are as defined in the legend to Fig. 2. Cells were enumerated by the MPN technique. Data are the means for duplicate soil columns. N in panel A represents number.

Numbers of *A. globiformis* cells. The numbers of heterotrophic bacteria were 15 times higher at 4 mM glucose than at 0 mM glucose (Fig. 5). Above 4 mM glucose, a slight increase in numbers was observed up to 10 mM glucose. For an unknown reason, the numbers of heterotrophic bacteria at 12 mM glucose were lower than those at 10 mM glucose.

pH and organic matter concentrations. The pH in the percolated samples from soil columns percolated with mineral medium for 10 weeks was 6.9, whereas the pH in the percolated samples from soil columns percolated with medium containing glucose increased almost linearly to 8.2 with increasing glucose concentrations up to 12 mM glucose.

The pH of the soil suspensions of layer A steadily decreased from 6.6 at 0 mM glucose to 6.1 at 8 mM glucose and remained constant above 8 mM glucose. The pHs of the soil suspensions of layers B and R were equal at all glucose concentrations. Up to 4 mM glucose in the reservoir, the pH was constant at 7.0. An increase in pH of 0.3 to 0.4 was observed between 4 and 6 mM glucose, but at higher glucose concentrations, the pHs of the suspensions of layers B and R were constant again.

The organic matter concentrations in soil columns percolated with mineral medium for 10 weeks were equal for all layers and amounted to 4.2% (Fig. 6). In layer A, the organic matter concentration rapidly increased between 0 and 2 mM glucose. Above 2 mM glucose, it was constant up to 8 mM glucose. At higher glucose concentrations, the organic matter concentration increased again. The organic matter concentrations in layers B and R were equal at all glucose concentrations, except 12 mM glucose, at which a small increase in the organic matter concentration in layer B was observed. Between 0 and 6 mM glucose, the organic matter concentrations in layers B and R increased slightly. Apart from layer B at 12 mM glucose, they were constant above 6 mM glucose.

Dissolved oxygen concentrations. In the uppermost 2 cm of the soil columns percolated with 10 mM glucose for 10 weeks, the dissolved oxygen concentrations decreased from 95 to 50% of air saturation. Between 2 and 4 cm from the top, the dissolved oxygen concentrations were constant at ca. 50% of air saturation. The same was true at a depth of 7 cm. It was therefore assumed that below a depth of 2 cm, the dissolved oxygen concentrations were constant at ca. 50% of air saturation.

TABLE 1. Ratios between the numbers of *N. europaea* and *N. winogradskyi* cells, grown in the presence of *A. globiformis*, in the three layers of soil columns percolated for 10 weeks with medium containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C

Glucose in reservoir (mM)	Ratio in layer[a]		
	A	B	R
0	0.95	2.78	1.22
2	1.28	3.45	1.14
4	1.53	4.14	0.96
6	1.50	2.72	0.83
8	1.27	1.96	0.54
10	1.15	2.12	0.59
12	0.25	0.37	0.47

[a] Data are means for duplicate soil columns.

TABLE 2. Ratios between the potential ammonium- and nitrite-oxidizing activities of *N. europaea* and *N. winogradskyi*, respectively, grown in the presence of *A. globiformis*, in the three layers of soil columns percolated for 10 weeks with medium containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C

Glucose in reservoir (mM)	Ratio in layer[a]		
	A	B	R
0	0.41	0.92	2.76
2	0.61	1.07	1.51
4	0.35	0.61	1.34
6	0.22	0.38	1.31
8	ND	0.17	0.82
10	ND	ND	0.66
12	ND	ND	0.65

[a] Data are means for duplicate soil columns. ND, not determined because of unmeasurable potential nitrite-oxidizing activities (see the text).

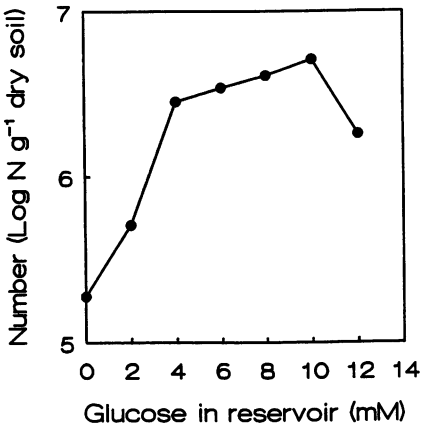

FIG. 5. Numbers of *A. globiformis* cells, grown in the presence of *N. europaea* and *N. winogradskyi*, in layer A (0 to 3 cm) of soil columns percolated for 10 weeks with medium containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C. Cells were enumerated by the FA technique. Data are the means for duplicate soil columns. N represents number.

DISCUSSION

In the rhizosphere of plants, with their usually high C/N ratios, nitrification often has been shown to be suppressed by the allelopathic effects of organic compounds originating from plant roots (33, 36, 49) or by the process of immobilization of heterotrophic bacteria (24, 35, 39, 41). Allelopathic effects probably did not play a role in the experiments described here because plants were not included and the soil columns were percolated with demineralized water for 2 weeks after gamma irradiation. Concerning suppression by the immobilization process, Jansson (24) demonstrated that mixed heterotrophic populations were successful in competition with nitrifying bacteria for limiting amounts of ammonium. According to Rosswall (41), nitrifying populations, with their high Michaelis-Menten constants (K_m) and thus low affinities for ammonium compared with those of heterotrophic bacteria, only used ammonium not needed by heterotrophic bacteria.

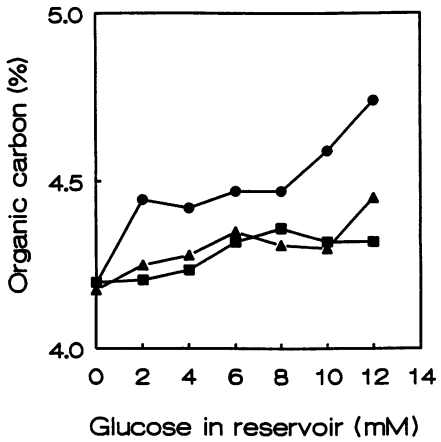

FIG. 6. Organic carbon concentrations in the three layers of soil columns after percolation for 10 weeks with medium containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C. Symbols are as defined in the legend to Fig. 2. Data are the means for duplicate soil columns.

Soil columns often have been used to study the effects of environmental factors on the activity and survival of nitrifying bacteria (1, 22, 28, 46). In the type of soil column experiment performed in this study, competition for ammonium between nitrifying and heterotrophic bacteria takes place provided that the numbers of heterotrophic bacteria increase during the percolation. When the numbers of *A. globiformis* cells reach their maximum, the glucose in the medium meets the maintenance requirements of the heterotrophic bacteria. Then, all the ammonium in the medium is available to the nitrifying bacteria again, and nitrification rates increase again. Apparently, this point was not reached in the experiments described here. *A. globiformis* was the most competitive organism for limiting amounts of ammonium as glucose concentrations increased in the medium. This conclusion was reached on the basis of the low potential ammonium-oxidizing activities at higher glucose concentrations. The decrease in nitrate concentrations in the percolated samples with increasing glucose concentrations in the reservoir could have been due to smaller amounts of ammonium being available to the nitrifying bacteria at higher glucose concentrations but also to nitrate consumption by the heterotrophic bacteria. The latter possibility, however, is less likely, because in similar competition experiments in chemostats, the heterotrophic bacteria did not use nitrate in addition to ammonium (50). At 10 and 12 mM glucose, nitrate concentrations in the percolated samples were zero, and the activities of the ammonium-oxidizing population in the uppermost 3 cm of the soil columns were also nonexistent at these glucose concentrations. These results indicate that the heterotrophic bacteria completely inhibited the nitrifying bacteria by competition and that the heterotrophic bacteria used only ammonium as an N source. It may be considered whether the heterotrophic bacteria were really nitrogen limited at higher glucose concentrations, because ammonium was present in layer A at all glucose concentrations supplied (Fig. 2A). From the measured potential ammonium-oxidizing activities, which were nonexistent in layer A at high glucose concentrations, we assume that the heterotrophic bacteria were indeed limited by ammonium at high glucose concentrations and that the ammonium measured in KCl extracts of the soil layers was not attainable or available to the heterotrophic and nitrifying bacteria. The reason for these relatively high ammonium concentrations in layer A is not clear. One possible explanation is that the nitrifying bacteria, possibly growing in microcolonies, were locally separated from their ammonium substrate. The formation of thin channels in the soil, through which the medium containing glucose flowed, might have played a role in such a scenario. Incomplete nitrification in a continuous-flow nitrification column was reported by Cox et al. (16).

The outcomes of the experiments described here may have been influenced by the possible inhibition of *N. europaea* by either glucose or products of glucose metabolism from *A. globiformis*. In previous experiments, it was shown that glucose, up to a final concentration of 1 mM, and an ammonium-free supernatant of an early-stationary-phase culture of *A. globiformis* did not inhibit or stimulate nitrate production (50). It is not clear whether glucose stimulated or inhibited nitrate production in the experiments described here, because the glucose concentrations in the layers of the soil columns could not be measured during percolation. In all soil columns, the glucose added was completely used up, because the glucose concentrations in the percolated sam-

ples were always below the detection level. In addition, glucose and other organic compounds, such as formate, acetate, pyruvate, and glycerol, have been reported not to affect nitrate formation (25, 27). Growth responses to individual amino acids or vitamins in log-phase cultures of *N. europaea* were observed by Clark and Schmidt (15). Some amino acids and vitamins stimulated nitrite formation and protein synthesis, whereas others were inhibitory. The inhibition of ammonium-oxidizing bacteria by organic compounds originating from plant roots is widely documented (33, 36, 49). These allelopathic effects have been ascribed to tannins and tannin-derived compounds.

In this study, the activities and survival of *N. europaea* cells, as indicated by viable MPN counts, were affected differently as the numbers of heterotrophic bacteria increased at higher glucose concentrations in the medium. The numbers of ammonium-oxidizing bacteria decreased slightly and at the same rates in the three layers of the soil columns as those at which the glucose concentrations increased (Fig. 3A). Despite the slight decrease in numbers, the potential activities of the ammonium-oxidizing bacteria decreased sharply at higher glucose concentrations (Fig. 3B). In the uppermost layer, layer A, the activities were nonexistent at 10 and 12 mM glucose, even though more than 10^7 ammonium-oxidizing bacteria per g of dry soil were still present. This result indicated that there is no relationship between the numbers and the activities of nitrifying bacteria in soil, as has often been reported (5, 7, 8, 12). In previous experiments, competition between the same species was studied in continuous cultures at two dilution rates (50). The persistence of nitrifying bacteria at high C/N ratios was ascribed to the adherence of these bacteria to the walls of the culture vessels, combined with a continuous flow of released cells into the vessels. It was suggested that adherence to the glass walls was a way to survive high C/N ratios. Activity determinations could not be done in those experiments. A comparison of the competition experiments performed with soil columns and with chemostats demonstrated clearly that the adsorption of nitrifying bacteria to solid particles apparently had no effect on their overall competitive power. However, it may have had an effect on the survival of nitrifying bacteria in the absence of available ammonium. The nitrifying bacteria may have survived as inactive, but viable, cells. This possibility may be a reason why there is no relationship between the numbers and the activities of nitrifying bacteria.

A comparison of the decreases in the numbers of *N. europaea* and *N. winogradskyi* cells revealed a larger decrease for the ammonium-oxidizing than for the nitrite-oxidizing bacteria at higher glucose concentrations. The only substantial decrease in the numbers of *N. winogradskyi* cells occurred in layer A at glucose concentrations between 0 and 4 mM. In layers A and B, the numbers of nitrite-oxidizing bacteria even increased at 12 mM glucose in the medium. Three possible explanations can be put forth for the different behaviors of *N. europaea* and *N. winogradskyi*. (i) There may have been chemoorganotrophic growth of *N. winogradskyi* on organic substrates released by the heterotrophic bacteria. As a result of the growth of the nitrite-oxidizing bacteria on organic substrates, the decrease in the numbers of *N. winogradskyi* cells was not coupled to the decrease in the numbers of *N. europaea* cells. Because glucose was found in none of the percolated samples from the soil columns, we propose that at high glucose concentrations, the heterotrophic bacteria, although limited by nitrogen, took up the surplus of glucose and excreted it in another organic form. Such a process was observed before in similar competition experiments performed with chemostats (50). Some of these unidentified organic compounds may have been used by *N. winogradskyi* for growth. The stimulation of nitrite-oxidizing bacteria by organic compounds in the environment often has been reported (10–13, 47). (ii) The nitrite-oxidizing bacteria may have used nitrate instead of oxygen for the oxidation of organic compounds excreted by the heterotrophic bacteria, for nitrate reduction. The dissolved oxygen concentrations below 2 cm from the top of the soil column percolated with medium containing 10 mM glucose were about 50% of air saturation. Denitrification at reduced oxygen concentrations has been reported for various bacteria (40). (iii) There may have been a higher death rate for the ammonium-oxidizing bacteria at high glucose concentrations. This explanation is less likely, because the smaller decrease in the numbers of *N. winogradskyi* cells was also observed in competition experiments with *N. europaea* and *A. globiformis* in the presence of *N. winogradskyi* in dual energy-limited chemostats, in which the presence of cells is always coupled to growth (50).

In layer A above 6 mM glucose and in layer B above 8 mM glucose in the reservoir, sharp increases in potential nitrite-oxidizing activities were observed (Fig. 4B). However, the fast disappearance of nitrite from the incubation medium apparently was not due to the higher potential activities of the nitrite-oxidizing bacteria. The heterotrophic bacteria, nitrogen limited at high glucose concentrations, were assumed to be responsible for the fast disappearance of the small amounts of nitrite from the incubation medium. For potential ammonium-oxidizing activities, no sharp increases were observed. This result was due to the fact that a surplus of ammonium was present, so that ammonium usage by the heterotrophic bacteria did not interfere with potential ammonium-oxidizing activity measurements.

Nitrogen balances were calculated for the soil columns. For these calculations, it was assumed that the increases in organic matter concentrations measured in the layers of the soil columns were totally in the form of cell material (Fig. 6). The increases in organic matter concentrations in the three layers of the soil columns were compared with the total amounts of ammonium supplied in 10 weeks of percolation and the increases in ammonium concentrations in the different layers of the soil columns (Fig. 2A). For the soil columns percolated with medium containing 10 and 12 mM glucose, N balances fit at bacterial C/N ratios of 9.1 and 12.1, respectively. These appear to be realistic values for C/N ratios of bacteria, because Rosswall (41) reported bacterial C/N ratios of 12.5 at N starvation levels. However, if the increases in organic matter concentrations measured in the layers of the soil columns were not totally in the form of cell material and only a portion of the excreted organic carbon was taken up by *N. winogradskyi*, N balances would have fit at bacterial C/N ratios lower than those mentioned above. For example, if only 50% of the increases in organic matter concentrations in the layers was in the form of cell material, N balances would fit at bacterial C/N ratios of 4.5 and 6.1 for 10 and 12 mM glucose, respectively. In chemostat experiments examining competition between the same bacterial species, a bacterial C/N ratio of 3.5 was found with low glucose concentrations in the medium (50).

For determination of the potential ammonium-oxidizing activity of a layer, sodium chlorate was added to the incubation medium to inhibit the activity of *N. winogradskyi*. It inhibited nitrite oxidation via the formation of chlorite by the nitrite oxidizer (4). Also, *N. europaea* was found to be very sensitive to chlorite, whereas it was found

to be insensitive to 10 mM chlorate (23, 50). It is not clear whether the formation of chlorite by the nitrite-oxidizing bacteria had an effect on the measured potential ammonium-oxidizing rates. We do not know how much chlorate was converted into chlorite in the incubation mixtures before complete inhibition of *N. winogradskyi* appeared or whether chlorite was excreted into the suspensions by the nitrite-oxidizing bacteria. For determination of the potential nitrite-oxidizing activity of a layer, nitrapyrin was added to the incubation mixtures to inhibit the activity of *N. europaea*. The extent of inhibition of ammonium oxidation by nitrapyrin depends on the ammonium-oxidizing species and the soil used. Inhibition rates of between 69 and 97% have been reported for soils (14). Inhibition of a *Nitrosomonas* species by nitrapyrin was very effective (6). For soil suspensions of an ammonium-amended silt loam soil, an inhibition rate of 98% was found (43). Inhibition of ammonium oxidation by nitrapyrin is more effective in liquid cultures and soil suspensions than in soil (34, 44). Therefore, it was assumed that ammonium oxidation by *N. europaea* was almost completely inhibited when the potential nitrite-oxidizing activities in the ammonium-amended sandy soil used in this study were determined.

Finally, it may be concluded that in the rhizosphere of plants, in which high C/N ratios are common, inhibition of the nitrification process by a mixture of more competitive heterotrophic microorganisms may occur. However, inactive nitrifying bacteria survive these unfavorable circumstances. The mechanism of this survival strategy of viable nitrifying bacteria is unknown. In times of favorable C/N ratios, i.e., under conditions of net mineralization, nitrifying bacteria may become active again and the formation of nitrate may begin again because of the lack of a carbon source for the heterotrophic bacteria.

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