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Competition for Ammonium between Nitrifying and Heterotrophic Bacteria in Dual Energy-Limited Chemostats

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The absence of nitrification in soils rich in organic matter has often been reported. Therefore, competition for limiting amounts of ammonium between the chemolithotrophic ammonium-oxidizing species *Nitrosomonas europaea* and the heterotrophic species *Arthrobacter globiformis* was studied in the presence of *Nitrobacter winogradskyi* in continuous cultures at dilution rates of 0.004 and 0.01 h^{-1}. Ammonium limitation of *A. globiformis* was achieved by increasing the glucose concentration in the reservoir stepwise from 0 to 5 mM while maintaining the ammonium concentration at 2 mM. The numbers of *N. europaea* and *N. winogradskyi* cells decreased as the numbers of heterotrophic bacteria rose with increasing glucose concentrations for both dilution rates. Critical carbon-to-nitrogen ratios of 11.6 and 9.6 were determined for the dilution rates of 0.004 and 0.01 h^{-1}, respectively. Below these critical values, coexistence of the competing species was found in steady-state situations. Although the numbers were strongly reduced, the nitrifying bacteria were not fully outcompeted by the heterotrophic bacteria above the critical carbon-to-nitrogen ratios. Nitrifying bacteria could probably maintain themselves in the system above the critical carbon-to-nitrogen ratios because they are attached to the glass wall of the culture vessels. The numbers of *N. europaea* decreased more than did those of *N. winogradskyi*. This was assumed to be due to heterotrophic growth of the latter species on organic substrates excreted by the heterotrophic bacteria.

The absence of nitrification in grassland soils that are rich in organic matter has often been reported (12, 14, 27, 34, 35). The numbers and activities of the nitrifying bacteria are also lower in soils covered with grasses than in soils with other types of vegetation (4, 24–26). Some authors attribute this inhibition of nitrification to allelopathic effects of organic compounds originating from grass roots (28, 29, 33, 41). Rice and Pancholy (33) reported that all herbaceous species, including grasses, contained considerable amounts of condensed tannins. These and derived compounds would inhibit nitrification eventually after they had accumulated in soil. Another explanation for the absence or low rates of nitrate production in grassland soils was given by others (18, 32, 36–38). At the C/N ratios encountered in the rhizosphere of grasses, competition for limited amounts of ammonium occurs between nitrifying and heterotrophic bacteria. The absence or low rate of nitrate formation is ascribed to the suppression of the nitrification process by more competitive heterotrophic bacteria.

The fate of ammonium depends on the carbon-to-nitrogen ratio of the environment (18). At the so-called critical C/N ratio, the heterotrophic bacteria consume all mineral nitrogen present in the system for assimilation in cell material. At carbon-to-nitrogen ratios below this critical value, heterotrophic populations will be carbon limited and a surplus of ammonium is available for nitrification. At carbon-to-nitrogen ratios above this critical value, heterotrophic and nitrifying populations become nitrogen limited. Usually storage of organic carbon in the form of glycogen or poly-β-hydroxybutyrate occurs in the heterotrophic bacteria at these carbon-to-nitrogen ratios.

Other, mostly mathematical studies on competition between different species of bacteria have demonstrated that in a system with n limiting substrates at most n bacterial populations, each limited by a different substrate, can coexist in a steady-state situation (13, 40, 45). Hence, below a critical carbon-to-nitrogen ratio, where the heterotrophs are carbon limited and the nitrifiers are nitrogen limited, the two competing organisms are expected to coexist in steady-state situations. Above this carbon-to-nitrogen ratio, where both organisms are nitrogen limited, one of the organisms would be outcompeted by the other. However, in cases of spatial heterogeneities or even in nonmixed environments, cell wall attachment, production of an autoinhibitor, or differences in cell motility properties may influence the outcome of the competition experiments (1, 11, 22).

The aim of this study was to investigate the competition for ammonium between pure cultures of the chemolithotrophic ammonium-oxidizing species *Nitrosomonas europaea* in combination with the nitrite-oxidizing species *Nitrobacter winogradskyi* and the heterotrophic species *Arthrobacter globiformis* in a chemostat with increasing carbon-to-nitrogen ratios in the medium and to establish the critical C/N ratio. This critical value was determined at two dilution rates.

MATERIALS AND METHODS

Microorganisms and culture conditions. The heterotrophic bacterium used in this study was isolated from the rhizosphere of ribwort plantain (*Plantago lanceolata*). For its isolation, a sterile seedling of *P. lanceolata*, obtained after hypochlorite treatment of the seeds, was planted into a pot containing nonsterile soil from the top 0- to 10-cm layer of an extensively used, calcareous grassland near the village of Brummen (52°5′ north, 6°9′ east) in The Netherlands. A dilution series of a rhizosphere suspension was plated on an agar medium with glucose as the carbon source and ammonium sulfate as the nitrogen source. After incubation for 14 days at 20°C, a number of bacterial colonies were picked up from the most diluted positive agar plate and subcultured on

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the same medium until pure cultures were obtained. One of the isolated strains was selected for further experiments. According to identification tests performed at The National Collections of Industrial and Marine Bacteria (Aberdeen, Scotland), the isolated strain was placed into the genus *Arthrobacter*, probably belonging to the *A. globiformis* group. Batch cultures of *A. globiformis* were grown in a liquid medium. The composition of the medium was identical to that of the agar medium mentioned above, containing the following (per liter): $(NH_4)_2SO_4$, 330 mg; KH_2PO_4, 100 mg; $MgSO_4 \cdot 7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 500 mg; glucose $\cdot$ H_2O, 1 g (glucose autoclaved separately); and trace elements solution, 1 ml. The trace elements solution contained the following (per liter): $FeCl_2 \cdot 4H_2O$, 2 g; disodium EDTA (Titriplex III), 4.3 g; $MnCl_2 \cdot 4H_2O$, 0.1 g; $CoCl_2 \cdot 6H_2O$, 24 mg; $NiCl_2 \cdot 6H_2O$, 24 mg; $CuCl_2 \cdot 2H_2O$, 17 mg; $ZnCl_2$, 68 mg; $Na_2WO_4 \cdot 2H_2O$, 33 mg; Na_2MoO_4, 24 mg; and H_3BO_3, 62 mg. The pH of the medium was adjusted with 0.1 N NaOH so that after sterilization in the autoclave the final pH was 7.8. The *Arthrobacter* strain was subcultured every 2 weeks by inoculating 50 ml of fresh medium with 1 to 1.5 ml of a mid-stationary-phase culture. Cultures were incubated at 15°C without shaking and without pH adjustment. Cultures were tested for purity every two subcultures on an agar medium containing the following (per liter): $(NH_4)_2SO_4$, 330 mg; KH_2PO_4, 19 mg; K_2HPO_4, 104 mg; yeast extract (Difco Laboratories, Detroit, Mich.), 1 g; tryptose (Oxoid Ltd., Basingstoke, Hampshire, England), 2 g; bacteriological agar (Oxoid), 16 g; and glucose $\cdot$ H_2O, 200 mg (glucose autoclaved separately). *A. globiformis* was cultured for 3 days and then inoculated on a rotary shaker at 20°C into a medium identical to the batch culture medium described above except for the amount of glucose. To the preinoculation medium 200 mg of glucose $\cdot$ H_2O was added per liter. All glucose was used by the heterotrophic bacteria before inoculation of the chemostats.

N. europaea ATCC 19718 and *N. winogradskyi* ATCC 25391 were used as the nitrifying bacteria in the competition experiments conducted in this study. *N. europaea* and *N. winogradskyi* probably belong to the dominant nitrifying bacteria in the grassland soil from which the *Arthrobacter* species was isolated, as was indicated by a positive reaction to specific antibodies against these bacteria in the most diluted positive tubes of a most-probable-number (MPN) enumeration. Mixed batch cultures of *N. europaea* and *N. winogradskyi* were grown for 4 weeks to increase cell numbers before inoculation. The composition of the medium for these batch cultures was as follows (per liter): $(NH_4)_2SO_4$, 330 mg; KH_2PO_4, 200 mg; $MgSO_4 \cdot 7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 500 mg; and trace elements solution, 1 ml (see above). Bromothymol blue (0.04%, 5 ml) was added as a pH indicator. The pH was adjusted with 0.1 N NaOH to a final pH after sterilization of 7.5. Incubation of the mixed batch cultures was at 20°C without shaking. During the incubation, the pH was adjusted with sterile 0.1 N NaOH to 7 to 7.5, as indicated by the pH indicator. After five or six pH adjustments the chemostats were inoculated.

Pure culture experiments. For comparison with the mixed bacterial cultures, pure cultures of *A. globiformis* were grown at both dilution rates on 2 mM glucose and 2 mM ammonium. To investigate its preference for a N source, pure cultures of *A. globiformis* were also grown at both dilution rates on 2 mM glucose with 2 mM NH_4^+ and 2 mM NO_3^- in the medium.

Pure cultures of the nitrifying species *N. europaea* and *N. winogradskyi* were grown at both dilution rates before the heterotrophs were added, and the numbers of nitrifying bacteria and the K_ms for ammonium oxidation were determined.

Competition experiments. Competition between *N. europaea* and *A. globiformis* for limiting amounts of ammonium was studied in continuous cultures at two dilution rates. This was done by analyzing steady-state situations in the culture vessels with glucose concentrations increasing stepwise from 1 to 5 mM in the reservoir. The experiments were performed in the presence of the nitrite-oxidizing species *N. winogradskyi* to prevent possible toxic effects of nitrite on the competing organisms.

The competition experiments were conducted by using Biostat M fermenters (B. Braun, Melsungen, Germany) with culture volumes of 1,250 ml. The dilution rates studied were 0.004 and 0.01 h^{-1}. The composition of the medium was as follows (per liter): $(NH_4)_2SO_4$, 132 mg; $MgSO_4 \cdot 7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 500 mg; and trace elements solution (see above), 1 ml. Sterilization of 9.5 liters of this medium was performed in a 10-liter vessel for 40 min at 121°C. The buffer solution, containing 19 mg of KH_2PO_4 and 104 mg of K_2HPO_4 per liter (pH 7.5), was autoclaved separately. The glucose solution, containing 198, 396, 595, 793, or 991 mg of glucose $\cdot$ H_2O per liter, depending on the desired glucose concentration, was also sterilized separately. After the reservoir was cooled, the concentrated buffer and glucose solutions were added to the mineral medium. After the medium was mixed, the pH was 7.5 and glucose concentrations ranged from 0 to 5 mM. During the experiments the pH was constantly maintained at 7.5 by autotitration with either 0.5 N NaOH or 0.1 N HCl. The temperature and O_2 tension, respectively, were maintained at 25°C and at least 60% of air saturation. The culture vessels were stirred at 300 rpm.

In the culture vessels, 1 liter of sterile mineral medium was inoculated with 250 ml of the 4-week-old mixed batch culture of *N. europaea* and *N. winogradskyi* described above. The inoculum contained 6×10^6 ammonium-oxidizing bacteria and 4×10^6 nitrite-oxidizing bacteria per ml. At first, the mixed cultures were grown batchwise in the fermenters until the 2 mM ammonium in the medium was totally converted into nitrate. Then the influent and effluent pumps were started, and the experiment was continued until the nitrifying bacteria reached a steady-state situation. After analysis of the culture medium and enumerations of the nitrifying bacteria, 50 ml of 3-day-old, glucose-free, early-stationary-phase cultures of *A. globiformis* were added to the culture vessels. The inoculum contained 2.1×10^7 heterotrophic bacteria per ml. New medium vessels containing 0.2 mM glucose were connected, and the experiment was continued until a new steady state was reached. After the steady state was reached, a new medium vessel was connected, whereupon a new steady state was awaited.

Samples were taken at every steady state to determine the ammonium, nitrite, nitrate, glucose, total organic carbon, and dissolved organic carbon concentrations and the pH. Also the numbers of nitrifying bacteria were determined by the MPN and the fluorescent antibody (FA) enumeration techniques. The heterotrophic bacteria were counted by using the FA and the plate count enumeration techniques. Enumerations of the heterotrophic bacteria by means of plate counting on a rich agar medium were also used to test the purity of the cultures. Also, samples of the reservoirs were taken to determine the ammonium and glucose concentrations and the pH. Samples were stored at −25°C. The steady-state situations with 0, 1, and 2 mM glucose were repeated in separate experiments for both dilution rates.

Enumerations. (i) MPN technique. The nitrifying bacteria in the culture vessels were enumerated by using an MPN technique (39). The nitrifying bacteria present in a sample were diluted in an appropriate medium. From the results of the dilutions after incubation, the number of nitrifying bacteria originally present in the sample could be deduced. The enumeration medium contained the following (per liter): $(NH_4)_2SO_4$ (330 mg) or $NaNO_2$ (35 mg); KH_2PO_4, 100 mg; $MgSO_4 \cdot 7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 500 mg; and trace elements solution (see above), 1 ml. The pH was adjusted with 0.1 N NaOH. In preparing the medium for the enumeration of the nitrite-oxidizing organisms, the buffer solution was sterilized separately to prevent precipitation. After sterilization, the pH was 7.5.

For the enumerations with the MPN technique, samples (0.25 ml) taken from the culture vessels were diluted 10-fold in sterile microtiter plates (tissue culture cluster, 24 wells; Costar, Cambridge, United Kingdom) containing 2.25 ml of the ammonium or nitrite oxidizer medium per well. Twelve replicates were made per dilution. After dilution, the microtiter plates were packed in aluminum foil and incubated at 20°C for 9 weeks. Ammonium and nitrite oxidations were determined by using 0.04% bromothymol blue solution (pH indicator) and Griess Ilosvay reagents, respectively. The MPNs of nitrifying bacteria were determined by comparing the results of the dilutions with statistical tables generated by a computer program (31).

(ii) FA enumeration technique. Nitrifying and heterotrophic bacteria were enumerated by using an indirect specific FA technique (42). Antisera against pure cultures of the nitrifying and heterotrophic bacteria were prepared from blood obtained from immunized rabbits. Antisera raised against pure cultures of the bacteria used were checked for their cross-reactivity. No cross-reaction was found.

A sample (1.00 ml) taken from the culture vessel and diluted with phosphate-buffered saline (PBS) was filtered over a black polycarbonate membrane filter (Nuclepore Corp., Pleasanton, Calif.) with a diameter of 25 mm and a pore size of 0.2 μm. The filter was held by a stainless steel, fine-meshed frit filter holder. After the filter was rinsed twice with 2 ml of PBS, 20 μl of rhodamine isothiocyanate gelatin was spread over the filter to reduce background fluorescence (7). After slight air drying, the filter was incubated with 20 μl of one of the specific antisera (diluted 1:400 with PBS) for 30 min in a humid petri dish. After the filter was rinsed twice with 2 ml of PBS to remove excess antiserum, the filter was incubated with commercially available goat anti-rabbit immunoglobulin G (Sigma) diluted 1:100 with PBS for 30 min in a humid petri dish in the dark. This goat anti-rabbit antiserum was conjugated with the green-fluorescent label fluorescein isothiocyanate. After the filter was rinsed twice with 2 ml of PBS, it was placed on a glass slide, whereupon 20 μl of 0.1% paraphenylenediamine solution in glycerol (pH 8) was added as a fluorescence brightener. Finally, the filter was protected by a cover glass and sealed with glyceel (BDH, Poole, England). Filters were stored at −25°C and analyzed within a week. Cells were enumerated by using a Leitz Diaplan epifluorescence microscope (Ernst Leitz, Wetzlar, Germany). The enumeration was continued until 800 to 1,000 fluorescent cells were counted.

(iii) Plate count enumeration technique. Heterotrophic bacteria were enumerated by using a plate count technique. The composition of the medium was as follows (per liter): $(NH_4)_2SO_4$, 330 mg; yeast extract, 1 g; tryptose, 2 g; bacteriological agar, 15 g; KH_2PO_4, 125 mg; and glucose $\cdot$ H_2O, 200 mg (glucose sterilized separately). The pH was adjusted with 0.5 N NaOH to a final pH after sterilization of 7.5.

Sterile samples (0.1 ml) taken from the culture vessel were diluted 10-fold in sterile test tubes containing 0.9 ml of PBS. A series of 10 dilutions was made from each sample, and 100 μl of each dilution was plated on agar medium as described above. After incubation for 14 days at 20°C, the number of CFU was determined.

Analytical methods. The concentrations of mineral nitrogen compounds were determined by using a Technicon Traacs 800 autoanalyzer (Technicon Instrument Corp., Tarrytown, N.Y.). Total and dissolved organic carbon concentrations were determined with a total organic carbon analyzer (model 700; O.I. Corp., College Station, Tex.) with potassium hydrogen phthalate as a standard and potassium persulfate as an oxidizing agent. The concentration of dissolved organic carbon was determined in the supernatant after centrifugation at 15,000 × *g* for 15 min in a Biofuge A table centrifuge (Heraeus-Christ, Osterode am Harz, Germany). The net cell organic carbon concentration for each glucose concentration was calculated from the total organic carbon minus the dissolved organic carbon. The C efficiency for the pure and mixed cultures was calculated by dividing the net cell organic carbon by the amount of glucose supplied and multiplying by 100%. The bacterial C/N ratio of the pure and mixed cultures was calculated by dividing the net cell organic carbon by the ammonium used.

The amounts of glucose in the culture vessels were determined with a test combination for glucose (Boehringer Mannheim Diagnostica, Mannheim, Germany) based on the photometric determination of glucose with glucose oxidase and peroxidase (43). In contrast to the original method, the A_{440} of the samples was measured on a Vitatron MCP spectrophotometer (Vital Scientific, Dieren, The Netherlands).

Determination of kinetic parameters. The K_m for ammonium oxidation by *N. europaea* was determined with cells grown in the absence or presence of *A. globiformis*. The mixed cultures of nitrifiers and heterotrophs received 2 mM ammonium and 2 mM glucose. Determinations of kinetic parameters were performed by using a biological oxygen monitor (model 781; Strathkelvin Instruments, Glasgow, United Kingdom) with a Clarck-type microcathode oxygen electrode. To obtain measurable respiration rates, 600-ml samples were concentrated 24-fold by centrifugation at 30,000 × *g* with a Sorvall RC5C high-speed centrifuge (Du Pont, Wilmington, Del.). After concentration, there were 1×10^7 to 7×10^7 *N. europaea* cells per ml and the pH of the cell suspension was 7.5.

Samples (1.00 ml) of the concentrated cell suspension were incubated in the reaction chamber at 25.0°C. To prevent disturbing effects of oxygen consumption by *N. winogradskyi* on the nitrite produced, 10 μl of 1 M sodium chlorate was added to the suspensions, giving a final concentration of 10 mM. Sodium chlorate inhibited the activity of nitrite-oxidizing bacteria because chlorite was formed by these microorganisms (3). After the suspensions were aerated for 1 min, 20 μl of a concentrated ammonium sulfate solution was added to the samples, yielding final concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5.0, and 10 mM ammonium. After the substrate was added, the respiration rates of the stirred suspensions were determined by measuring the linear decreases in oxygen concentration for 15 min. The measured oxygen consumption rates were corrected for endogenous respiration, which was measured for 30 min in the presence of sodium chlorate but in the absence of

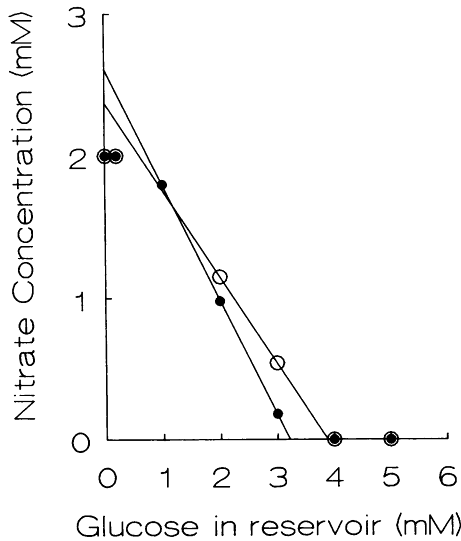

FIG. 1. Steady-state concentrations of nitrate in culture vessels containing *N. europaea*, *N. winogradskyi*, and *A. globiformis* with increasing glucose concentrations in the reservoir at dilution rates of 0.004 h^{-1} (O) and 0.01 h^{-1} (●).

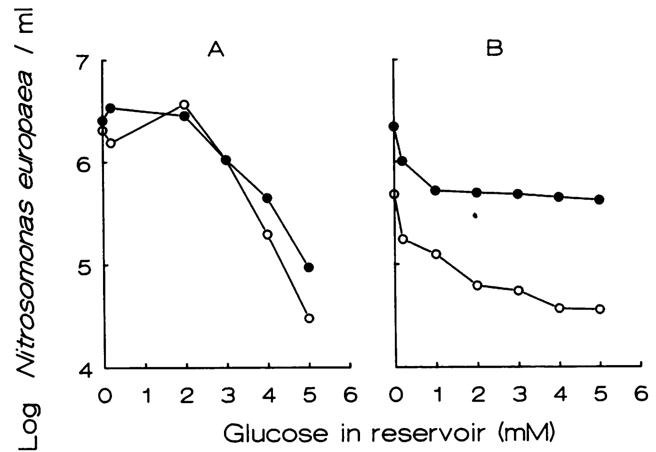

FIG. 2. Steady-state numbers of *N. europaea* cells grown in the presence of *N. winogradskyi* and *A. globiformis* with increasing concentrations of glucose in the reservoir at dilution rates of 0.004 h^{-1} (A) and 0.01 h^{-1} (B). Cells were enumerated with the FA (●) and MPN (O) techniques.

substrate. The K_m and V_{max} for ammonium oxidation by *N. europaea* were calculated with the computer program Enzpack version 2.0 (P. A. Williams, Bangor, United Kingdom) by using the direct linear method.

RESULTS

Mineral nitrogen concentrations. The nitrate concentrations measured in the culture vessels with increasing glucose concentrations in the reservoirs are given in Fig. 1. In the absence of glucose in the reservoir, the 2 mM of ammonium supplied was converted into 2 mM nitrate by the nitrifying bacteria. With glucose at a concentration of 0.2 mM in the reservoir, no measurable difference in nitrate concentration compared with that in the mineral medium was found. However, nitrate concentrations decreased as the glucose concentration increased further and were 0 at the critical C/N ratios. Critical C/N ratios of 11.6 and 9.6 were established for dilution rates of 0.004 and 0.01 h^{-1}, respectively. At glucose concentrations of 4 and 5 mM, no nitrate was detected in the culture vessels. Neither ammonium nor glucose was detected in the culture vessel with any glucose concentration supplied during the competition experiments. Pure cultures of *A. globiformis* growing on 2 mM glucose–2 mM NH_4^+–2 mM NO_3^- only used ammonium as an N source. At both dilution rates, no decreases in nitrate concentrations were measured.

Numbers of *N. europaea* cells. The steady-state numbers of *N. europaea* cells in relation to the glucose concentrations in the reservoirs are presented in Fig. 2. For both growth rates, the numbers of *N. europaea* cells decreased as the glucose concentrations increased in the reservoirs. According to the enumerations with the FA technique, the numbers of *N. europaea* cells with 5 mM glucose had decreased to 4 and 19% of the original numbers at 0 mM glucose for dilution rates of 0.004 and 0.01 h^{-1}, respectively.

At a dilution rate of 0.004 h^{-1}, the numbers were almost constant with up to 2 mM glucose in the reservoir and decreased as the glucose concentrations increased above 2 mM. In addition, a good agreement was found between the numbers of *N. europaea* cells obtained with the MPN and FA techniques. Apparently, all cells present were active in the culture vessel or became active during incubation of the MPN enumerations.

At a dilution rate of 0.01 h^{-1}, the numbers of *N. europaea* cells decreased drastically with concentrations of glucose in the reservoir between 0 and 1 mM. A slight decrease was shown with concentrations of glucose higher than 1 mM. Comparison of the numbers of the ammonium-oxidizing bacteria obtained with the two enumeration techniques showed that at a dilution rate of 0.01 h^{-1} only 8 to 24% of the *N. europaea* cells in the culture vessel were or became active during the incubation for the MPN enumerations.

Comparison of the two growth rates indicated that the decrease in numbers of *N. europaea* cells was most pronounced at a dilution rate of 0.004 h^{-1}. *N. europaea* cells at a dilution rate of 0.01 h^{-1} appeared to be better competitors for limiting amounts of ammonium than were cells growing at a dilution rate of 0.004 h^{-1}, at least with glucose concentrations above 2 mM in the reservoir.

Numbers of *N. winogradskyi* cells. The steady-state numbers of *N. winogradskyi* cells in relation to the glucose concentrations of the influent are presented in Fig. 3. As with the ammonium-oxidizing bacteria, the numbers of *N. winogradskyi* decreased with increasing glucose concentrations in the reservoir. According to total counts with the FA technique, the numbers of the nitrite-oxidizing organism at 5 mM glucose decreased to 17 and 64% of the original numbers with 0 mM glucose for dilution rates of 0.004 and 0.01 h^{-1}, respectively.

Again, a difference between the results of the two enumeration techniques was found. This indicates that only parts of the *N. winogradskyi* cells were or became active during the incubation for the MPN enumerations. The parts of the nitrite-oxidizing population that were detectable with the MPN technique varied between 15 to 49% and 10 to 33% of the total numbers present, obtained with the FA method, for dilution rates of 0.004 and 0.01 h^{-1}, respectively. As with *N. europaea*, the decrease in numbers of *N. winogradskyi* was most pronounced at the lower dilution rate of 0.004 h^{-1}, at

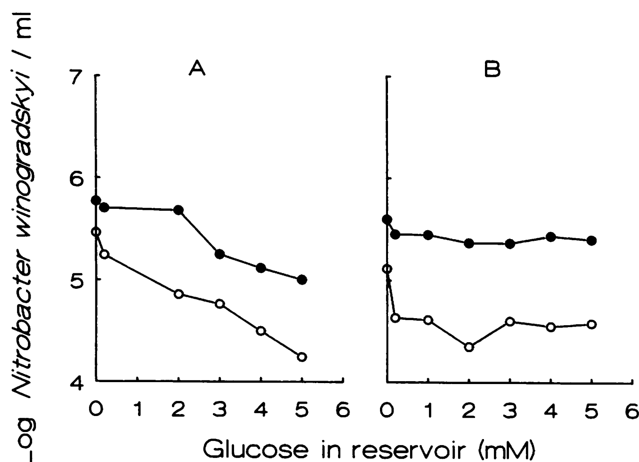

FIG. 3. Steady-state numbers of *N. winogradskyi* cells grown in the presence of *N. europaea* and *A. globiformis* with increasing concentrations of glucose in the reservoir at dilution rates of 0.004 h^{-1} (A) and 0.01 h^{-1} (B). Cells were enumerated with the FA (●) and MPN (○) techniques.

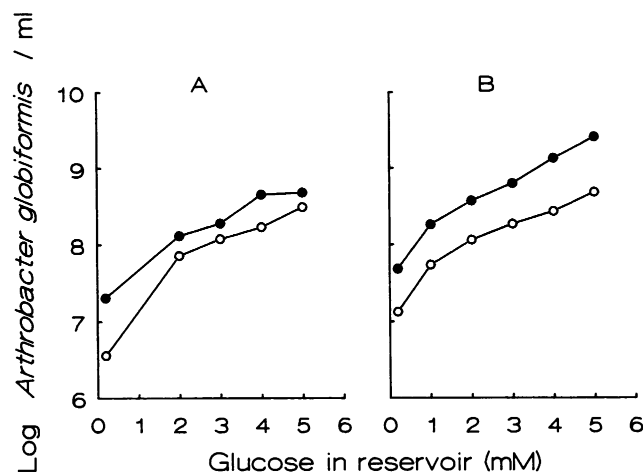

FIG. 4. Steady-state numbers of *A. globiformis* cells grown in the presence of *N. europaea* and *N. winogradskyi* with increasing concentrations of glucose in the reservoir at dilution rates of 0.004 h^{-1} (A) and 0.01 h^{-1} (B). Cells were enumerated with the FA (●) and plate count (○) techniques.

least with glucose concentrations above 2 mM in the reservoir.

Ratios between numbers of nitrifying bacteria. The ratios between the numbers *N. europaea* and *N. winogradskyi* cells in steady-state situations with different glucose concentrations in the reservoirs are given in Table 1. Comparison of the changes in the ratios between both populations revealed that the numbers of *N. europaea* cells decreased more than did the numbers of *N. winogradskyi* cells. At both dilution rates, the ratios decreased as the glucose concentrations increased until, with 5 mM glucose in the reservoir, the ratio between the two nitrifying populations was almost 1.

Numbers of *A. globiformis* cells. The results of the enumerations of *A. globiformis* in steady-state situations in the chemostats are presented in Fig. 4. As expected, the numbers of heterotrophic bacteria grew as the glucose concentrations increased in the reservoirs. The numbers of heterotrophic bacteria at a dilution rate of 0.004 h^{-1} were always lower than those at a dilution rate of 0.01 h^{-1} with similar glucose concentrations, probably due to the higher maintenance energy of the heterotrophs at the lower dilution rate. The numbers of *A. globiformis* cells obtained with the FA technique were always higher than those obtained with the plate count technique.

Organic carbon. The total organic carbon and the cell organic carbon contents of the culture vessels increased with increasing glucose concentrations in the reservoirs for both dilution rates (Fig. 5). As with the numbers of heterotrophic bacteria, the amounts of total and net organic carbon of the mixed culture growing at a dilution rate of 0.004 h^{-1} were lower than those of the culture growing at a dilution rate of 0.01 h^{-1} at all glucose concentrations supplied. Above the critical C/N ratios, i.e., with glucose concentrations of 4 and 5 mM in the medium vessels, the amounts of the dissolved organic carbon increased, whereas the amounts of cell organic carbon remained almost constant.

Bacterial C/N ratios and C efficiencies. The C/N ratios and C efficiencies of cultures of *A. globiformis* grown in the presence and absence of the nitrifying species *N. europaea*

TABLE 1. Ratios between *N. europaea* and *N. winogradskyi* grown in the presence and absence of *A. globiformis* in ammonium-limited chemostats at pH 7.5 and 25°C at two dilution rates[a]

A. globiformis	C source (mM glucose)	No. of bacteria at the following dilution rate (h^{-1}): 0.004 FA	0.004 MPN	0.01 FA	0.01 MPN
−		4.3	7.1	5.6	3.7
+	0.2	6.8	8.9	3.6	4.1
+	1	ND	ND	1.9	3.0
+	2	5.9	51.0	2.1	2.8
+	3	5.9	18.1	2.1	1.4
+	4	3.4	6.3	1.6	1.0
+	5	0.9	1.7	1.6	0.9

[a] The N source was 2 mM NH_4^+ for all chemostats. The numbers of nitrifying bacteria were determined by enumerations with the FA and MPN techniques. ND, not determined.

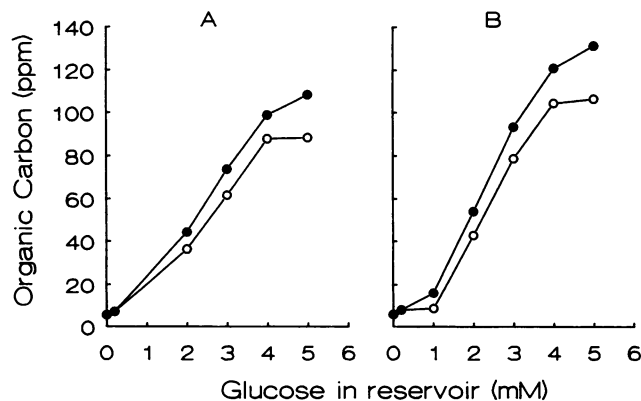

FIG. 5. Steady-state concentrations of total organic carbon (●) and cell organic carbon (○) in culture vessels containing *N. europaea*, *N. winogradskyi*, and *A. globiformis* with increasing concentrations of glucose in the reservoir at dilution rates of 0.004 h^{-1} (A) and 0.01 h^{-1} (B).

TABLE 2. Bacterial C/N ratios and C efficiencies of *A. globiformis* grown in the presence and absence of *N. europaea* and *N. winogradskyi* in glucose-limited chemostats at pH 7.5 and 25°C at two dilution rates

Organisms[a]	Dilution rate (h^{-1})	N source(s) (2 mM each)	C source (mM glucose)	C/N ratio	C efficiency (%)
H	0.004	NH_4^+	2	3.47	35.5
		NH_4^+ + NO_3^-	2	3.45	37.5
H	0.01	NH_4^+	2	3.48	38.0
		NH_4^+ + NO_3^-	2	3.48	41.1
H + N	0.004	NH_4^+	0.2	ND[b]	ND
			2	3.56	25.2
			3	3.52	28.6
H + N	0.01	NH_4^+	0.2	ND	ND
			1	3.58	11.9
			2	3.50	29.8
			3	3.61	36.5

[a] H, pure cultures of *A. globiformis*; H + N, mixed cultures of heterotrophic and nitrifying bacteria in the competition experiments.
[b] ND, not determined.

and *N. winogradskyi* are presented in Table 2. Because the mixed cultures during the competition experiments contained at least 97% *A. globiformis*, the bacterial C/N ratios and the C efficiencies determined in these mixed cultures are dominated by the heterotrophic bacteria and can be compared with the values for *A. globiformis* obtained in pure cultures. The C/N ratios and the C efficiencies of pure cultures of *A. globiformis* growing on 2 mM glucose with equal amounts of ammonium and nitrate present in the reservoir are also given in Table 2.

In comparison with the continuous cultures of *A. globiformis* growing on only ammonium as an N source, the cultures growing on 2 mM glucose–2 mM NH_4^+–2 mM NO_3^- gave similar C/N ratios and C efficiencies for both dilution rates. In the cultures with an excess of ammonium and nitrate in the medium, no decrease in the nitrate concentrations in the culture vessels was detected. These results confirm that *A. globiformis*, similar to many other heterotrophic bacteria, prefers ammonium to nitrate as a source of N.

The C efficiencies were low in mixed cultures compared with those in pure cultures with low glucose concentrations in the reservoirs. The C efficiencies increased as the glucose concentrations increased until values comparable to those in the pure culture experiments were reached. There is as yet no explanation for these observations.

Kinetic parameters. Since the affinity of *N. europaea* for ammonium might be affected by the dilution rate and by the presence of *A. globiformis*, the kinetic parameters for steady-state cultures of *N. europaea* cultured without and with heterotrophic bacteria growing on 2 mM glucose were determined (Table 3). *N. winogradskyi* cells were always present in the samples used for the K_m measurements. To prevent disturbing effects of oxygen consumption by *N. winogradskyi*, sodium chlorate was added to the cell suspensions in the reaction chamber of the biological oxygen monitor.

In pure cultures, no significant difference was found between the K_ms for ammonium of *N. europaea* at the different dilution rates. However, in the presence of *A. globiformis* growing on 2 mM glucose, the K_m at a dilution rate of 0.004 h^{-1} increased significantly up to 0.89 mM, whereas at a dilution rate of 0.01 h^{-1} no significant increase

TABLE 3. K_ms for ammonium oxidation of *N. europaea* grown in combination with *N. winogradskyi* in the presence and absence of *A. globiformis* in ammonium-limited chemostats at pH 7.5 and 25°C at two dilution rates[a]

Dilution rate (h^{-1})	*A. globiformis*	C source (mM glucose)	K_m for ammonium oxidation (mM)
0.004	−	0	0.385
	+	2	0.893
0.01	−	0	0.285
	+	2	0.470

[a] The N source was 2 mM NH_4^+ in all chemostats.

in the K_m was found. Hence, the affinity of *N. europaea* for ammonium decreased more at the lower growth rate than at the higher growth rate when heterotrophic bacteria were present in the system.

As mentioned above, sodium chlorate was added to the cell suspension when the kinetic parameters were determined. Sodium chlorate inhibits the activity of the nitrite-oxidizing bacteria during the respiration measurements because of formation of chlorite by the nitrite oxidizer itself (3). With this result in mind, the sensitivity of *N. europaea* to sodium chlorite and sodium chlorate was tested in the biological oxygen monitor. The ammonium oxidizers were found to be very sensitive to chlorite. The activities of the ammonium oxidizers were inhibited by more than 80% with a chlorite concentration of 10 μM. However, the oxidation of NH_4^+ by *N. europaea* was insensitive to 10 mM sodium chlorate.

DISCUSSION

Nitrification in the carbon-rich, ammonium-limited rhizosphere of plants has been shown to be repressed by heterotrophic processes (18, 32, 36–38). Jansson (18) demonstrated that the heterotrophic flora competed successfully with the nitrifiers for limiting amounts of ammonium. The nitrifiers used only the ammonium nitrogen not needed by the heterotrophs. According to Rosswall (38), this was due to differences in affinity for ammonium between the two bacterial processes. The Michaelis-Menten constants for the immobilization and nitrification processes were found to be 0.014 mM and 0.57 to 1.29 mM, respectively. In the experiments described here, corresponding K_m values for ammonium of *N. europaea* of 0.39 and 0.29 were found for both dilution rates in the absence of heterotrophs, and K_ms of 0.89 and 0.47 mM were found in the presence of heterotrophs growing on 2 mM glucose (Table 3).

In the model experiments performed in this study, the heterotrophic species *A. globiformis* also won the competition for limiting amounts of ammonium supplied to the system. Linear decreases in nitrate concentrations in the culture vessels were found as the glucose concentrations increased in the reservoirs. The ammonium not needed by the heterotrophs was apparently converted into nitrate. The numbers of nitrifying bacteria also decreased as the glucose concentrations increased. This indicates that below the critical C/N ratio the heterotrophic bacteria were using mainly ammonium instead of nitrate as an N source. Critical C/N ratios of 11.6 and 9.6 were established at *D*s of 0.004 and 0.01 h^{-1}, respectively. The difference in the critical C/N

ratio was probably due to a higher maintenance energy of the heterotrophic bacteria at the lower dilution rate.

The outcome of the competition experiments may be influenced by the inhibition of *N. europaea* by glucose or by products of glucose metabolism from *A. globiformis*. Therefore, batch culture experiments were conducted in which glucose, dissolved in demineralized water, and ammonium-free supernatant, obtained after centrifugation of an early-stationary-phase culture of *A. globiformis*, were added to actively nitrifying mixed cultures of *N. europaea* and *N. winogradskyi*. In the control experiments, equal amounts of demineralized water were added. The rate of nitrate formation was followed for 2 weeks, and the pH of the batch cultures was adjusted regularly to 7.5 with sterile 0.1 N NaOH. Neither inhibition or stimulation of nitrate formation was observed with a final glucose concentration of up to 1 mM. During the competition experiments described in this study the glucose concentrations in the culture vessels were below the detection level at all glucose concentrations supplied. Thus, inhibition of the nitrifying bacteria by glucose during the competition experiments can be excluded. These results agree with those of Krümmel and Harms (21), who examined the effect of various organic compounds, i.e., formate, acetate, pyruvate, glucose, and peptone, on nitrite formation of two *Nitrosomonas* spp. They found that the organic compounds tested scarcely affected the growth and activity of the *Nitrosomonas* strains. With the addition of glucose, when autotrophically grown *Nitrosomonas* cells had oxidized 3 mM of ammonium the nitrite formation of mixotrophically grown cultures amounted to 3.00 mM. Jensen (19) found that organic compounds (e.g., glucose, glycerol, acetate, and butyrate) generally were not inhibitory to *Nitrosomonas* spp. but that mannose at 12.5 mM was toxic to growth but did not inhibit the oxidation of ammonium (30). Martiny and Koops (23) investigated the incorporation of organic compounds into cell protein by a *Nitrosomonas* sp. They found that the *Nitrosomonas* sp. assimilated only 0.5 nmol of glucose per mmol of ammonium oxidized. The addition of 10% ammonium-free supernatant of a culture of *A. globiformis* had no effect on the rate of nitrate formation by the nitrifying bacteria. Neither stimulation nor inhibition by the excreted products of glucose metabolism from *A. globiformis* on the rate of nitrate formation was observed. Therefore, it was expected that the organic compounds excreted by *A. globiformis* had no effect on the rate of nitrate formation in the competition experiments in chemostats. Growth responses to individual amino acids or vitamins in log-phase cultures of *N. europaea* were observed by Clark and Schmidt (10). Nitrite formation and protein synthesis were increased by L-glutamic acid, L-aspartic acid, L-serine, and L-glutamine. However, L-lysine, L-histidine, L-threonine, L-valine, L-methionine, and L-arginine were inhibitory. The inhibition of ammonium-oxidizing bacteria by organic compounds originating from plant roots is widely documented (28, 29, 41). These allelopathic effects were ascribed to tannins and tannin-derived organic compounds (33).

By definition, no nitrate was found in the culture vessels at the critical C/N ratios. If the heterotrophs were using only ammonium as an N source and the cultures were totally homogeneous, then the nitrifying populations would have decreased to zero at the critical C/N ratios. Yet small parts of the original nitrifying populations were still present above the critical C/N ratios in steady-state situations. There are two hypotheses which may explain these results.

(i) *N. europaea* might have been partly attached to the walls of the culture vessels and a continuous release of cells that were counted in the enumerations may have taken place. Attached ammonium- and nitrite-oxidizing cells are more active than free-living cells (2, 20). Hence, these attached chemolithotrophic cells might survive in the presence of the heterotrophic cells and use a small part of the ammonium present. The small amounts of nitrate formed were probably taken up by the heterotrophic bacteria, which were N limited above the critical C/N ratios. Once released into the liquid culture, the nitrifying bacteria might become inactive. However, in the mineral medium used for the MPN enumerations they become active again. These inactive cells would be observed in the FA enumerations as well as in the MPN counts. Baltzis and Frederickson (1) described a model of competition for a single resource between two microbial populations in a chemostat when one of them is (partly) attached to the wall. They proved that coexistence of the two populations was possible in spite of the fact that there was only one limiting substrate. During the experiments described here, no cell wall attachment was observed, but the possibility cannot be excluded that microlayers of nitrifying organisms were present on the walls of the culture vessels.

(ii) *A. globiformis* might have won the competition for limiting amounts of ammonium but might not have completely outcompeted the nitrifying organisms. The heterotrophic bacteria might have mainly used ammonium as an N source, but with increasing glucose concentrations in the reservoirs small parts of nitrate might have been consumed. However, this second explanation is less likely, since the specific affinity of the heterotrophic population, reflected by the V_{max}/K_m ratio (9, 15), increases with increasing cell numbers.

To test the latter hypothesis, calculations were made of the numbers of *N. europaea* expected in the culture vessels below the critical C/N ratios. In these calculations, C/N ratios of 3.47 and 3.48 for *A. globiformis*, determined for pure cultures growing on 2 mM NH_4^+, were used for the dilution rates of 0.004 and 0.01 h^{-1}, respectively. In subsequent calculations, it was assumed that below the critical C/N ratios the heterotrophic bacteria only used ammonium for their nitrogen supply. From the measured cell organic carbon and the bacterial C/N ratio, the nitrogen requirements of the heterotrophs were calculated for all glucose concentrations in the reservoirs below the critical C/N ratios. Ammonium not needed by the heterotrophs was supposed to be available for nitrification. The numbers of ammonium oxidizers that could be produced by the amounts of available ammonium were then calculated. From the heterotroph-free cultures with 0 mM glucose it was calculated that 1 mM NH_4^+ gave 1.3×10^6 and 1.1×10^6 *N. europaea* cells ml^{-1} for dilution rates of 0.004 and 0.01 h^{-1}, respectively, according to enumerations with the FA technique.

The calculated and measured numbers of *N. europaea* cells are presented in Fig. 6. Deviations from the calculated ideal line could be caused by different C/N ratios of *A. globiformis* in pure and mixed cultures, nitrate uptake by the heterotrophic bacteria, and inaccuracies in the enumeration techniques. Bacterial C/N ratios in the competition experiments agreed well with those in pure culture experiments of the heterotrophic bacteria (Table 2). The contribution of the nitrifiers to the ratios can be neglected because the ratios between nitrifying and heterotrophic bacteria growing with 2 mM glucose in the reservoir were 1:40 and 1:500 for dilution rates of 0.004 and 0.01 h^{-1}, respectively. If the deviations

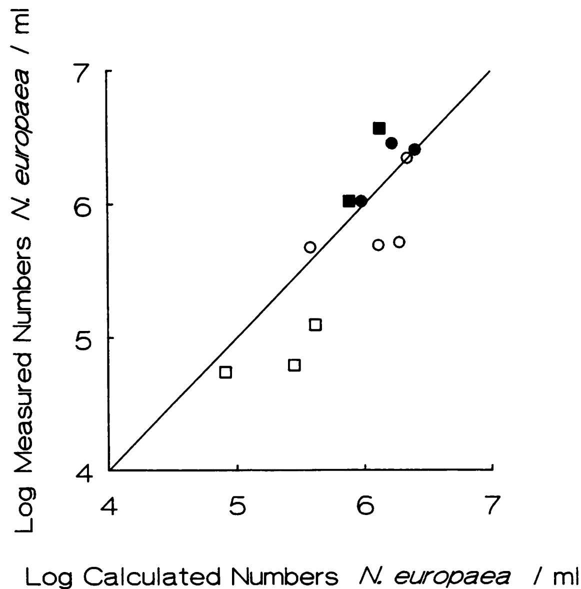

FIG. 6. Calculated versus measured numbers of *N. europaea* cells grown in the presence of *N. winogradskyi* and *A. globiformis* in competition experiments at dilution rates of 0.004 h^{-1} (●, ■) and 0.01 h^{-1} (○, □). Cells were enumerated with the FA (○, ●) and MPN (□, ■) techniques.

from the calculated line were only caused by nitrate uptake by the heterotrophs, all measured numbers of *N. europaea* cells would be above the calculated numbers. Therefore, it is proposed that the differences between the measured and calculated numbers of *N. europaea* cells were mainly caused by inaccuracies in the enumeration methods.

The decrease in numbers of *N. europaea* cells with increasing glucose concentrations in the reservoirs in the presence of *A. globiformis* was most pronounced at the lower dilution rate of 0.004 h^{-1} with glucose concentrations above 2 mM in the reservoir. This phenomenon is not in agreement with the observed lower affinity for ammonium of *N. europaea* at lower glucose concentrations at this dilution rate (Table 3). This lower affinity for ammonium at the lower dilution rate may be a reason for the greater decrease in numbers of *N. europaea* at higher glucose concentrations. Hence, the outcome of the competition at both dilution rates is determined more by the activity of the heterotrophic bacteria than by the affinity for ammonium of the chemolithotrophic bacteria. Apart from that, a decrease in affinity for an inorganic substrate in the presence of organic compounds was also observed in chemostat-grown *Nitrobacter* species (7a).

With respect to the decreased numbers of *N. europaea* and *N. winogradskyi* cells, there was a larger decrease for the ammonium oxidizers than for the nitrite oxidizers as the numbers of heterotrophic bacteria increased during the competition experiments (Table 1). A possible explanation for this observation is heterotrophic growth of *N. winogradskyi* on organic substrates probably released by the heterotrophs. In this way, the decrease in numbers of the nitrite oxidizers was no longer completely coupled to the decrease in numbers of the ammonium oxidizers due to the amounts of nitrite formed. The amounts of organic compounds excreted by *A. globiformis* are shown in Fig. 5. The amounts of dissolved organic carbon above the critical C/N ratios increased as the glucose concentrations in the reservoirs increased, whereas the amounts of cell organic carbon remained almost constant. This was probably due to the fact that the heterotrophic bacteria became nitrogen limited above the critical C/N ratio. Since glucose was not detectable after growth on glucose at any concentration, it is supposed that above the critical C/N ratios the surplus of glucose was still taken up by the heterotrophic bacteria and was excreted in some other organic form. The stimulation of nitrite-oxidizing bacteria by organic compounds has often been reported (5, 6, 8, 16). Hockenbury et al. (16) found that the addition of the filtrate of a mixed heterotrophic population to a batch culture of *Nitrobacter* sp. increased its activity. A reduction of the lag period and an increased growth rate were observed after the addition of 10% of a heterotrophic *Pseudomonas* sp. culture filtrate to continuous cultures of a *Nitrobacter* sp. (5). In addition, an increased release of attached *N. winogradskyi* cells from the vessel wall compared with that of attached *N. europaea* cells may be responsible for changing the ratios between the species as the growth conditions change (20).

The proportion of carbon in the substrate that ended up as cell biomass depended on the C efficiency of the heterotrophs. The efficiency of microorganisms for utilizing glucose for biomass production was reported to be 37% (44). The C efficiencies of 36 and 38% found in this study for pure cultures of *A. globiformis* growing on ammonium at dilution rates of 0.004 and 0.01 h^{-1}, respectively (Table 2), agreed well with this value. Although the carbon content of microbial biomass generally is about 50%, the nitrogen content of microorganisms grown on laboratory culture media varies from 8 to 12%, depending on growth conditions (38). As a consequence of this variable nitrogen content, bacterial C/N ratios vary between 4.2 and 6.3. With an assumed carbon content of 50%, the C/N ratios of 3.47 and 3.48 found in this study for pure cultures of *A. globiformis* growing on 2 mM glucose and 2 mM NH_4^+, respectively (Table 2), indicate a nitrogen content of 14.4% for both dilution rates, illustrating the excess of nitrogen available to the heterotrophs in relation to the amount of available glucose.

In determining the kinetic parameters in this study, sodium chlorate was added to the cell suspension in the reaction chamber of the biological oxygen monitor with a final concentration of 10 mM to prevent disturbing effects of oxygen consumption by *N. winogradskyi*. The ammonium-oxidizing bacteria were found to be very sensitive to chlorite, which is formed by *N. winogradskyi* (3). Their activity was inhibited for more than 80% with sodium chlorite at a concentration of 10 μM. Hynes and Knowles (17) found complete inhibition of the ammonium oxidation by *N. europaea* in the presence of 10 μM sodium chlorite, whereas the oxidation of NH_4^+ was insensitive to 10 mM sodium chlorate. They also demonstrated the formation of chlorite from chlorate by *N. winogradskyi*. In the determinations of K_ms for ammonium oxidation of *N. europaea* in the study described herein, it is not clear whether the formation of chlorite by the nitrite oxidizers has influenced the results. It is unknown how much chlorite was formed before complete inhibition of the nitrite oxidizers took place and whether chlorite was excreted into the medium by the nitrite oxidizers.

The numbers of *A. globiformis* obtained with the FA technique were always higher than those obtained with the plate count technique. The more or less constant differences between the two enumeration techniques were either due to the fact that not all heterotrophic cells in the culture vessels were viable at the time of sampling or that the heterotrophic cells often occur in a double of triple coccoid form.

Also in the rhizosphere of plants, with their commonly high C/N ratios, nitrification is expected to be repressed by more competitive heterotrophic microorganisms. However, adsorption to solid surfaces has a stimulating effect on the activity and survival of nitrifying bacteria. Adherence to soil particles may be a way for the nitrifiers to survive times when the C/N ratios are high, e.g., when the tip of a root is in their vicinity. Therefore, it would be interesting to study the competition for ammonium between nitrifying and heterotrophic bacteria in soil columns continuously percolated with media with different C/N ratios.

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