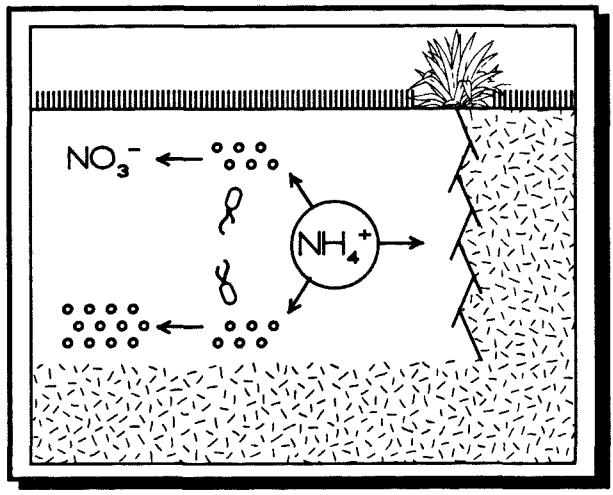
NITRIFICATION VERSUS IMMOBILIZATION OF AMMONIUM IN GRASSLAND SOILS AND EFFECTS OF PROTOZOAN GRAZING



FRANK J.M. VERHAGEN

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IN HET BIJZONDER DE BIOLOGIE

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Aan mijn moeder

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CONTENTS

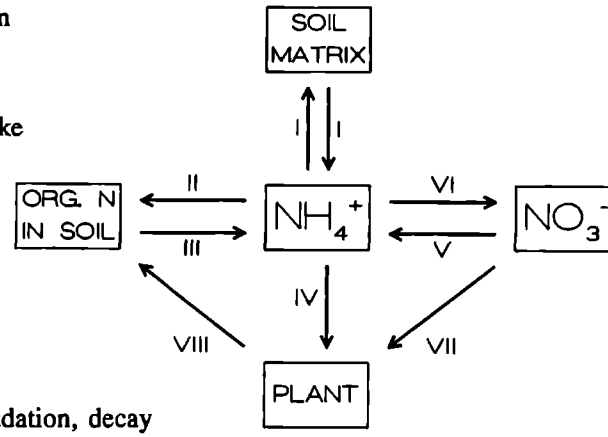
CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The study described in this thesis was performed in order to get more information about the fate and turnover of ammonium in grassland soils. In soil, ammonium can be utilized according to Fig. 1:

I = chemical fixation
II = immobilization
III = mineralization
IV = ammonium uptake



V = nitrate reduction
VI = nitrification
VII = nitrate uptake
VIII = cortex cells, exudation, decay

Fig. 1. Possible routes of ammonium in soil.

Three of these pathways for ammonium, namely nitrification, immobilization, and uptake by plant roots are biological processes, whereas fixation in clay minerals of the soil matrix is a physical immobilization of ammonium, which rate depends on the type of clay minerals in the soil.

In this thesis, the distribution of ammonium over the nitrification and immobilization processes and uptake of ammonium by plant roots are studied. In addition, the contribution of bacteria-feeding protozoa to this distribution was investigated, as protozoa create a recycling of immobilized nitrogen within the system. As the rhizosphere of grassland soils is an extremely complex system with several trophic levels and biological and physical interactions, the distribution of ammonium over the processes mentioned above is studied in model systems using different combinations of pure cultures of nitrifying and heterotrophic microorganisms, a bacteria-feeding flagellate species and a plant species. The model systems used are energy-limited chemostats, continuously percolated non-water-saturated soil columns, and pots with and without plants.

NITRIFICATION IN GRASSLAND SOILS.

Since the 1920s, the nitrification process in grassland soils has been the subject of numerous studies. Mineral nitrogen concentrations tend to remain at low levels in soil under grass (48,104,143). This low level of mineral nitrogen under grass can be explained on the basis of the removal of nitrogen by grass roots since grass roots remove nitrogen from soil at a high rate (149). In grassland soils, it was also often observed that the nitrate concentrations were unexpectedly low in relation to those found in other soils (47,60,82,84,85,103,107,132,144), and that in contrast to arable soils, the major proportion of the mineral nitrogen occurring in grassland soils is in the form of NH_4^+-N rather than NO_3^--N. Not only the nitrate concentrations, but also the numbers and activities of the nitrifying bacteria in grassland soils were found to be low in relation to those found in other soils such as arable land (12,13,14,16,83,108,119,133). In trying to explain these observations, scientists have split into two groups, putting forward two different explanations. The first group advocates that the observations mentioned above arise from inhibitory effects of organic compounds originating from plant roots on the nitrification process, as was first proposed in 1951 by Theron (141). The second group explains the observations by assuming that there is a competition for ammonium in soil between nitrifying bacteria, heterotrophic bacteria and plant roots. This leads to the suppression of the nitrification process by the uptake of ammonium by plant roots or by the immobilizing activities of the more competitive heterotrophic bacteria at high C/N ratios, which are usually found in grassland soils.

Inhibition of nitrification by allelochemicals.

Theron (141) concluded that some plants, and particularly perennial grasses, excrete small quantities of a toxic substance which specifically inhibit the activities of the nitrifying bacteria. According to Rice (99), the suppression of nitrification should have a selective advantage for the plant, because no energy is necessary for the reduction of nitrate to ammonium inside the plant and nitrogen losses by the leaching of mobile nitrate or denitrification are avoided. Munro (88) reported the extraction of a compound, inhibitory to both ammonium- and nitrite-oxidizing bacteria, from roots of various grass species. Rice and Pancholy (100,101,102) extended the idea of inhibition by reporting

that nitrification was inhibited more by plant species of climax ecosystems than by thoseof pioneer vegetations, due to an accumulation of inhibiting compounds over a period of time, and ascribed the inhibitory effect to tannins and some other compounds such as phenolic acids and flavenoids. All herbaceous species, including the grasses, were found to contain considerable amounts of condensed tannins (102). It has also been shown that plant roots contain compounds such as phenols, terpenes, and alkaloids, which are toxic to nitrifying bacteria. The effect of organic substances originating from plants on the nitrification process is widely documented (8,11,27,87,99,135). However, the results of these studies are not always convincing, as most of them were based on experiments, in which plant extracts supposed to contain the inhibitory substances were added only once to the soil and not repeatedly. By the repeated addition of the inhibitory plant extracts the appropriate microorganisms, which rapidly decompose the toxic compounds, will be enriched. In the experiments of Newman and Miller (90), inhibitory plant extracts were added to the soil repeatedly, but the accumulation of toxic compounds to inhibitory levels in soil under natural conditions could not be demonstrated. Moreover, nitrification started immediately after the removal of grasses (28,69,152). Purchase (95) found that washings from living or decaying grass roots did not inhibit nitrification when applied to soil daily. Also macerated roots mixed with soil did not inhibit nitrification in the soil during perfusion with ammonium sulfate. Conclusive experiments on inhibitory plant substances should be carried out in the presence of non-limiting amounts of ammonium in order to eliminate suppressing mechanisms other than those by allelochemicals. Moreover, the reported low numbers of nitrifying bacteria in grassland soils were obtained by MPN estimates, which may have been severe underestimations of the actual population sizes of nitrifying bacteria (26). In addition, the reported low nitrate concentrations in grassland soils are only an indication of the product pool size but do not give information about the nitrate production and consumption rates. Based on literature, it may be concluded that the effects of organic compounds originating from plants on cultures of nitrifying bacteria is dependent on the plant species used and varies from inhibition to stimulation to no effect at all (24,86,88). Organic compounds from *Plantago* species do not inhibit nitrification, as nitrate production in the rhizosphere of three *Plantago* species was demonstrated indirectly by Smit and Woldendorp (131). They found a correlation between the MPN-numbers of nitrifying bacteria and the nitrate reductase activity in the plant material.

Suppression of nitrification by NH_4^+ immobilization of heterotrophic bacteria.

In 1963, Robinson (107) and Theron (142) launched the idea that the low numbers of nitrifying bacteria in grassland soils and the failure of these soils to accumulate nitrate were due to a limiting effect of the ammonium concentration on the population size and activity of the nitrifying bacteria. Nitrification appeared to be controlled by the availability of ammonium, which suggests that a competitive, not a toxic, effect was operative (95,106). In the rhizosphere, with its usual high C/N ratio, competition for limiting amounts of ammonium occurs between nitrifying and heterotrophic bacteria and plant roots, as was described by some authors, but their results were not conclusive. Jansson (70) considered roots of higher plants as weak competitors for ammonium and placed their ability compared with other organisms in the following order: heterotrophic bacteria > nitrifying bacteria > higher plants. His results were based on planted pot experiments, in which the mineral nitrogen was immobilized completely in the presence of excess organic carbon. However, plant roots are known to interfere with the nitrogen mineralization-immobilization turnover by heterotrophic bacteria in soil. Rosswall (109) on the contrary, who based his findings on Km values of 14, 20 to 271, and 571 to 1286 μM N for ammonium uptake by heterotrophic bacteria, plant roots, and nitrifying bacteria, respectively, concluded that the nitrifying bacteria were the weakest competitors for ammonium. It can be questioned whether Km values obtained in homogeneously mixed culture solutions apply to soil. Nevertheless, his results were supported by Zak et al. (157) and Riha et al. (105) who found that plant and microbial uptake of NH_4^+ could reduce the quantity of substrate available for nitrification, depending on the C/N ratio of the environment.

Models of competition.

Three possible mechanisms can be distinguished theoretically with respect to the competition for limiting amounts of ammonium between nitrifying and heterotrophic bacteria. In the mechanistic schemes presented in Fig. 2, the encircled N and H stand for the Nitrifying and Heterotrophic populations in the mixed bacterial community, respectively.

In model 1, the heterotrophic bacteria utilize only ammonium as a N-source and they win the competition for ammonium. The nitrate concentrations and numbers of

nitrifying bacteria in the system decrease as the concentration of organic carbon in the medium increases. Finally, at the critical C/N ratio, both become low or zero, depending on the competitive strengths of both bacterial species.

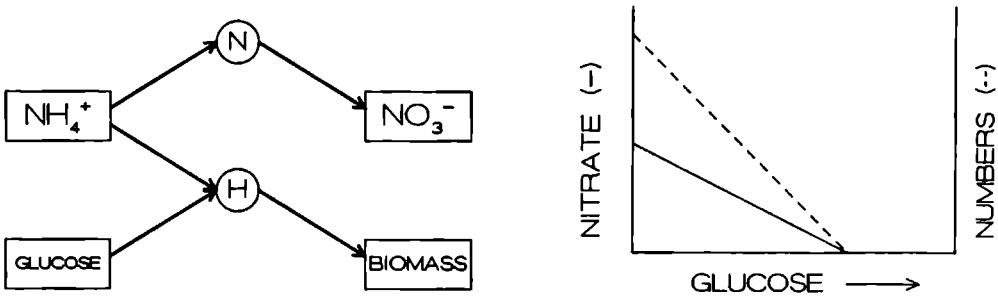


Fig. 2A. Mechanism I. The heterotrophic bacteria win the competition for ammonium.

These decreases are due to limited amounts of ammonium available for nitrification, as only ammonium not needed for assimilation by the heterotrophic bacteria, is being nitrified. At the critical C/N ratio, the heterotrophic bacteria use all nitrogen supplied and consequently, the nitrifying bacteria are outcompeted.

In model 2, the nitrifying bacteria consume all ammonium and the heterotrophic bacteria utilize only nitrate as a N-source. Also in this model, the nitrate concentrations in the system decrease with increasing concentrations of organic carbon in the medium. However, the numbers of nitrifying bacteria are not affected by the organic carbon

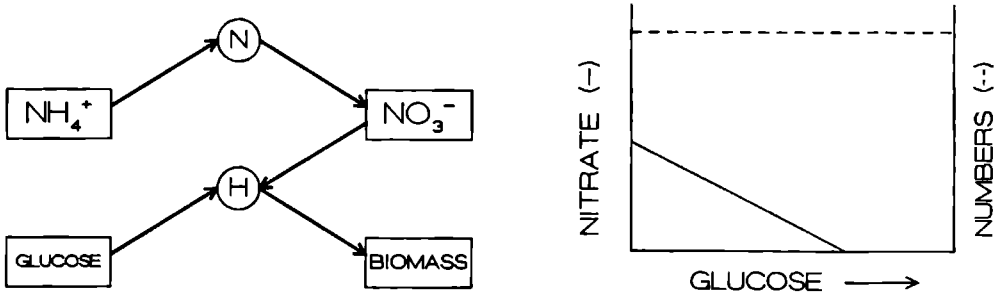


Fig. 2B. Mechanism II. All ammonium present is converted into nitrate, after which the nitrate is partly or totally used as a N-source by the heterotrophic bacteria.

concentrations. First of all, all ammonium present is converted into nitrate by the nitrifying population. After that, the nitrate formed is totally or partly, depending on the amount of organic carbon available, used as a nitrogen source by the heterotrophic bacteria. At the critical C/N ratio, the heterotrophic bacteria use all nitrate present.

In model 3, the heterotrophic bacteria outcompete the nitrifying bacteria to a less extent than in model 1. Nitrate is used in addition to ammonium as a nitrogen source by the heterotrophic bacteria. The nitrate concentrations again decrease with increasing organic carbon concentrations and become zero at the critical C/N ratio.

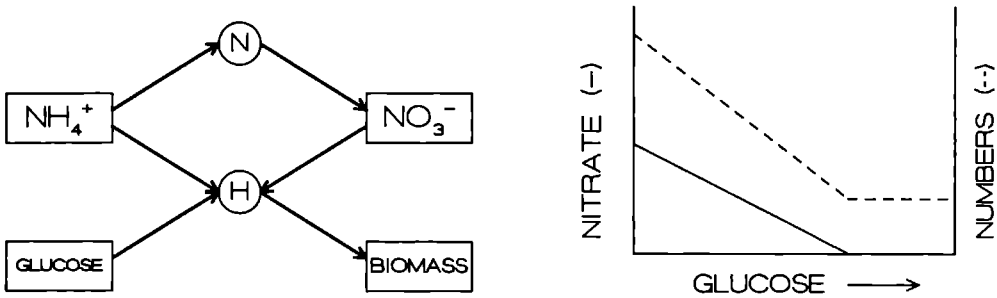


Fig. 2C. Mechanism III. The heterotrophic bacteria utilize nitrate in addition to ammonium as a N-source.

The numbers of nitrifying bacteria also decrease, but at the critical C/N ratio, where no nitrate is found in the system, they do not fully disappear or become totally inactive, but small parts of the ammonium are still being nitrified.

In conclusion, distinction between the three mechanisms can not be made on basis of the observed nitrate concentrations in the system, since in all models the measured nitrate concentrations decrease as the concentrations of organic carbon in the medium increase and become zero at the critical C/N ratio. However, the observed numbers of nitrifying bacteria with increasing organic carbon concentrations differentiate between the mechanisms and give an indication of the model operating in the studied system.
In order to get more insight in the mutual influences of nitrifying and heterotrophic bacteria, the individual processes will be discussed shortly.

The chemolithotrophic nitrification process.

The nitrification process, i.e., the microbial oxidation of ammonium to nitrate, consists of two reactions, which are carried out by two different groups of microorganisms, namely the ammonium- and nitrite-oxidizing bacteria (150). Both groups belong to the family *Nitrobacteraceae* and are chemolithotrophic, i.e., able to use the oxidation of mineral nitrogen compounds as an energy source. Both groups can use CO_2 as sole carbon source and therefore they are also called autotrophic (154). The first group, the ammonium-oxidizing bacteria, transforms ammonium into nitrite via hydroxylamine as an intermediate. The reaction has the following stoichiometry:

$$NH_4^+ + 1\frac{1}{2}\ O_2 \longrightarrow NO_2^- + H_2O + 2H^+$$

Ammonium-oxidizing bacteria are obligatory aerobic organisms. The oxidation of ammonium to nitrite under standard conditions has a free energy change of -260 kJ per mole ammonium oxidized. In soil, most ammonium-oxidizing bacteria belong to the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus*, or *Nitrosovibrio* (121,150).
The second group, the nitrite-oxidizing bacteria, oxidizes the nitrite formed in the first reaction to nitrate:

$$NO_2^- + \frac{1}{2}\ O_2 \longrightarrow NO_3^-$$

Nitrite-oxidizing bacteria are non-obligatory aerobic organisms. Growth of *Nitrobacter* species under anaerobic conditions or at reduced concentrations of oxygen by the reduction of nitrate has been reported by Bock (22,23). The oxidation of 1 mole of nitrite to nitrate under standard conditions has a free energy change of -75 kJ. Until now, all nitrite-oxidizing species isolated from soil belong to the genus *Nitrobacter* (121) or *Nitrospira* (150). Of the first genus, three species have been described from soil: *N. winogradskyi*, *N. hamburgensis*, and *N. vulgaris* (21). Nitrite-oxidizing bacteria are not obligatory autotrophic. Mixotrophic and chemo-organotrophic growth of *N. agilis* with acetate plus casein hydrolysate, acetate alone, pyruvate, yeast extract, and peptone have been reported (19,29,134). *N. hamburgensis* showed highest growth rates under mixotrophic conditions when compared to autotrophic or heterotrophic conditions (22,140).

The kinetics of both oxidation reactions, the biochemistry of the nitrification process and the physiology and taxonomy of the microorganisms performing these reactions have been intensively studied (9,10,20,26,55,71,150).

The N-immobilization process.

By definition, nitrogen immobilization is the incorporation of nitrogen into microbial biomass by heterotrophic bacteria. The immobilization process in natural nitrogen-deficient grassland soils is coupled to the mineralization process. Mineralization of soil organic nitrogen occurs in all soils (97). Heterotrophic bacteria use the energy gained during this mineralization process, which leads to the re-immobilization of a part of the mineralized nitrogen. This mineralization-immobilization cycle has been called the nitrogen turnover (70). For each degradable organic substrate the immobilization ratio may be calculated. This is the amount of immobilized nitrogen divided by the amount of decomposed carbon. For glucose, the immobilization ratio showed to be about 45 to 48 mg N g^{-1} C (96). Clearly, the rate of immobilization depends on the amounts of mineral nitrogen and degradable (nitrogen-containing) organic carbon available to the heterotrophic bacteria. A relative shortage of carbonaceous compounds leads to an increase of the total ammonium pool, which may lead to a higher nitrification rate. A surplus of degradable organic compounds without nitrogen, however, leads to a decrease of the total inorganic nitrogen level (ammonium plus nitrate) (153). It has been found that in liquid cultures, when heterotrophic bacteria grow on an organic substrate in the presence of excess ammonium and nitrate, hardly any of the added nitrate was immobilized (70,96,98,152). When ammonium and nitrate are both available at the same place in soil, immobilization depletes first or exclusively the ammonium pool, nitrate being immobilized after ammonium has been exhausted (96). Rice and Tiedje (98) studied the concept of preferential assimilation of ammonium and elucidated the regulation of nitrate assimilation by ammonium in bacteria isolated from soil. The preferential immobilization of NH_4^+ was attributed to the inhibition of nitrate uptake by ammonium (96).

PROTOZOA.

Protozoa are unicellular, eukaryotic, organisms ranging in size from 2 μm (nanoflagellates) to over 6 mm (some *Sarcodina* species) (54). Protozoa multiply by division, with minimum generation times varying between 2 to 5 hours for small flagellates (53) and 2 to 10 days for thecate amoebae (124). Protozoa can be parasitic or free-living. Free-living protozoa belong either to the ciliates, the flagellates, or the

rhizopods (amoebae). In grassland as well as in arable soils, small heterotrophic free-living flagellates and small naked amoebae are the most numerous protozoa (137,139), whereas ciliate activity is usually low or absent in normally aerated soils. The reason for this is that ciliates, which are usually larger than flagellates and amoebae, need both relatively large amounts of water to be able to swim (45) and high concentrations of bacteria available for consumption (15,52). Many protozoa are able to form cysts in order to survive periods of unfavourable conditions (41). For more information about the general characteristics of soil protozoa the reader is referred to reviews by Darbyshire (44), Fenchel (54), Stout (136), Stout and Heal (138), Stout et al. (139), and Waksman (147).

Protozoan grazing.

Protozoa are the major consumers of bacteria in soil (42,43). In an arable field soil, Stout and Heal (138) calculated that protozoa consumed 150 to 900 g of bacteria per m^2 per year, although the protozoan biomass is estimated to be only 0.3 (50), 2 (138), or 5 (122) g per m^2. However, the importancy of protozoa is related to the rate of biomass production and not to the biomass itself (59). Protozoa feed by phagocytosis and their major food is bacteria (42), but many species also consume yeasts (67), hyphae of fungi (1,42), algae (42,156), spores (91) and detritus (123). Some heterotrophic protozoa can utilize dissolved organic matter (145), whereas others are predatory feeding on smaller protozoa, as well as on small nematodes and other metazoans (120). It was shown that protozoa selectively ingest bacteria from mixed bacterial populations, which has to do with toxic bacterial pigments or toxic bacterial metabolic products (65,118,127,128, 129,130). There is also growing evidence for a strong selective pressure by protozoa on large bacteria (31,57). In soil, probably a large part of the bacteria grows in aggregates or is adsorbed to soil particles and lives in so-called microniches. Caron (30) showed that two heterotrophic microflagellate species efficiently grazed on unattached or non-aggregated bacteria but showed little or no ability to graze on attached or aggregated ones, whereas two other species showed a marked preference for attached and aggregated bacteria and a limited ability to graze on unattached cells, although the densities of unattached bacteria were 5 to 90 times those of attached ones. This food selectivity was also found by Sibbald and Albright (126). Bacterial growth in microniches may be advantageous for the bacteria, since it was shown that microniches may protect bacteria against predation by protozoa in soil (45,93,94,146).

Role of protozoa in nutrient cycling and controlling bacterial densities.

Numerous studies (2,34,37,39,61,125,155) have demonstrated that grazing by protozoa on bacterial populations has a stimulating effect on the mineralization of C, N, P, and S in soil, as was first reported for nitrogen by Waksman and Starkey in 1931 (148). For example, Coleman et al. (40) observed in microcosms with sterilized soil inoculated with microflora and microfauna, up to 35 % more P mineralization and up to 50 % more NH_4^+-N mineralization, as compared with soil microcosms inoculated with only bacteria. In soil, bacteria and protozoa together are responsible for 90 % of the nitrogen mineralization (72). The N, P, and S in excess of the protozoan needs for assimilatory processes is excreted (37,39,137). For example, when bacteria are consumed, part of the bacterial nitrogen is excreted as ammonia or nitrogen-containing organic compounds (65,125). This is due to the fact that a considerable part of the ingested bacterial carbon is respirated and is therefore not available for assimilation.

The effect of predation on bacterial numbers depends on the conditions in the system (68). In a carbon-limited system, the increased mineralization of nitrogen and phosphorus did not result in an increase in bacterial numbers as compared to nitrogen- or phosphorus-limited systems, since the heterotrophic bacteria present were limited by the organic carbon source. In a nitrogen-limited system, however, the higher mineralization rates decreased the negative effects on bacterial numbers by protozoan consumption, and stimulated respiration and ammonium uptake by the bacteria. It is difficult to predict the net effect of protozoan grazing on microbial populations. On one hand, there is the negative effect of a drastical decrease in bacterial numbers by protozoan consumption of bacteria (3,17,32,63,64). Nevertheless, Sambanis and Frederickson (117) found that in batch as well as in homogeneous continuous cultures 10^4 to 10^7 viable bacteria persisted in the presence of protozoa. The decrease in bacterial numbers may be (partly) compensated by the recycling of nutrients as a consequence of higher mineralization rates by the protozoa or by the excretion of organic compounds. The rate of compensation depends on the limiting compound for the bacteria (see above). As a result, some studies reported higher bacterial numbers in the presence of predators than in their absence (4). On the other hand, positive effects on bacterial numbers are that protozoa may contribute to an improved distribution of nutrients or bacteria through the soil or that the protozoa excrete growth factors, as was suggested by Griffiths (62) in case of the higher nitrification rates observed in the presence of protozoa.

Protozoa in the rhizosphere.

A diagrammatic representation of a plant root is shown in Fig. 3 (49). The root is divided into five zones and represents a time period of about 2 to 3 weeks.

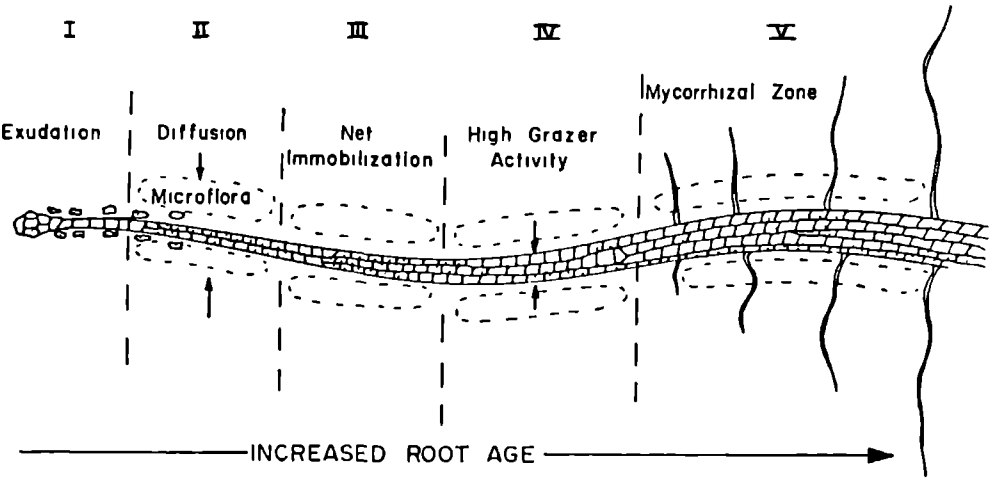


Fig. 3. Diagrammatic model of a spatial-temporal series of events occurring along a growing root.

In zone I, considerable quantities of organic carbon compounds are released into the soil by plant roots (38,89). Carbon losses may occur from lysis of root cell walls (56,78) rather than through active exudation and therefore may consist of cell constituents such as carbohydrates, amino acids, organic acids, nucleotides, flavonones and enzymes (112,116). The amounts of carbon lost by active exudation are small as compared to total carbon losses from roots, which are mainly determined by high numbers of scoured off cortex cells (115). Actually, plant roots are the highest inputs of organic carbon into the soil. These inputs are equal to about 20 % of the total plant dry matter (114) or about 40 % of the carbon translocated to the roots (78). The exudation of organic compounds into the rhizosphere in the zone of elongation of plant roots (113) is well documented (25,77,79,80,81,111,151). In Zone II, microbial populations begin to develop on the plant organic carbon and nutrients diffuse into the rhizosphere. As a consequence of root exudation, bacteria usually have higher densities near roots than in root-free soil (32,46,

76,110), but only small proportions (5 to 10 %) of the root surface are colonized by bacteria (114). In some studies, it was found that bacteria enhance root exudation rates (6,7). In Zone III of the rhizosphere, bacterial species compete for inorganic nutrients such as nitrogen and phosphorus, that have been mineralized by the bacteria or have diffused into the rhizosphere (58,66,92). When high bacterial numbers are reached, a population of bacterial grazers, consisting of protozoa and nematodes, begins to develop (Zone IV). It has been shown that the numbers of protozoa are higher in rhizosphere soil than in bulk soil (5,18,32,46). Because of the high grazer activity and lack of readily available carbon, net mineralization and plant uptake of available inorganic nitrogen and phosphorus may occur in Zone IV. The plant benefits from the heightened concentrations of nutrients. There is a higher uptake of nitrogen by the plant roots in Zone IV, higher nitrogen concentrations within the plants and a higher plant biomass production, as has been reported in many studies (35,36,51,73,74,75). For example, nitrogen mineralization was 50 to 100% higher in the presence of protozoa (50), the nitrogen taken up by plants in the presence of protozoa increased by 20 % (73) to 75 % (35), plants contained 20 % more nitrogen (33), and their weights were 80 % higher in the presence of protozoa than in their absence (33).
Finally, in Zone V mycorrhiza start to develop.

OUTLINE OF THIS THESIS.

In non-rhizosphere soil with its low C/N ratio, the available ammonium is normally oxidized to nitrate by chemolithotrophic bacteria, since the heterotrophic bacteria present are limited by carbon. However, as stated earlier in this chapter, when a plant root grows by creating a carbon-rich rhizosphere with a high C/N ratio, a heterotrophic population starts to develop and needs, in addition to the organic compounds, inorganic compounds such as nitrogen and phosphorus. They compete with the nitrifying organisms for the available ammonium. When starting this study, it was questioned whether the nitrification process would be competitive to other ammonium-utilizing processes. This thesis contains the results of a series of model experiments with increasing complexity, in which the competition for limiting amounts of ammonium was studied between nitrifying and heterotrophic bacteria. Moreover, the effect of grazing by protozoa on the fate of ammonium is studied.

The new free-living soil flagellate *Adriamonas peritocrescens*, gen. nov., sp. nov., is described in Chapter 2.

The competition for ammonium between nitrifying and heterotrophic bacteria by supplying increasing amounts of glucose and equal amounts of ammonium, imitating the exudation process of plant roots, is studied in chemostats (Chapter 3) and in continuously percolated non-water-saturated columns with γ-sterilized soil (Chapter 5). The three possible models of competition mentioned earlier in this chapter are also investigated for both model systems.

The effect of grazing by protozoa on the fate of ammonium is studied by the addition of a bacteriovorous flagellate species to the mixed bacterial cultures in the chemostats (Chapter 4) and in the γ-sterilized non-water-saturated soil columns (Chapter 6). By the addition of flagellates, an internal recycling of immobilized nitrogen is likely created in the system, which increases the turnover rate of nitrogen.

The most complex model system of this study is described in Chapter 7, in which an axenic plant (Ribwort plantain) grows in a pot containing γ-sterilized soil. Pots are inoculated with the competing bacterial species and with protozoa mentioned above. The effects of fertilization were also studied and all possible combinations within the experimental factors, i.e., presence of a plant, fertilization with ammonium, and grazing by flagellates, are included in this experiment.

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CHAPTER 2

ADRIAMONAS PERITOCRESCENS GEN. NOV., SP. NOV., A NEW FREE-LIVING SOIL FLAGELLATE, (PROTISTA INCERTAE SEDIS).

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SUMMARY

The free-living flagellate *Adriamonas peritocrescens*, a new genus from calcareous pasture soil, has been studied by light microscopy and scanning and transmission electron microscopy. The cell is elongate and possesses two anterior, subapically inserted subequal flagella. A mouth is situated in the antero-ventral part of the cell. Three flagellar roots, of which two are comprised of relatively few microtubules and one of many, arise from the flagellar basal bodies. Two of these roots, a major right and a minor left one, run parallel and posteriorly to the right under the ventral cell membrane. They both end in the lips of the mouth. The major root turns 180° around the opening of the mouth. The third root runs anteriorly and ends in a electron dense polar strip in the antero-dorsal part of the cell. This polar strip gives rise to a corset of microtubules, which lies under the dorsal surface. The cell has one or two concentric extrusomes and a contractile vacuole in the anterior part of the cell. Mitochondria have tubular cristae. A fragmenting nuclear envelope was observed during mitosis. *Adriamonas peritocrescens* has minimum doubling times of 2-3 hours in dense pure cultures of *Arthrobacter globiformis* at pH 7.5 and 20°C. The ultrastructure reveals some affinities with those of the Pseudodendromonads, although scales and a stalk are absent. Its phylogenetic position is discussed and the new genus is consequently placed *incertae sedis* in the kingdom Protista.

INTRODUCTION

Soil flagellates have been little studied and most descriptions from light microscopical studies are ambiguous (11,47). Recently, the ecological importance of free-living flagellates has been recognized (4,8,44). By their grazing and subsequent mineralizing activities, they stimulate or reduce bacterial growth and numbers or release nutrients to the ecosystem (5,6,21,22,23,46, 56). Because of their ecological importance, more study is needed to establish which species are involved in these processes.

The introduction of electron microscopy has greatly changed flagellate taxonomy (28,29,33,42,51). The characterization of soil flagellates using electron microscopical techniques has lead to the creation, for example *Hemimastix* (10), and elimination, for example *Spiromonas* (43), of genera. As a result of electron microscopical studies,

several new flagellate species could not be assigned to any described genus and consequently new genera, often consisting of 1 or 2 species, were created, for example *Cafeteria* (9), *Massisteria* (41), *Jakoba* (39), *Discocelis* (55) and *Psalteriomonas* (1). The soil flagellates have become a group within the Protists with an extremely high diversity (11).

Base sequences of ribosomal RNA of many flagellates are now being studied in order to get a comprehensive view of the relationships within this diverse group (7,12,24,26,48,49, 53). The combination of molecular biological studies with electron microscopy will probably give a thorough knowledge about the identities of organisms, the structures and relationships within a genus and the relatedness of genera to other kinds of Protists. This may ultimately lead to an unambiguous classification of previously described and newly isolated flagellates. Until that time, new flagellate species will be isolated of which some cannot be assigned to any existing well circumscribed group.

This paper reports the isolation, culturing and monoxenisation of a hitherto undescribed heterotrophic flagellate. It was isolated from the calcareous soil of a pasture in The Netherlands and it has a remarkable growth rate capacity in liquid cultures of *Arthrobacter globiformis*. On basis of a study of its internal structure with electron microscopical techniques, it is shown that it has some relationships with the genus *Pseudodendromonas*, but outside this it shows no affinities with any well characterized suprafamilial groups of Protists.

MATERIALS AND METHODS

Isolation

Soil samples were collected from the top 0-10 cm layer of an extensively used pasture near Brummen (52°05' N, 06°09' E) in the Netherlands on 5 May 1988. The samples were passed through a 2 mm sieve to remove roots and stones and were well mixed. The soil is sandy and contains 21.3 % coarse sand, 69.3 % fine sand, 4.1 % silt and 5.3 % clay. The pH-H_2O of the sieved mixed soil was 7.8, the pH-KCl was 7.3, the content of calcium carbonate was 0.8 % and of organic carbon 5.3 %.

To 100 ml of a 5 % (w/v) soil suspension in 5 mM phosphate buffer (pH 7.5) 20 mg proteose peptone was added to increase the number of flagellates by stimulating bacterial growth. The soil suspension was gently shaken 2-3 times per day. After 4 days,

the liquid above the soil was divided into 50 tubes containing pure cultures of *Arthrobacter globiformis* (pH 7.5). This selected for flagellate species able to grow on this bacterium. After 3 days, single flagellates were transferred using a micropipette to pure cultures of *A. globiformis* to obtain monocultures. Since then, the cultures have been maintained at the Institute for Ecological Research, Heteren, the Netherlands. The flagellate species is deposited at the Culture Collection of Algae and Protozoa, Ambleside, UK and video sequences are available at the BOFS Video Database of the Department of Zoology, University of Bristol, UK.

Culturing

Flagellates were maintained in 5-day old cultures of *A. globiformis*. For this purpose, *A. globiformis* was grown in a liquid medium containing (per l): $(NH_4)_2SO_4$, 0.33 g; KH_2PO_4, 0.75 g; $MgSO_4.7H_2O$, 0.04 g; NaCl, 0.5 g; glucose.H_2O, 1 g (glucose autoclaved separately) and solution of trace elements, 1 ml. The pH of the medium was adjusted with 1 N NaOH; after autoclaving the pH was 7.8. After inoculation with *A. globiformis* and incubation on a rotary shaker at 25°C for 4 days, the pH was adjusted to 7.5 with sterile 1 N NaOH. On day 5, 1 ml of a culture of flagellates was added to 40 ml of a culture of *A. globiformis* in a 100 ml flask, stopped with cotton wool. The culture of bacteria contained 10^8-10^9 bacteria per ml. Cultures of flagellates were gently shaken 2-3 times per day. After 3-4 days, dense cultures of flagellates were obtained.

Monoxenisation

Sterile medium A (30 ml), containing (per l): $(NH_4)_2SO_4$, 0.56 g; KH_2PO_4, 0.33 g (pH 7.0 after sterilization); $MgSO_4.7H_2O$, 67 mg; $CaCl_2$, 33 mg, NaCl, 0.83 mg, and 1.7 ml of a solution of trace elements was added to 20 ml of a commercial (Sigma) sterile 0.9 % (w/v) NaCl solution (Sigma) containing the following mixture of antibiotics (per ml): penicillin, 5000 U; streptomycin, 5 mg and neomycin, 10 mg. After mixing, 5 ml of a dense culture of flagellates (10^7 cells ml^{-1}) was added and incubated for 2 days at 20°C. Starved flagellates, free of bacteria within the cells, were filtered using a sterile mixed-cellulose-ester membrane filter with a pore size of 3 μm. Subsequently, 50 ml of a filter-sterilized solution of 20 mg lysozyme (49,100 U mg^{-1} protein, Sigma) in 200 ml medium, containing the same components as medium A but at 0.6 times their concentrations, was poured slowly through the filter in 10 minutes to remove the remaining bacteria. The flagellates were rinsed with 10 ml sterile 60 % strength medium

A. Treatments with sterile lysozyme and rinsing solution were repeated twice. The bacteria-free flagellates were resuspended by sucking 10 ml of the rinsing solution into a sterile syringe and the suspension of flagellates was subsequently divided over 50 tubes containing pure cultures of *A. globiformis* (pH 7.5). After incubation for 5 days at 20°C tubes were tested for growth of flagellates. Positive tubes were tested for monoxenity by plating on an agar medium, containing (per l): $(NH_4)_2SO_4$, 0.33 g; KH_2PO_4, 0.125 g; yeast extract (Difco), 1 g; tryptose (Oxoid), 2 g, and glucose.H_2O, 0.2 g (glucose autoclaved separately). The pH of the agar medium was adjusted with 1 N NaOH, after autoclaving it was 7.5.

Growth features

To determine the growth rate of the flagellates, 40 ml in a 100 ml flask of a culture of *A. globiformis* (pH 7.5), containing 6.5 x 10^9 bacteria ml^{-1}, was inoculated with 1 ml of a culture of flagellates. Control experiments were not inoculated with flagellates. Incubation was at 20°C and the flasks were gently shaken 2-3 times per day. Samples were taken regularly and diluted 1:1 with Lugol's Iodine. Samples with fixed flagellates were stored at 4°C and counted within 3 days. No loss of flagellates or bacteria by fixation and storage at 4°C was observed. Numbers of flagellates and bacteria were determined using counting chambers. Determination of the growth features were performed in triplicate.

Microscopy

Living cells were examined using a Zeiss Standard 18 light microscope, equipped with phase contrast optics and a dedicated electronic flash device for photography (37).

For scanning electron microscopy, cells were concentrated by centrifugation and subsequently fixed according to Parducz (36). Fixed cells were transferred to glass cover slips coated with poly-L-lysine. Dehydration of the cells was achieved by passing the cover slips through a graded series of ethanols and, subsequently, cells were critical point dried. After coating with gold, cells were examined with a JEOL JSM-U3 scanning electron microscope.

For transmission electron microscopy, cells were concentrated by centrifugation and subsequently fixed in a cocktail of 2 % glutaraldehyde and 2 % OsO_4 in 10 mM Hepes buffer (pH 7.2) for 1 hr at 0°C (27). After rinsing the cells three times with 10 mM Hepes buffer (pH 7.2), cells were embedded in 2 % (w/v) agarose. Cells were

dehydrated by applying a graded series of ethanols. En-bloc staining with 2 % (w/v) uranyl acetate in 70 % ethanol for 2 hours at 20°C was followed by embedding in Araldite CY212 resin (Agar Sc., UK). Sections were cut with a diamond knife and a LKB Ultratome 4801A ultramicrotome and were subsequently stained with 2 % (w/v) lead citrate. Cells were examined using a JEOL 1200 EX transmission electron microscope.

The orientations in the description and drawings of the organism were based on axonemal rotation (51).

OBSERVATIONS

Light microscopy

Normally, cells have a somewhat oval, elongate shape, 8.3 ± 1.0 μm (n=38) long and 4.3 ± 0.9 μm (n=38) wide with one pair of flagella (Figs. 1-3,43). Larger dividing cells were frequently observed, mostly bearing two pairs of two flagella. The two subequal flagella are acronematic and insert subapically at the anterior end of the cell. The flagella are somewhat shorter than the body. Swimming cells move quickly, regularly changing direction. When they change direction, they shake continuously and regularly turn around the longitudinal axis. The anterior flagellum F1 is directed forward and is probably the cause of motion, as it beats rapidly when the cell moves around. The posterior flagellum F2 is directed to the ventral posterior part of the cell, covering the mouth region. Non-swimming cells have a more or less round shape, with sometimes one of the flagella inactive.

Cells often circle around a bacterium, embracing the bacterium with one of the flagella. Somewhat later, these bacteria disappeared into the cell.

Most cells have one large nucleus, 2.5 ± 0.2 μm (n=27) in diameter, although 2-4 nuclei were observed in some cells from logarithmically growing cultures (Fig. 12). The nucleus has a spherical to droplet shape and is located dorsally near the flagellar apparatus. There is one round nucleolus, 1.2 ± 0.1 μm (n=27) in diameter. A curved mitochondrion lies close to the nucleus and the flagellar apparatus in the ventral side of the cell. The diameter of the mitochondrion is 0.5 ± 0.1 μm (n=12). There is an obvious refractile granule in the centre of the cell. It is 1.4 ± 0.2 μm (n=22) in diameter and consists probably of storage material. There are a number of dense granules at the

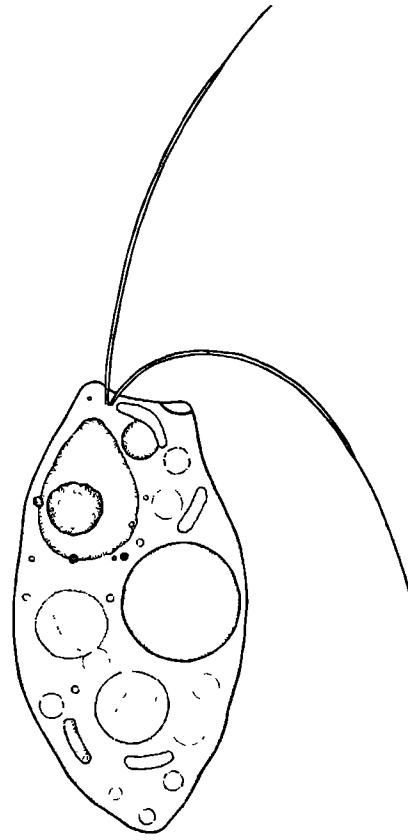


Fig. 43. Diagram showing general aspects of the cell, with two anterior, subapically inserted acronematic flagella, a dorsally located nucleus with nucleolus near the flagellar apparatus, a antero-ventrally located mouth, a centrally situated refractile granule, and vacuolated cytoplasm.

posterior end of the cell. The position and variable number of these granules indicate that these are probably food vacuoles.

The cell has no scales. The organisms are not connected to each other or to organic material by means of stalks.

Cysts are more of less globular without pores or surface structures (Fig. 40). They are 3.9 $\pm$ 0.4 μm (n=20) in diameter.

Scanning electron microscopy

Scanning electron microscopy confirms that the flagella insert subapically near the anterior end of the cell and that they are acronematic (Fig. 5). One flagellum (F1) is inserted more dorsally than and to the right of the other (F2). The anterior flagellum (F1) usually is about 0.9 μm longer than the posterior one (F2), 7.7 $\pm$ 0.6 μm and 6.8 $\pm$ 0.5 (n=13), respectively, but the difference in length is not statistically significant. The 9+2 structure relative to the narrow tip, which is assumed to contain only the central pair, is longer in the longer anterior flagellum. The transition from the 9+2 structure into the tip in F1 occurs at about 80 % of the total length of the flagellum, whereas in F2 the transition is situated at about 50 % of the total length of the flagellum. The difference may have something to do with feeding, as the posterior flagellum (F2) was often observed to encircle bacteria (see above).

The mouth is situated at the ventral side of the organism and is usually surrounded by extended lips (Fig. 4). The diameter of the cytostome is 0.5 to 1.0 μm.

Dividing cells usually have a triangular shape with two pairs of two flagella, each pair located at one tip of the triangle (Fig. 6). Halfway between the two pairs of flagella an indentation may be seen.

Transmission electron microscopy

i) *The flagella*

F1 is the right flagellum and F2 is the left one. The basal bodies of the two flagella insert subapically. B1 (i.e. the basal body of F1) lies anterior and to the right of B2, and is directed antero-ventrally. They are 300 nm long. The angle between the two basal bodies is 60-70°. The transition zone includes a single transverse plate (Figs. 8,33,37-39). The central pair of microtubules of the axoneme makes contact with the transverse plate. The other end of B1 is associated with a dense rhizostyle, which is about 800 nm long and ends on the nuclear envelope (Fig. 26). The rhizostyle is also connected to B2. Central fibrous structures, which are possibly microtubules, were observed within this rhizostyle.

In many cells 3 or 4 basal bodies were observed (Fig. 13). On basis of the short doubling time of the flagellate, it is assumed that these belong to a replicating flagellar apparatus.

Plate 1. Figs. 1-3. Light microscopy, phase contrast optics (Figs. 1,2) and differential interference contrast optics (Fig. 3). Views of living cells showing cell shape, flagella, nucleus, nucleolus, refractile granule and food vacuoles. Fig. 3, courtesy Dr. Claudius K. Stümm, Catholic University Nijmegen, Nijmegen, The Netherlands. Figs. 4-6. Scanning electron microscopy. Fixed, gold coated cells showing the mouth (Fig. 4) and the anteriorly inserted, subequal, acronematic flagella (Fig. 5). Triangular shaped, dividing cells usually bear two pairs of two flagella (Fig. 6). An indentation is seen in the middle between the flagellar pairs. Courtesy Professor Sassen, Catholic University Nijmegen, the Netherlands. Fig. 7. This and all subsequent micrographs are transmission electron micrographs of thin sectioned material. Fig. 7 represents a longitudinal, dorso-ventral section showing the cell shape, one of the two flagella (F), food vacuoles (FV), mitochondria (M), the mouth (Mo) and the nucleus (N). Scale bars: 5 μm (Figs. 1-3); 4 μm (Fig. 6); 2 μm (Fig. 4); 1 μm (Figs. 5,7).

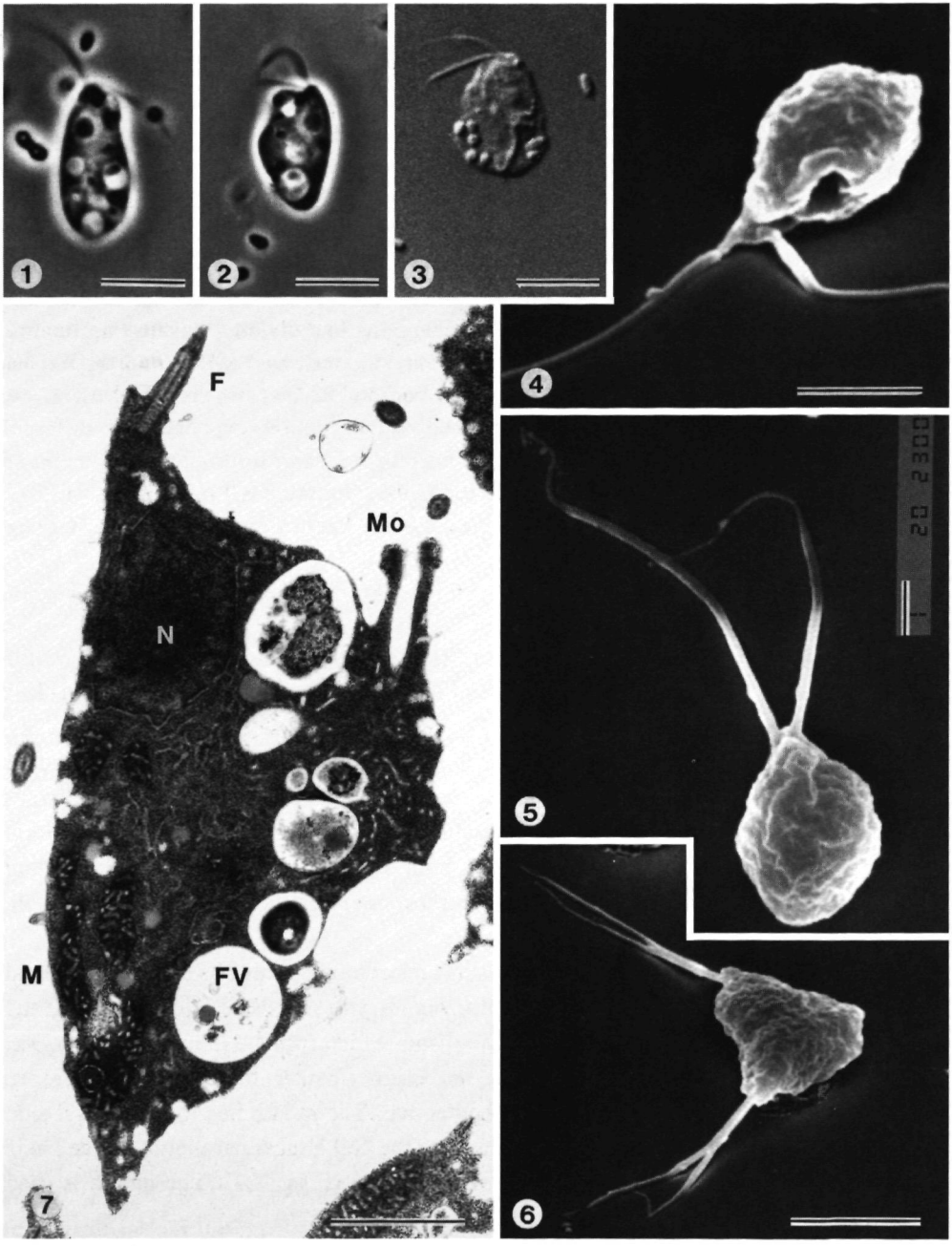
No flagellar hairs, paraxial rods, transition helices or other elaborations of the flagella were observed.

ii) *The cytoskeleton*

The cytoskeleton of *A. peritocrescens* consists of one major ventral root (MV) supporting the mouth, one minor ventral root (mV) ending at the dorsal side of the mouth, one short dorsal root (DR) running to the anterior end of the cell and a dorsal corset of microtubules (DC) supporting the cell membrane.

Ventral roots.

The first root is the major ventral mouth root (MV). It is a band of 11 or 12 microtubules (Figs. 17-23,24). They attach obliquely to the right side of the basal body of F1 (Figs. 8,24,25,27). The root is attached to the right side of the basal body of F2 by a fibrous connective structure (Fig. 13). The microtubules run to the right along the antero-ventral side of the cell. It may be observed that the major ventral mouth root splits before reaching the mouth, either due to a predivision stage, in which the root has already divided, or to the flexibility of microtubular root structures, which causes variety in shape, length and direction of the root. At the mouth, some of the microtubules of the root, mostly 6-7, turn 180° supporting the extended lips of the mouth (Figs. 9,10,19-23). The microtubules end on the left side of the mouth. One of the microtubules lies to the right outside the row formed by the others. This single microtubule also curves around


1
2
3
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F
Mo
N
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FV
7
5
6

Plate 2. Fig. 8. Dorso-ventral section showing the anterior part of the cell: basal body of flagellum 1 (B1), dorsal corset of microtubules (DC), extrusome (E), flagellum 2 (F2), mitochondrion (M) and major ventral mouth root (MV). Fig. 9. Lengthwise section through the mouth. The shape of the cytopharynx, the extended lips of the cytostome, the cross-sectioned microtubules of the major ventral mouth root (arrow), and the single microtubule turning around the mouth outside the rest can be seen. Fig. 10. Micrograph of a cross-sectioned cytostome, showing the 180° turn around the mouth (Mo) of the major ventral mouth root. Fig. 11. Mitosis with fragmenting nuclear envelope (arrow). Fig. 12. Cultures with logarithmically growing cells sometimes contain cells with 4 nuclei. Fig. 13. In some cells 3-4 basal bodies were observed, probably belonging to a dividing flagellar apparatus. The basal body of flagellum 1 (B1), the major ventral mouth root (MV), and the fibrous connective structure between MV and the basal body of F2 (arrow) are visible. Fig. 14. Cross sectioned posterior part of the cell with food vacuoles containing bacteria in different stages of digestion. Fig. 15. Numerous mitochondrial profiles with tubular cristae. Fig. 16 shows the fibrous structure (arrow) between the Golgi-apparatus (G) and the nucleus (N). It probably plays a role during mitosis (see text). Scale bars: 1 μm (Figs. 12,14); 0.5 μm (Figs. 8,9,15); 0.25 μm (Figs. 10,11,13,16).

the mouth more ventrally outside the others (Fig. 9). This microtubule might be the onlyone extending 360° around the mouth and ends at the dorsal side of the mouth. It is cross-sectioned in Fig. 9.

The second root is the minor ventral mouth root (mV). It consists of 2 or 3 microtubules originating in electron-dense material between the two basal bodies (Figs. 17-23,24). The microtubules run parallel external to those of the major ventral root. The microtubules of the minor and the major ventral root diverge before reaching the mouth. The microtubules of the minor ventral root end in the dorsal lip of the mouth (Figs. 20-23).

A variable appearance of one or two short microtubules was observed at the dorsal face at the end of and parallel to the cytopharynx. If present, these microtubules at the tip are the source of a delicate electron-dense sheath which forms a funnel (cytopharynx) leading into the cell (Figs. 21,22). It is not clear whether these microtubules are connected to one of the mouth roots described above. The mouth lies to the ventral side of the large curved mitochondrion. It extends into the cell almost parallel to the cell axis (Fig. 7). The cytostome is surrounded by extended lips (Figs. 7,9). The mouth is used for the ingestion of bacteria (Fig. 41).

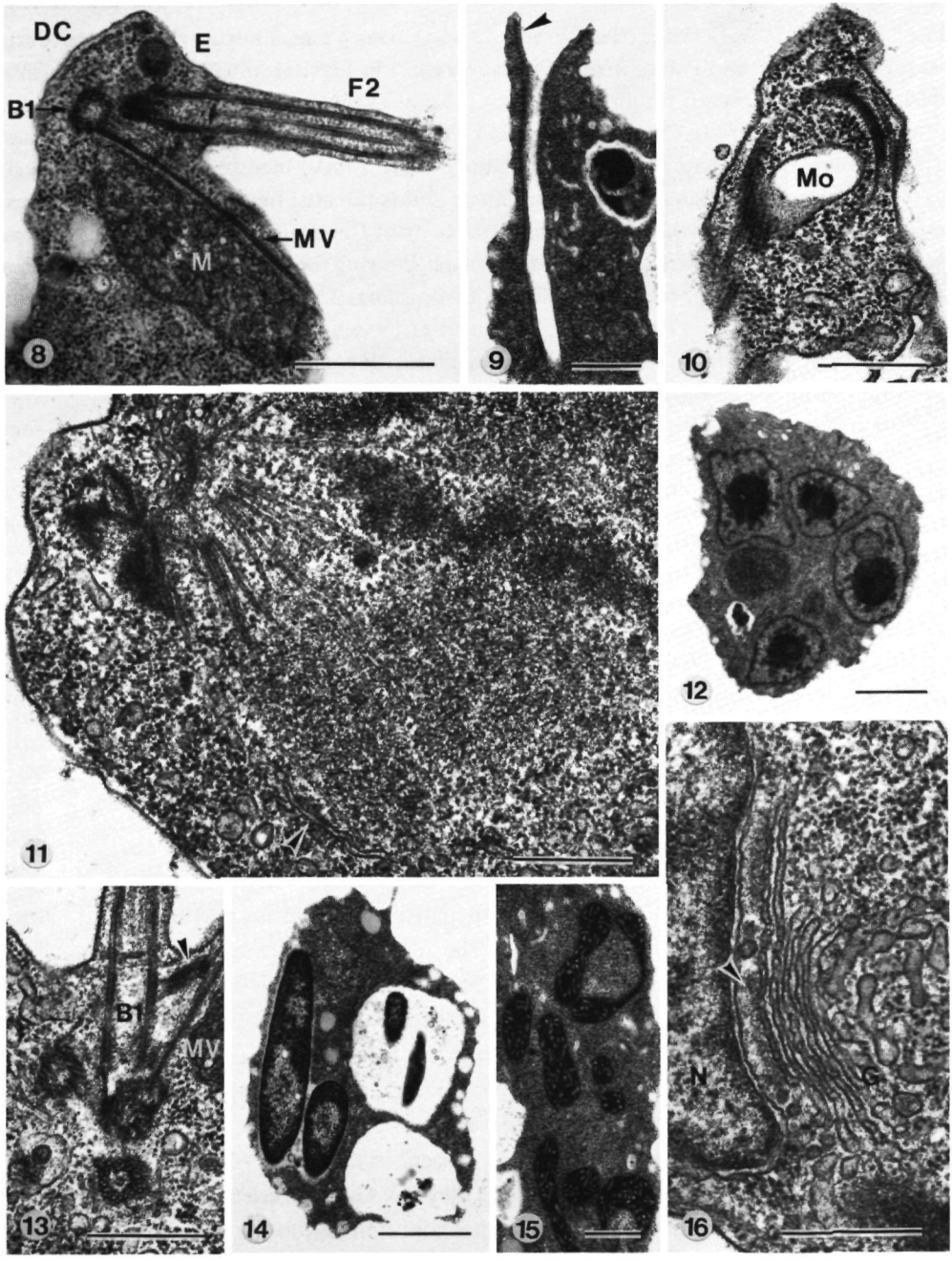
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Plate 3. Figs. 17-23. Serial sections in the dorso-ventral plane, showing the courses of the major (MV) and the minor ventral (mV) mouth root. In Figs. 21 and 22 the electron-dense mouth-supporting sheath (S) and the mouth (Mo) are visible, respectively. Figs. 21-23 are turned 90° in relation to Figs. 17-20. Fig. 24. Dorso-ventral section of the anterior part of the cell, showing the major (MV) and the minor ventral (mV) mouth roots, a large curved mitochondrion (M) and the nucleus (N). Fig. 25 shows the attachment of the major ventral mouth root (MV) to the right side of the basal body of flagellum 1 (F1). Also the electron-dense rhizostyle (R) is visible. Fig. 26. Micrograph showing the association (arrow) between the tip of the rhizostyle, which is in line with flagellum 1 (F1), and the envelope of the nucleus (N). Fig. 27. Cross sectioned attachment of the major ventral mouth root (MV) to the basal body of flagellum 1 (B1) and the close association with a mitochondrion (M). Scale bars: 0.5 μm (Figs. 17-26); 0.25 μm (Fig. 27).

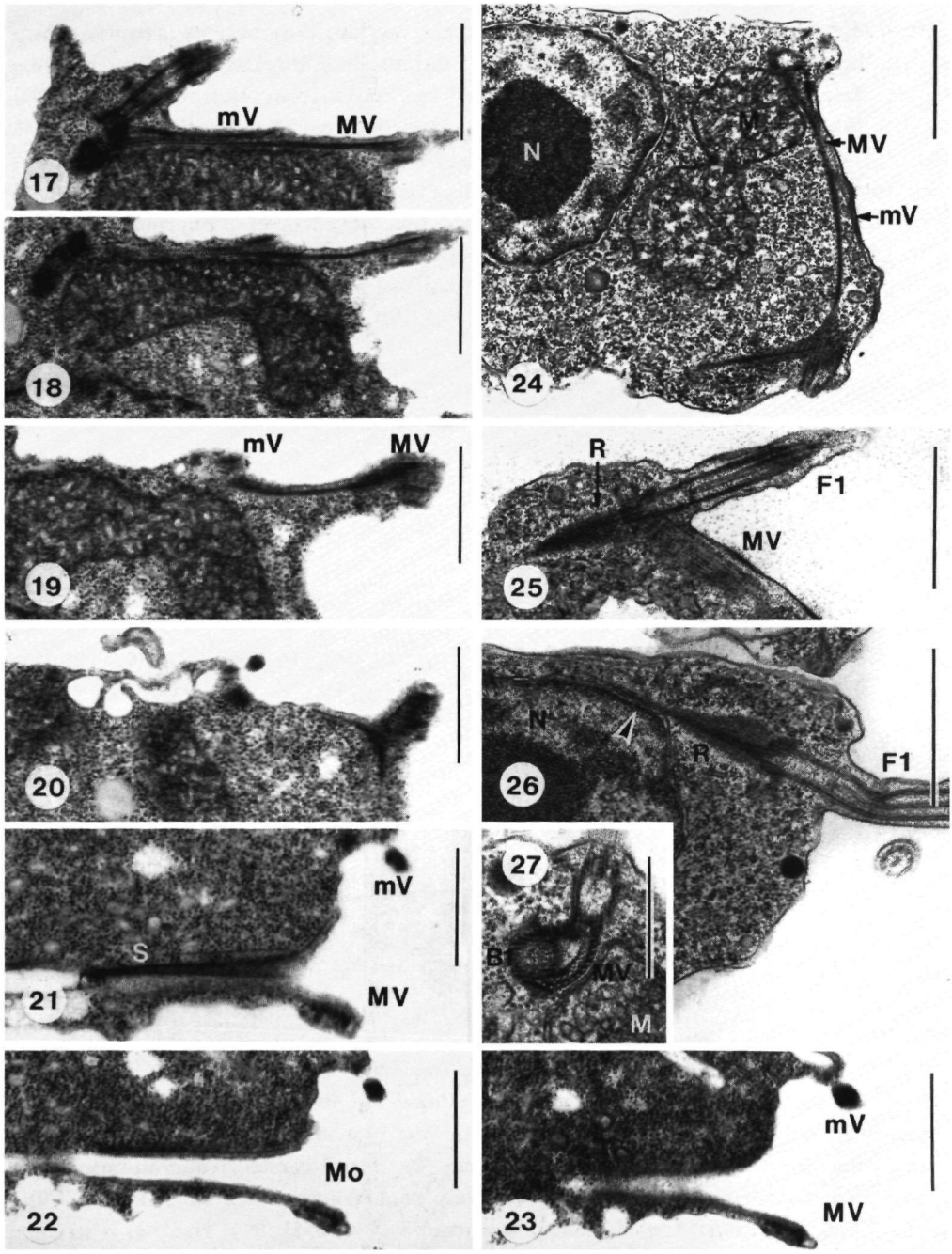
Dorsal root.

The third root is the dorsal root (DR). It is comprised of a thin electron-dense band (dorsal connector) with 2 microtubules on one side and one on the other (Figs. 28-33, 34-36). All arise in electron-dense material located adjacent and dorsal to the basal body of F1 and extend to a band of electron-dense material (polar strip) at the anterior pole of the cell (Figs. 28,29,34,37-39). The polar strip gives rise to about 12 microtubules forming the dorsal corset (DC), which run under the dorsal surface to the posterior and ventral parts of the cell (Figs. 8,29,34,35). They support the plasma membrane and give the cell its shape.

iii) *Other cell organelles*

A single large spherical nucleus lies dorsally in the anterior part of the cell, close to the flagellar apparatus (Figs. 7,24,26). The nucleus contains a nucleolus, which is surrounded by electron dense chromatic granules. During mitosis a fragmenting nuclear envelope was observed (Fig. 11). The spindle microtubules arise from a fibrous structure, which normally lies between the Golgi apparatus and the nucleus (Fig. 16). A Golgi apparatus consisting of 5-6 dictyosomes, is located at the antero-ventral side of the nucleus. Endoplasmic reticulum normally surrounds the nucleus.

Numerous mitochondrial profiles are present in the cell (Fig. 7,15). The number of mitochondria per cell ranges from 1-5 and they have tubular cristae. They may appear in all parts of the cell. One large curved mitochondrion lies to the anterior ventral side of all cells (Figs. 8,18,24). It is closely associated with the flagellar apparatus and the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F1
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26
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R
F1
27
B1
MV
M

Plate 4. Figs. 28-33. Serial sections showing the cross sectioned dorsal root (arrows), which ends in the polar strip (P) in the anterior end of the cell. The polar strip gives rise to a dorsal corset of microtubules (DC). The dorsal root arises from electron-dense material located adjacent and dorsal to the more or less cross-sectioned basal body of flagellum 1 (B1). Flagellum 2 (F2) is sectioned lengthwise. Figs. 34-36. Serial sections showing a lengthwise sectioned dorsal root (DR), originating from the basal body of flagellum 1 (B1) and ending in the polar strip (P), which gives rise to a dorsal corset of microtubules (DC). Also an extrusome (E) is visible in Fig. 34. Figs. 37-39. Serial sections through a cross-sectioned transition zone of one of the flagella. An extrusome (E) and the polar strip (P) are also visible. Scale bars: 0.5 μm (Figs. 28-33, 37-39); 0.25 μm (Figs. 34-36).

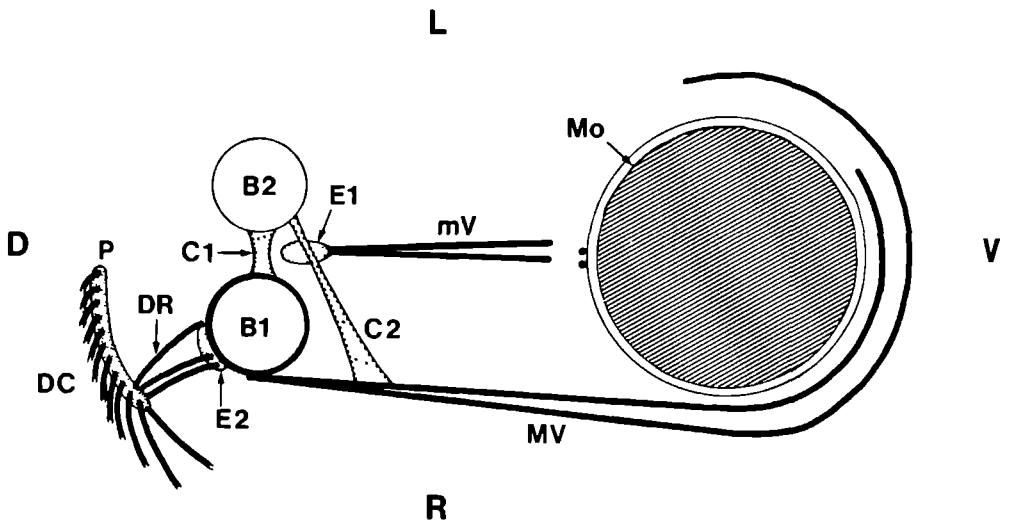
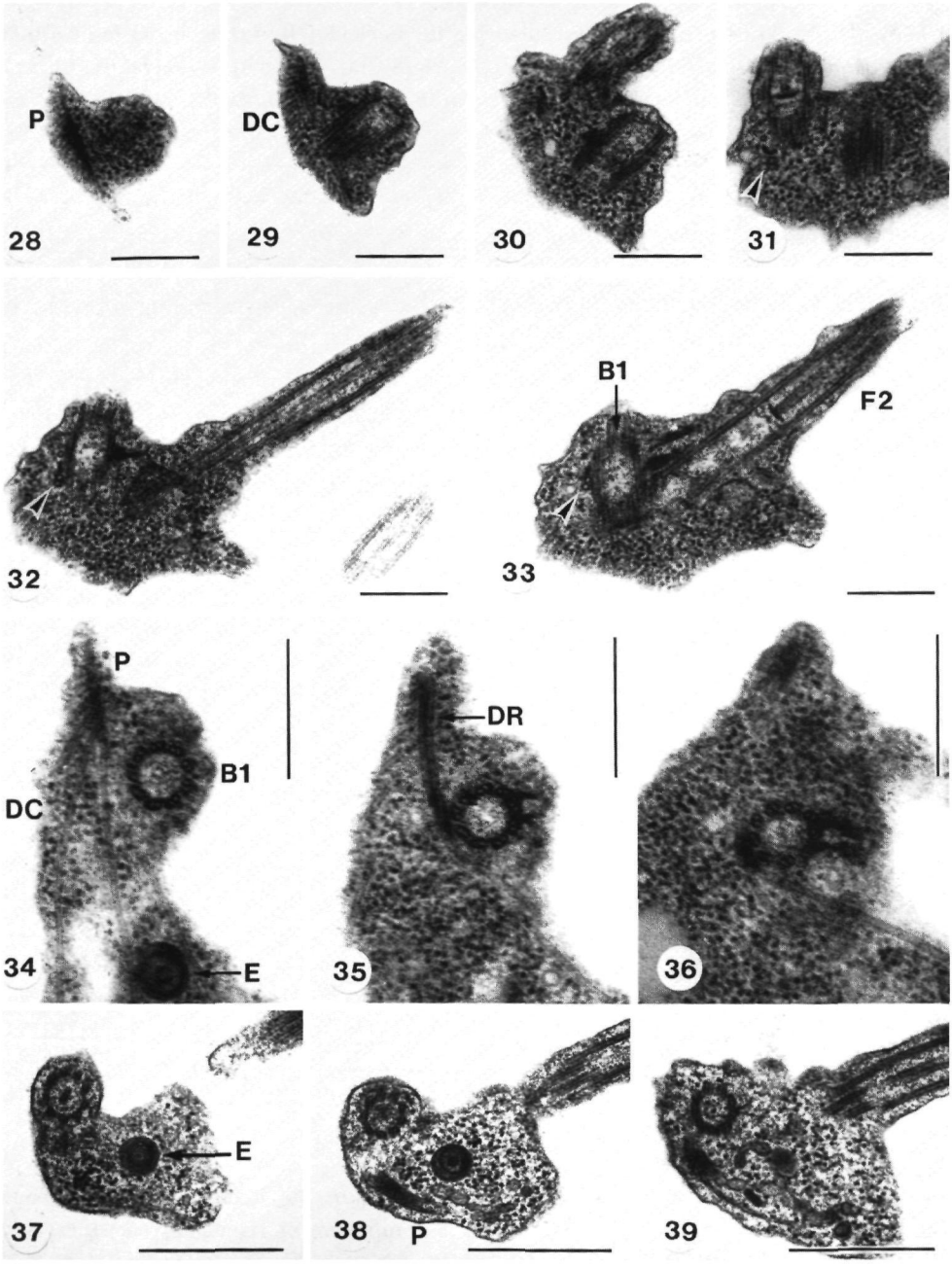


Fig. 45. Diagrammatic reconstruction of the cytoskeleton around the basal bodies of the flagella (B1 and B2) and the mouth (Mo). Major ventral mouth root (MV), minor ventral mouth root (mV), dorsal root (DR), polar strip (P), dorsal corset of microtubuli (DC), electron dense material (E1 and E2), fibrous connecting structures (C1 and C2), funnel-shaped sheath (S). Orientation: D = dorsal; V = ventral; R = right; L = left.


P
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B1
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38
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39

Plate 5. Fig. 40. Micrograph of a cyst, showing the nucleus (N), a mitochondrion (M) and the cyst wall. Fig. 41. Mouth containing a bacterium (Ba). The major ventral mouth root (MV) in the lips of the mouth, a mitochondrion (M) and the contractile vacuole (CV) are visible. Scale bars: 0.5 μm.

two ventral microtubular mouth roots.

Most cells have one or two concentric extrusomes at the anterior end of the cell, dorsal to the flagellar apparatus (Figs. 8,37,38). Extrusomes were also found lower than the insertion of the flagella but never in the posterior half of the cell.

A contractile vacuole lies anteriorly in the ventral part of the cell (Fig. 41). The posterior part of the cell contains a number of food vacuoles (Fig. 7,14). Some of these contain bacterial cell wall material remained behind after digestion. Newly ingested bacteria are tightly enclosed by food vacuole membrane (Fig. 14).

Growth curve

A lag phase of 6 hours was observed after inoculation of the bacterial cultures with *A. peritocrescens* (Fig. 42).

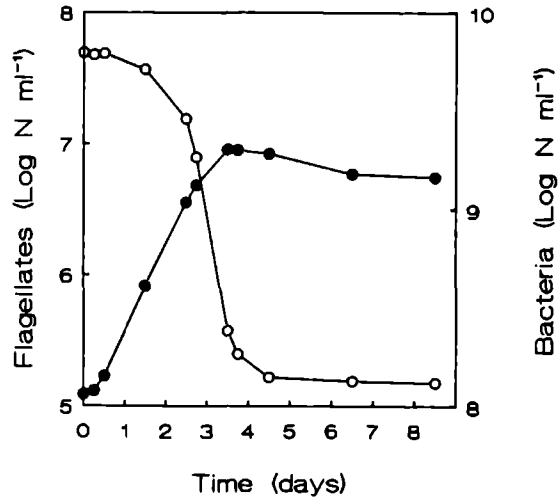
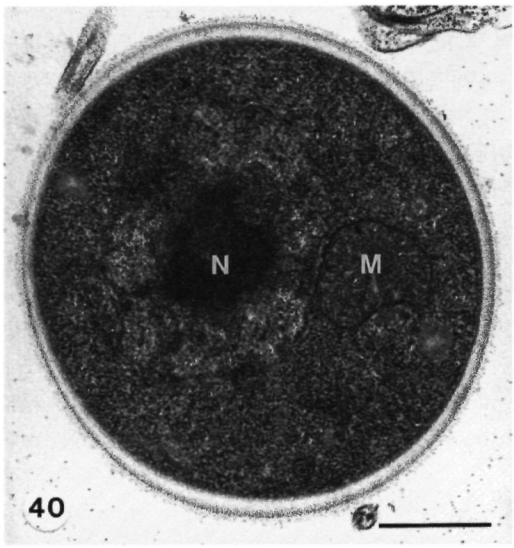
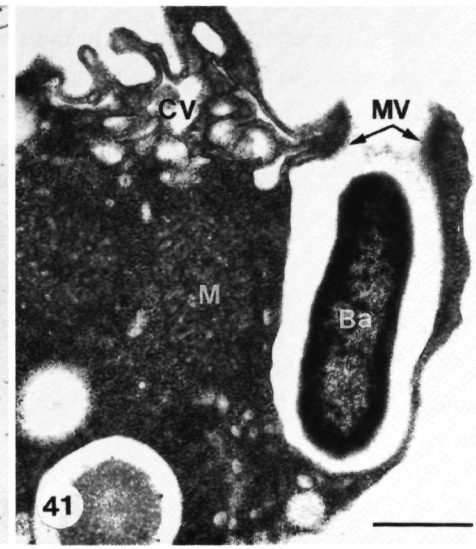


Fig. 42. Growth curve of *A. peritocrescens* growing on the heterotrophic bacterium *Arthrobacter globiformis* at pH 7.5 and 20°C. The numbers of flagellates (●) increased logarithmically till day 4 of the incubation period, whereas the numbers of bacteria (○) decreased logarithmically.





After that, the numbers of flagellates increased logarithmically and doubling times of 2-3 hours were calculated. A maximal density of 10^7 flagellates ml^{-1} was reached after 3.5 days. Cyst formation was observed from then on and after 8.5 days, 60 % of the original maximal numbers were found as cysts. The numbers of heterotrophic bacteria in the cultures decreased logarithmically during grazing, whereas the numbers of bacteria in the control experiments were constant. After 8.5 days, the numbers of bacteria in the cultures containing flagellates had decreased to 2 % of the original numbers.

DISCUSSION

A. peritocrescens was isolated from the rhizosphere of a grassland in a outer march of the river IJssel in the Netherlands. Nothing is known about the geographic distribution of *A. peritocrescens*. The organism has a optimum for growth at pH 7-8. Hence, *A. peritocrescens* may be found in calcareous, organic rich soils with high bacterial numbers, for example in pasture soils of outer marches of rivers.

A. peritocrescens is probably one of the dominant protozoa in the soil from which it was isolated, since the capacity to form cysts and to reproduce rapidly makes this

flagellate a successful competitor in the soil ecosystem. The capacity of cyst formation is advantageous to this organism, since the outer march from which it was isolated, is being submerged by the river usually twice a year, by which the soil may become anoxic. After these unfavourable circumstances, it may build up a protozoan population rapidly when bacterial numbers are high, for example generally in spring or when the tip of a root grows by excreting organic material into the soil (5,6), while other species of protozoa are still inactive or in the lag phase before growth.

Affinities of a flagellate with one or more genera are based on three categories of information: light microscopy, electron microscopy and base sequences in ribosomal RNA. The first one was the only available until about 1965. However, light microscopy proved to be insufficiently discriminating between genera and in establishing affinities between major adaptive lineages. As a result, groups were poorly described, for example Zoomastigophorea (47), or organisms were placed together in a group because of their superficial similarities but which were otherwise dissimilar, for example cercomonads with the bodonids (17).

Major adaptive lineages of Protists have distinctive assemblages and deployment of organelles, as was shown in ultrastructural studies using EM techniques. They have been referred to as ultrastructural identities (40). Each well circumscribed group of flagellates has a distinctive ultrastructural identity. For example the stramenopiles have tripartite flagellar hairs, mitochondria with tubular cristae, and paired kinetosomes with four microtubular roots (38), the euglenids have a plicate cortex, mitochondria with discoid cristae, paired flagella with three microtubular roots, and paraxial rods in the flagella (25). On basis of evidence from ultrastructural studies, affinities have been shown between the euglenids and the kinetoplastids (20) and between the opalines and other stramenopiles (38).

Base sequences in protozoan ribosomal RNA may confirm, corroborate, and extend the insight in affinities revealed by ultrastructural studies (7,26,48,49). Consistency of the two independent categories of information provides confidence in emerging hypotheses of affinities.

For *A. peritocrescens* only the first two categories of information are available: light and electron microscopy. This discussion is based on the assumption that overlap in ultrastructural identities may be used as evidence of phylogenetic affinities, and consideration must be taken of the sequence in which characters were formed.

Affinities of *Adriamonas* with a genus within the Pelobiontida, Diplomonadida, and Trichomonadida can be excluded by the presence of mitochondria in *Adriamonas*. Also affinities with mitochondriate groups or genera, for example bodonids, euglenids,

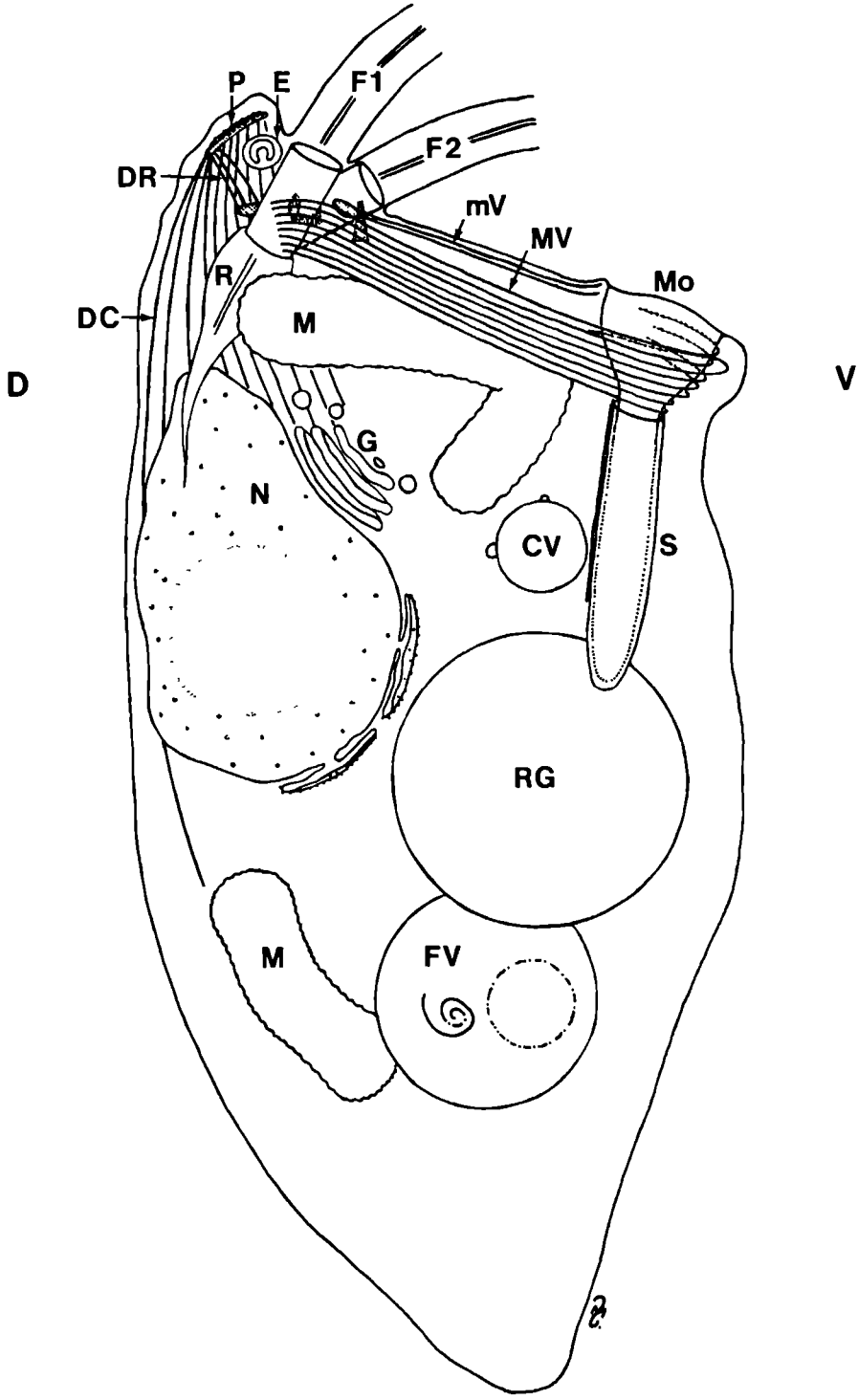
heteroloboseids, choanoflagellates, *Jakoba*, *Diplonema*, *Stephanopogon*, and *Hemimastix*, is unlikely on basis of distinctive mitochondrial morphologies and absence of concentric extrusomes in the just mentioned groups and genera (10,35,39,40). However, the heteroloboseids, *Stephanopogon*, and *Jakoba* have mouths supported by microtubules as in *A. peritocrescens*, but the configurations of the mouths are different (35,39,40). On basis of lack of the synapomorphic feature (presence of tripartite tubular hairs) it is concluded that membership of the stramenopiles is very unlikely (38). Also the cryptomonads and dinoflagellates are excluded for affinities with *Adriamonas* by lack of a cytoskeletal periplast and by lack of pellicular vesicles, dinokarya, and paraxial flagellar roots, respectively.

The greatest protistan radiation appears to have occurred within the organisms with tubulocristate mitochondria. The morphology of mitochondria and cristae is generally a reliable indicator. However, mitochondrial morphology gives some little uncertainty. Patterson and Brugerolle (40) drew attention to several cases of inconsistency in the morphology of cristae and higher categories. A match of characters, for example naked cell surface, dorsal nucleating site for pellicular microtubules, three microtubular roots, mouth supported by microtubules, and concentric extrusomes, may reveal affinities with *A. peritocrescens*. In the case of a number of groups flagellar rootlet systems can generally not be compared for lack of sufficient information. *A. peritocrescens* shows similarities in the flagellar apparatus with the apusomonads, for example two inclined basal bodies. However, affinity of *A. peritocrescens* with the apusomonads is very unlikely on basis of the presence of external organic thecae and absence of mouths in the latter group (19,54). Extrusomes similar to those of *A. peritocrescens* are found in the cercomonads, but the latter group lacks a discrete mouth and have fewer microtubular roots associated with the flagellar apparatus (31,34,50). The desmothoracids and dimorphids are heliozoon-like flagellates unlike *Adriamonas*. They have axonemes and adhesive extrusomes but they lack a discrete ingestion apparatus (2,3). The root system of *Phalansterium* was described by Hibberd (15) and is unlike the root system of *A. peritocrescens*. Moreover, *Phalansterium* does not have a mouth. Just like the latter, *Rhipidodendron* and *Spongomonas* lack mouths. They have an excreted mucus and a concentric anchorage (14,15). It is concluded that the these differences do not support membership of or affinities with *Adriamonas*. The thaumatomonads have scales on the outside of the cells and the form is in association with mitochondria (18). They lack a mouth but feed with pseudopodia emerging ventrally. The genera *Colponema* and *Colpodella* have no extrusomes unlike *Adriamonas* (32). They both have subsurface alveoli and *Colpodella* has an ingestion apparatus with a different configuration to

Fig. 44. Reconstruction of A. peritocrescens, gen. nov., sp. nov., based on serial sections, showing the flagella F1 and F2, the cell organelles and the course of the microtubular structures. Major ventral mouth root (MV), minor ventral mouth root (mV), dorsal root (DR), polar strip (P), dorsal corset of microtubuli (DC), extrusome (E), rhizostyle (R), nucleus with nucleolus (N), Golgi-apparatus (G), mitochondrion (M), mouth (Mo), funnel-shaped sheath (S), contractile vacuole (CV), refractile granule (RG), and food vacuoles (FV). Orientation: D = dorsal; V = ventral; right = in front of drawing plane; left = behind drawing plane.

Adriamonas. *Discocelis* lacks a mouth but the cells are naked and have three microtubular flagellar root systems like *Adriamonas* (55). However, the course of these roots and the number of microtubules in these roots are different to *Adriamonas*. The polar strip in the anterior end of the *A. peritocrescens* cell is absent in *Discocelis*.

A. peritocrescens has some remarkable similarities with the pseudodendromonads (13,16,30,45,52). Corresponding features are two subapically inserted flagella, a ventrally situated mouth, microtubular flagellar root systems turning 180° in the lips of the mouth, a large mitochondrion associated with the mouth roots, concentric extrusomes, comparable positions of nucleus and Golgi apparatus, and association of fibrous rhizostyle with the nuclear envelope. Especially the support of the lips of the mouth by flagellar microtubular roots, which turn around the mouth, and the association of a large mitochondrion with these roots in *Adriamonas* as well as in the pseudodendromonads clearly reveal affinities between them. However, all species in the genera *Pseudodendromonas* and *Cyathobodo* described up till now are connected to each other and to organic material by a stalk and the organisms have scales on the outside of the cells. No single unstalked and/or unscaled species within the pseudodendromonads has been described. We are uncertain as to how to evaluate this balance of similarities and differences. Hence, we feel that a dogmatic stance would be premature and we look forward to extension of the data base with ribosomal RNA data of *A. peritocrescens* or further ultrastructural details of *Pseudodendromonas* species. On basis of the two prominent differences with the pseudodendromonads (i.e. stalks and scales), the organism described in this paper is arranged in the new genus *Adriamonas*, with the type species *A. peritocrescens*. Consequently, *A. peritocrescens* is placed *incertae sedis* in the kingdom Protista.


P
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DIAGNOSES

Genus Adriamonas gen. nov.
Type species: *Adriamonas peritocrescens* Verhagen gen. nov. The genus name is dedicated to Adrianus J. Verhagen († January 20, 1991).

Cellulae sine chloroplastis cum duobus subapicalis flagellis nudis, tertia vittatis microtubuli et granulis extrusomis.

Flagellated colourless protists with two naked subapically inserting, subequal flagella. Both flagella insert at the antero-ventral part of the cell, one directed anteriorly, one ventrally. Kinetosomes with associated microtubular roots, of which two support the lips of a ventrally situated mouth. Cell body margin supported by a corset of microtubules. Association between tip of rhizostyle and nuclear envelope. Mitochondria with tubular cristae. Golgi apparatus present. Few concentric extrusomes in the anterior part of the cell. One species from soil, feeding on bacteria. Assigned to Protista, incertae sedis.

Adriamonas peritocrescens sp. nov. The species name is derived from the latin perite crescens = fast growing.

Cum characteribus generis. Cellula perite crescens, cum granulo refractivo. Ex solo.

With the characters of the genus. Cells solitary, 7-9 μm long and 3-5 μm wide. Both flagella about 7 μm long. Few anteriorly located extrusomes, 110 μm in diameter (only visible with EM). A refractile granule, diameter 1.5 μm, central in the cell. Doubling times 2-3 hours under optimal conditions.

Type material: Soil was collected from the top 0-10 cm layer of a pasture at Cortenoever near Brummen (52°05' N, 06°09' E) in the Netherlands on 5 May 1988.

Location of culture: Culture Collection of Algae and Protozoa, Ambleside, England and Centre for Terrestrial Ecology, Netherlands Institute of Ecology, Heteren, the Netherlands.

Type micrograph: Figs. 1-3.

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CHAPTER 3

COMPETITION FOR AMMONIUM BETWEEN NITRIFYING AND HETEROTROPHIC BACTERIA IN DUAL ENERGY-LIMITED CHEMOSTATS.

FRANK J.M. VERHAGEN AND HENDRIKUS J. LAANBROEK

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SUMMARY

The absence of nitrification in soils, rich in organic matter, has often been observed. Therefore, competition for limiting amounts of ammonium between the chemolithotrophic ammonium-oxidizing *Nitrosomonas europaea* and the heterotrophic *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 stepwise increasing the glucose concentration in the reservoir from 0 to 5 mM, while maintaining the ammonium concentration at 2 mM.

The numbers of *N. europaea* and of *N. winogradskyi* 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 at steady state situations. Although 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 due to being attached to the glass wall of the culture vessels. The numbers of *N. europaea* decreased more than 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 suppression of nitrification by more competitive heterotrophic bacteria in soils that are rich in organic matter is discussed as well as that this inhibition might be responsible for the absence or low rates of nitrate production in the carbon-rich, ammonium-limited rhizosphere of plants.

INTRODUCTION

The absence of nitrification in grassland soils that are rich in organic matter has often been reported (11,14,27,34,35). The numbers and activities of the nitrifying bacteria are also lower in soils covered with grasses compared to other types of vegetation (4,24,25,26). Some authors explain this inhibition of nitrification by 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 (18,32,36,37,38). At the C/N ratios encountered in the rhizosphere of grasses, competition for limiting amounts of ammonium occurs between nitrifying and heterotrophic bacteria. The absence or low rates 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 and that of bacteria (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 as well as nitrifying populations become nitrogen limited. Usually storage of organic carbon in the form of glycogen or PHB 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 at 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 at 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 inhomogeneities or even in non-mixed environments, cell wall attachment, production of an auto-inhibitor or differences in cell motility properties may influence the outcome of the competition experiments (1,12,22).

The aim of this study was to investigate the competition for ammonium between pure cultures of the chemolithotrophic ammonium-oxidizing *Nitrosomonas europaea* in combination with the nitrite-oxidizing *Nitrobacter winogradskyi* and the heterotrophic *Arthrobacter globiformis* in the 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 non-sterile soil from the top 0-10 cm layer of an extensively used, calcareous grassland near the village of Brummen (52°5'N,6°9'E) in the Netherlands. A dilution series of a rhizosphere suspension was plated on an agar medium with glucose as the carbon source and ammonium sulphate 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 the same medium until pure cultures were obtained. One of the isolated strains was selected for further experiments. According to identification tests performed by The National Collections of Industrial and Marine Bacteria (Aberdeen, Scotland), the isolated strain was placed into the genus *Arthrobacter*, probably belonging to the *Arthrobacter 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 (per liter): $(NH_4)_2SO_4$, 330 mg; KH_2PO_4, 100 mg; $MgSO_4.7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 500 mg; glucose.H_2O, 1 g (autoclaved separately) and trace elements solution, 1 ml. The trace elements solution contained (per liter): $FeCl_2.4H_2O$, 2 g; Na_2-EDTA (Titriplex III), 4.3 g; $MnCl_2.4H_2O$, 0.1 g; $CoCl_2.6H_2O$, 24 mg; $NiCl_2.6H_2O$, 24 mg; $CuCl_2.2H_2O$, 17 mg; $ZnCl_2$, 68 mg; $Na_2WO_4.2H_2O$, 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; after sterilization in the autoclave the final pH was 7.8. The *Arthrobacter* strain was subcultured every two weeks by inoculating 50 ml fresh medium with 1-1.5 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 (per liter): $(NH_4)_2SO_4$, 330 mg; KH_2PO_4, 19 mg; K_2HPO_4, 104 mg; yeast extract (Difco Laboratories, Detroit, Michigan, USA), 1 g; tryptose (Oxoid Ltd., Basingstoke, Hampshire, England), 2 g; bacteriological agar (Oxoid Ltd., Basingstoke, Hampshire, England), 16 g and glucose.H_2O, 200 mg (autoclaved separately). *A. globiformis* was cultured for three days prior to inoculation on a rotary shaker at 20°C in a medium identical to the batch culture

medium described above except for the amount of glucose. To the pre-inoculation medium 200 mg of glucose.H_2O was added per liter. All glucose was used by the heterotrophic bacteria before inoculation of the chemostats.

Nitrosomonas europaea ATCC 19718 and *Nitrobacter 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 MPN enumeration. Mixed batch cultures of *N. europaea* and *N. winogradskyi* were grown for four weeks to increase cell numbers prior to inoculation. The composition of the medium for these batch cultures was (per liter): $(NH_4)_2SO_4$, 330 mg; KH_2PO_4, 200 mg; $MgSO_4.7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 500 mg and trace elements solution, 1 ml (composition see above). Bromothymol blue (0.04 %), 5 ml, was added as a pH-indicator. The pH was adjusted with 0.1 N NaOH; after sterilization the final pH was 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 7-7.5, as indicated by the pH indicator. After 5-6 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^+ + 2 mM NO_3^- present in the medium. Pure cultures of the nitrifying couple *N. europaea* and *N. winogradskyi* were grown at both dilution rates prior to addition of the heterotrophs. Numbers of nitrifying bacteria and the K_m's for ammonium oxidation were determined.

Competition experiments.

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

The competition experiments were conducted using Biostat M fermenters (B. Braun, Melsungen, Germany) with culture volumes of 1250 ml. The dilution rates studied were 0.004 and 0.01 h^{-1}. The composition of the medium was (per liter): $(NH_4)_2SO_4$, 132 mg; $MgSO_4.7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 500 mg and trace elements solution, 1 ml (composition see above). Sterilization of 9.5 l of this medium was performed in 10 l vessels for 40 minutes at 121°C. The buffer solution, containing 19 mg KH_2PO_4 and 104 mg K_2HPO_4 per liter (pH 7.5), was autoclaved separately. The glucose solution, containing 198, 396, 595, 793 or 991 mg glucose.H_2O per liter, respectively, depending on the desired glucose concentration, was also sterilized separately. After cooling of the reservoir the concentrated buffer and glucose solutions were added to the mineral medium. After mixing, the medium had a pH of 7.5 and glucose concentrations ranged from 0-5 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. Temperature and O_2-tension were maintained respectively at 25°C and at least 60 % of air-saturation. The culture vessels were stirred at 300 rev per min.

In the culture vessels, 1 liter sterile mineral medium was inoculated with 250 ml of the four week-old mixed batch culture of *N. europaea* and *N. winogradskyi* described above. The inoculum contained 6 x 10^6 ammonium- and 4 x 10^6 nitrite-oxidizing bacteria per ml. At first, the mixed cultures were grown batchwise in the fermenters until the 2 mM ammonium present 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 x 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 reaching steady state, a new medium vessel was connected whereupon a new steady state was awaited. Samples were taken at every steady state to determine ammonium, nitrite, nitrate, glucose, total organic carbon, dissolved organic carbon and pH. Also the numbers of nitrifying bacteria were determined by the MPN and the FA enumeration techniques. Numbers of heterotrophic bacteria were determined by 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 as tests for purity of the cultures. Also samples of the reservoirs were taken to determine ammonium, glucose and pH. Samples were stored at -25°C. Steady state situations at 0, 1, and 2 mM glucose were repeated in separate experiments for both dilution rates.

Most Probable Number enumeration technique.

Enumerations of nitrifying bacteria present in the culture vessels were performed using a Most Probable Number (MPN) technique (39). In this technique, 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 (per liter): $(NH_4)_2SO_4$, 330 mg or $NaNO_2$, 35 mg; KH_2PO_4, 100 mg; $MgSO_4.7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 500 mg and 1 ml trace elements solution (composition see above). 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 according to the MPN technique samples (0.25 ml) taken from the culture vessels were diluted tenfold in sterile microtiter plates (tissue culture cluster, 24 wells, Costar, Cambridge, UK) 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 aluminium foil and incubated at 20°C for 9 weeks. Ammonium and nitrite oxidation were determined using 0.04 % bromothymol blue solution (pH indicator) and Griess Ilosvay reagents, respectively. The most probable numbers of nitrifying bacteria, according to the results of the dilutions, were obtained from statistical tables that were generated by a computer program (31).

Fluorescent Antibody enumeration technique.

Enumerations of nitrifying and heterotrophic bacteria were performed using an indirect specific Fluorescent Antibody (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, if needed diluted with Phosphate Buffered Saline (PBS), was filtered over a black polycarbonate membrane filter (Nuclepore Corp., Pleasanton, USA) 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 rinsing twice with 2 ml PBS, 20 μl 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 buffer) for 30 minutes in a humid petri-dish. After rinsing twice with 2 ml PBS buffer to remove excess antiserum, the filter was incubated with commercially available Goat Anti-rabbit IgG (diluted 1:100 with PBS buffer) for 30 minutes in a humid petri-dish in the dark. This Goat Anti-rabbit antiserum (Sigma) was conjugated with the green-fluorescent label fluoresceïne isothiocyanate (FITC). After rinsing twice with 2 ml PBS buffer, the filter was placed on a glass slide, whereupon 20 μl 0.1 % paraphenylene diamine solution in glycerol (pH 8) was added as a fluorescent 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 using a Leitz Diaplan epifluorescence microscope (Ernst Leitz, Wetzlar, Germany). The enumeration was continued until in total 800-1000 fluorescent cells were counted.

Plate Count enumeration technique.

Enumerations of heterotrophic bacteria were performed using a Plate Count technique. The composition of the medium was (per liter): $(NH_4)_2SO_4$, 330 mg; yeast extract (Difco Laboratories, Detroit, Michigan, USA), 1 g; tryptose (Oxoid Ltd., Basingstoke, Hampshire, England), 2 g; bacteriological agar (Oxoid Ltd.), 15 g; KH_2PO_4, 125 mg; glucose.H_2O, 200 mg (sterilized separately). The pH was adjusted with 0.5 N NaOH; after sterilization it was 7.5.

Sterile samples (0.1 ml), taken from the culture vessel, were diluted tenfold in sterile test tubes containing 0.9 ml PBS buffer solution. A series of 10 dilutions was made from each sample. Of each dilution 100 μl was plated on agar medium as described above. After incubation for 14 days at 20°C, the number of colony forming units (cfu's) was determined.

Analytical Methods.

Mineral nitrogen compounds were determined using a Technicon Traacs 800 autoanalyzer (Technicon Instr. Corp., Tarrytown, New York). Total and dissolved organic carbon were determined with a total organic carbon analyzer (model 700, O.I. Corp., College Station, Texas, USA) using potassium hydrogen phthalate as standard and potassiumpersulphate as oxidizing agent. Dissolved organic carbon was determined in the supernatant after centrifugation at 15,000 x g for 15 minutes in a Biofuge A table centrifuge (Heraeus-Christ, Osterode am Harz, Germany). The net cell organic carbon

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 one hundred percent. 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, Germany) based on the GOD-POD method (photometric determination of glucose with glucose oxidase and peroxidase) (43). In contrast to the original method, the absorbance of the samples was measured at 440 nm on a Vitatron MCP spectrophotometer (Vital Scientific, Dieren, the Netherlands).

Determination of kinetic parameters.

The kinetic parameter 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 (Strathkelvin Instr., model 781, Glasgow, UK) with a Clarck type microcathode oxygen electrode. In order to obtain measurable respiration rates, 600 ml samples were concentrated 24-fold by centrifugation at 30,000 x g using a Sorvall RC5C high speed centrifuge (Du Pont Comp., Wilmington, USA). After concentration, numbers of *N. europaea* were 1-7 x 10^7 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 1M sodium chlorate was added to the suspensions giving a final concentration of 10 mM. Sodium chlorate inhibited the activity of nitrite-oxidizing bacteria due to formation of chlorite by these microorganisms (3). After aeration of the suspensions for 1 minute, 20 μl of a concentrated ammonium- sulphate 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, respectively. After addition of the substrate, respiration rates of the stirred suspensions were determined by measuring the linear decreases in oxygen concentration for 15 minutes. The measured oxygen consumption rates were corrected for endogenous respiration, which was measured for 30 minutes in the presence of sodium chlorate but in the absence of substrate. The kinetic parameters K_m and V_{max} for ammonium oxidation by *N. europaea* were calculated by the computer program Enzpack 2.0 (P.A. Williams, Bangor, England) 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.

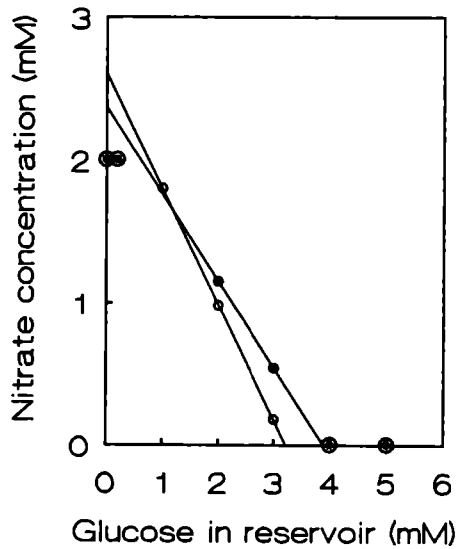

Fig. 1. Steady state concentrations of nitrate in the culture vessels containing *N. europaea*, *N. winogradskyi* and *A. globiformis*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (●) and 0.01 (○) h^{-1}.

In the absence of glucose in the reservoir, the 2 mM of ammonium supplied was converted into 2 mM nitrate by the nitrifying bacteria. At a glucose concentration of 0.2 mM in the reservoir, no measurable difference in nitrate concentration compared to the mineral medium was found. However, nitrate concentrations decreased as the glucose concentration increased further and were zero at the so-called critical C/N ratios. Critical C/N ratios of 11.6 and 9.6 were established for D = 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 or glucose was detected in the culture vessels at all glucose concen-

trations supplied during the competition experiments.
Pure cultures of *A. globiformis*, growing on 2 mM glucose and 2 mM NH_4^+ + 2 mM NO_3^-, only used ammonium as a N-source. At both dilution rates no decreases in nitrate concentrations were measured.

Numbers of *Nitrosomonas europaea*.

The steady state numbers of *N. europaea* in relation to the glucose-concentrations in the reservoirs are presented in Fig. 2.

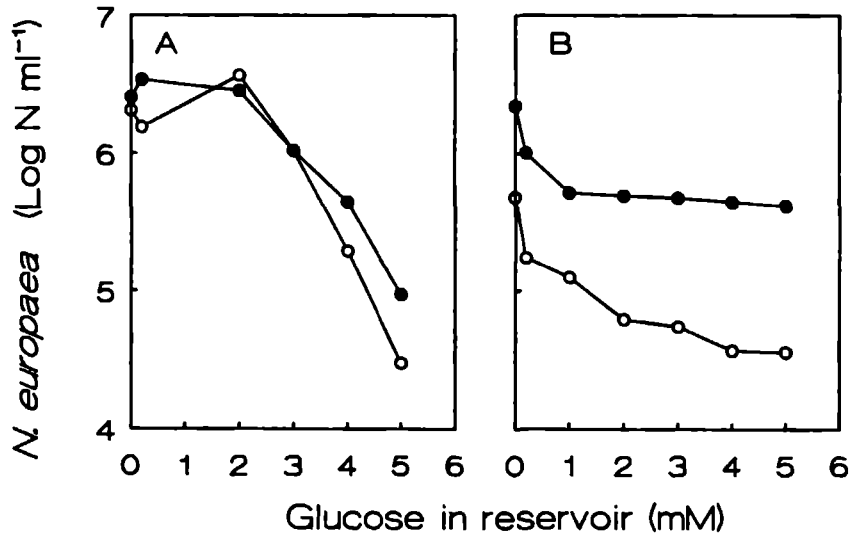

Fig. 2. Steady state numbers of *N. europaea*, grown in the presence of *N. winogradskyi* and *A. globiformis*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. Enumerations were performed according to the FA (●) and the MPN (○) technique. No heterotrophic bacteria were present at 0 mM glucose.

For both growth rates, the numbers of *N. europaea* decreased as the glucose concentrations increased in the reservoirs. According to the enumerations with the Fluorescent Antibody technique, the numbers of *N. europaea* at 5 mM glucose had decreased to 4 % and 19 % of the original numbers at 0 mM glucose for D = 0.004 and 0.01 h^{-1}, respectively.
At D = 0.004 h^{-1}, numbers were almost constant 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* obtained by the Most Probable Number enumeration technique and those counted by the Fluorescent Antibody enumeration technique. Apparently, all cells present were active in the culture vessel or became active during the incubation time of the MPN enumerations.
At D = 0.01 h^{-1}, numbers of *N. europaea* decreased drastically between concentrations of 0 and 1 mM glucose in the reservoir. A slight decrease was shown above 1 mM glucose. Comparison of the numbers of the ammonium-oxidizing bacteria obtained by the two enumeration techniques showed that at D = 0.01 h^{-1} only 8 % to 24 % of the *N. europaea* cells in the culture vessel were or became active during the incubation time of the MPN enumerations.

Comparison of the two growth rates indicated that the decrease in numbers of *N. europaea* was most pronounced at D = 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 as compared with cells growing at D = 0.004 h^{-1}, at least above 2 mM glucose in the reservoir.

Numbers of *Nitrobacter winogradskyi*.

The steady state numbers of *N. winogradskyi* 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 using the Fluorescent Antibody technique, the numbers of the nitrite-oxidizing organism at 5 mM glucose decreased to 17 % and 64 % of the original numbers at 0 mM glucose for D = 0.004 and 0.01 h^{-1}, respectively.
Again a difference between the two enumeration techniques was found. This indicates that only parts of the *N. winogradskyi* cells were or became active during the incubation time of the Most Probable Number enumerations. The MPN-detectable parts of the nitrite-oxidizing population varied between 15-49 % and 10-33 % of the total numbers present, obtained with the FA method, for D = 0.004 and 0.01 h^{-1}, respectively.
In accordance with *N. europaea*, the decrease in numbers of *N. winogradskyi* was most pronounced at the lower dilution rate of 0.004 h^{-1}, at least above 2 mM glucose in the reservoir.

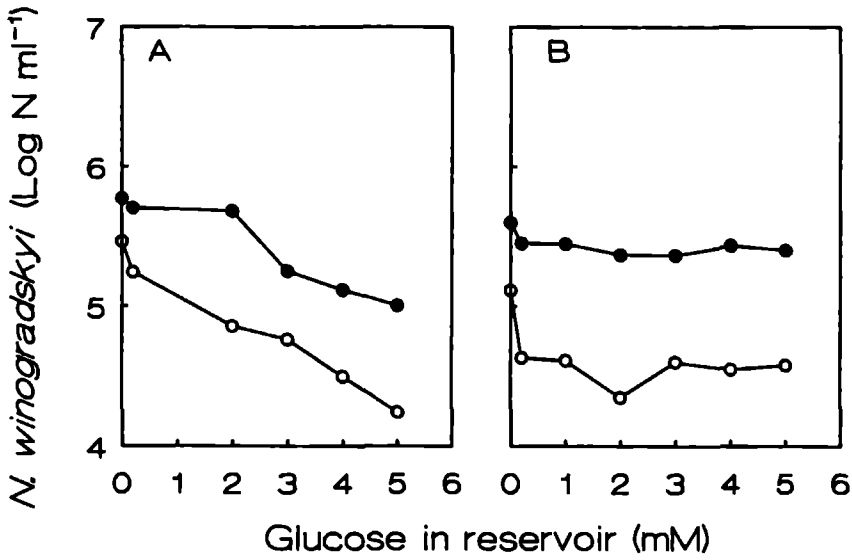

Fig. 3. Steady state numbers of *N. winogradskyi*, grown in the presence of *N. europaea* and *A. globiformis*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. Enumerations were performed according to the FA (●) and the MPN (○) technique. No heterotrophic bacteria were present at 0 mM glucose.

Ratios between numbers of nitrifying bacteria.

The ratios between *N. europaea* and *N. winogradskyi* at steady state situations at 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* decreased more than the numbers of *N. winogradskyi*. At both dilution rates, the ratios decreased as the glucose concentrations increased until, at 5 mM glucose in the reservoir, the ratios between the two nitrifying populations were almost 1.

Numbers of *Arthrobacter globiformis*.

The results of the enumerations of *Arthrobacter globiformis* at steady state situations in the chemostats are presented in Fig. 4. As expected, numbers of heterotrophic bacteria grew as the glucose concentrations increased in the reservoirs. The numbers of heterotrophic bacteria at D = 0.004 h^{-1} were always lower than those at 0.01 h^{-1}

Table 1. Ratios between *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, grown in the presence and absence of *Arthrobacter globiformis* in ammonium limited chemostats at pH 7.5 and 25°C at two dilution rates.

			Dilution rate (h^{-1})			
			0.004		0.01	
A. globiformis	N-source mM NH_4^+	C-source mM glucose	FA	MPN	FA	MPN
—	2	—	4.3	7.1	5.6	3.7
+	2	0.2	6.8	8.9	3.6	4.1
+	2	1	ND	ND	1.9	3.0
+	2	2	5.9	51.0	2.1	2.8
+	2	3	5.9	18.1	2.1	1.4
+	2	4	3.4	6.3	1.6	1.0
+	2	5	0.9	1.7	1.6	0.9

Numbers of nitrifying bacteria were determined by enumerations according to the Fluorescent Antibody (FA) and the Most Probable Number (MPN) technique.
ND, Not determined.

at similar glucose concentrations. The FA numbers of *A. globiformis* were always higher than those obtained by the Plate Count technique.

Organic carbon.

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 as well as net organic carbon of the mixed culture growing at D = 0.004 h^{-1} were lower than those of the culture growing at D = 0.01 h^{-1} at all glucose concentrations supplied.
Above the critical C/N ratios, *i.e.* at 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.

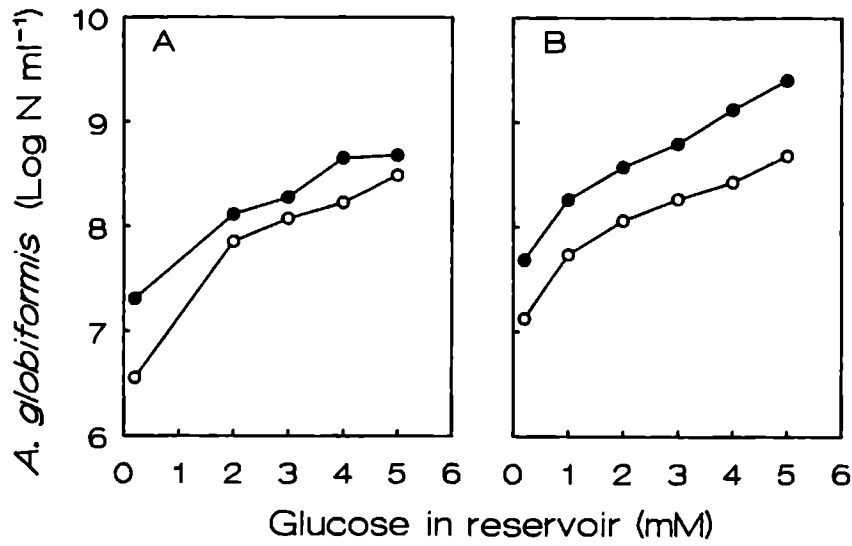

Fig. 4. Steady state numbers of *A. globiformis*, grown in the presence of *N. europaea* and *N. winogradskyi*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. Enumerations were performed according to the FA (●) and Plate Count (○) technique.

Bacterial C/N ratios and C-efficiencies.

Bacterial C/N ratios and C-efficiencies of cultures of *A. globiformis*, grown in the presence and absence of the nitrifying couple *N. europaea* and *N. winogradskyi*, are presented in Table 2. As the mixed cultures during the competition experiments were composed for at least 97 % of *A. globiformis*, the bacterial C/N ratios as well as the C-efficiencies, determined in these mixed cultures, are dominated by the heterotrophic bacteria and can be compared to the values of *A. globiformis*, obtained in pure cultures. The bacterial C/N ratios and the bacterial 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 N-source, the cultures growing on 2 mM glucose and 2 mM NH_4^+ + 2 mM NO_3^-, gave similar values with respect to the bacterial C/N ratio and C-efficiency for both dilution rates. In the cultures with an excess of ammonium and nitrate present

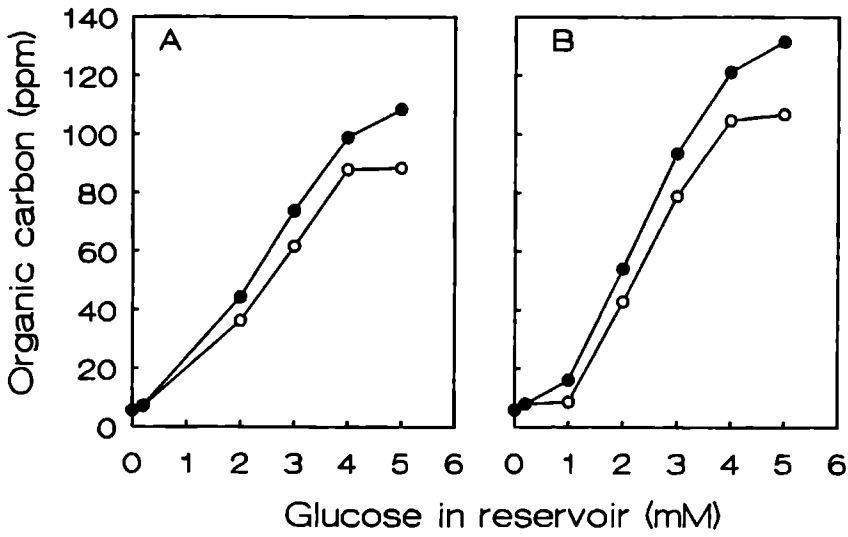

Fig. 5. Steady state concentrations of total organic carbon (●) and cell organic carbon (○) in the culture vessels containing *N. europaea*, *N. winogradskyi* and *A. globiformis*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. No heterotrophic bacteria were present at 0 mM glucose.

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

Kinetic parameters.

Since the affinity for ammonium of *N. europaea* might be affected by the dilution rate and by the presence of *A. globiformis*, 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 con-

Table 2. Bacterial C/N ratios and C-efficiencies of *Arthrobacter globiformis*, grown in the presence and absence of the nitrifying couple *Nitrosomonas europaea* and *Nitrobacter winogradskyi* in glucose limited chemostats at pH 7.5 and 25°C at two dilution rates.

Organisms	Dilution rate h^{-1}	N-source 2 mM each	C-source mM glucose	C/N_{bact}	C-efficiency %
H[1]	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[2]	0.004	NH_4^+	0.2	ND	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

[1] Pure cultures of *Arthrobacter globiformis*.
[2] Mixed cultures of Heterotrophic and Nitrifying bacteria in the competition experiments.
ND, Not determined.

Table 3. K_m values for ammonium oxidation of *Nitrosomonas europaea*, grown in combination with *Nitrobacter winogradskyi* in the presence and absence of *Arthrobacter globiformis in* ammonium limited chemostats at pH 7.5 and 25°C at two dilution rates.

Dilution rate h^{-1}	*A. globiformis*	N-source mM NH_4^+	C-source mM glucose	K_m for ammonium oxidation mM
0.004	—	2	0	0.385
	+	2	2	0.893
0.01	—	2	0	0.285
	+	2	2	0.470

sumption 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 in K_m for ammonium of *N europaea* was found between the dilution rates. However, in the presence of *A. globiformis*, growing on 2 mM glucose, the K_m value at D = 0.004 h^{-1} increased statistical significantly to 0.89 mM, whereas at D = 0.01 h^{-1} no significant increase in K_m was found. Hence, the affinity for ammonium of *N. europaea* decreased more at the lower 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 in determining the kinetic parameters. Sodium chlorate inhibites the activity of the nitrite-oxidizing bacteria during the respiration measurements by the 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 activity of the ammonium-oxidizers was inhibited for more than 80 % at a concentration of 10 μM chlorite. 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 often been shown to be repressed by heterotrophic processes (18,32,36,37,38). Jansson (18) demonstrated that the heterotrophic flora competed successfully with the nitrifiers for limiting amounts of ammonium. The latter 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 immobilisation- and nitrification processes were found to be 0.014 and 0.57-1.29 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_m values of 0.89 and 0.47 in the presence of heterotrophs growing on 2 mM glucose (Table 3).

In the model experiments performed in this study, the heterotrophic *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 as N-source instead of nitrate. Critical C/N ratios of 11.6 and 9.6 were established at D = 0.004 and 0.01 h^{-1}. The difference in 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 possible inhibition of *N. europaea* by either glucose or 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 up to a final glucose concentration of 1 mM. During the competition experiments described in this study the glucose concentration 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 the ones described by Krümmel and Harms (21). They examined the effect of various organic compounds, i.e. formate, acetate, pyruvate, glucose and peptone, on nitrite formation of two *Nitrosomonas* sp. They found that the organic compounds tested scarcely affected the growth and activity of the *Nitrosomonas* strains. In case of the addition of glucose, when autotrophically grown *Nitrosomonas* cells had oxidized 3 mM of ammonium the nitrite formation of mixotrophically grown cultures amounted to also 3.00 mM. Also Jensen (19) found that organic compounds generally (e.g. glucose, glycerol, acetate and butyrate) were not inhibitory to *Nitrosomonas* but mannose at 12.5 mM was toxic to growth but not to 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 *Nitrosomonas* assimilated only 0.5 nmol of glucose per mmol of ammonium oxidized. Also 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 or 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 excreted organic compounds by *Arthrobacter* 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* was observed by Clark and Schmidt (10). They found that 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 described 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 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 at steady state situations. There are two hypotheses which may explain these results.

- *N. europaea* might have been partly attached to the walls of the culture vessels and a continuous release of cells took place that were counted in the enumerations. 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 in the liquid culture, the nitrifying bacteria might become inactive. However, in the mineral medium of 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 it can not be excluded that a micro-layer of nitrifying organisms was present on the wall of the culture vessels.
- *A. globiformis* might have won the competition for limiting amounts of ammonium but it did not completely outcompete the nitrifying organisms. The heterotrophic bacteria might have mainly used ammonium as N-source, but with increasing glucose concentrations in the reservoirs small parts of nitrate were 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), will increase 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 bacterial C/N ratios of 3.47 and 3.48 of *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 the heterotrophic bacteria only used ammonium for their nitrogen-supply below the critical C/N ratios. 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. Subsequently were calculated the numbers of ammonium-oxidizers, that could be produced by the amounts of available ammonium. From the heterotroph-free cultures at 0 mM glucose it was calculated that 1 mM NH_4^+ gave 1.3 x 10^6 and 1.1 x 10^6 *N. europaea* cells ml^{-1} for D = 0.004 and 0.01 h^{-1}, respectively, according to enumerations using the FA enumeration technique. The calculated and measured numbers of *N. europaea* are presented in Fig. 6. Deviations from the calculated, ideal line could be caused by different bacterial 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

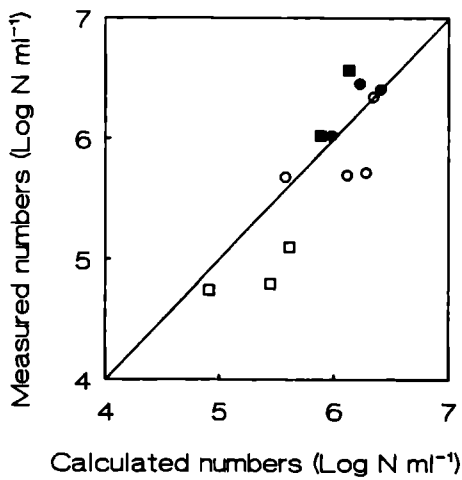

Fig. 6. Calculated against measured numbers of *N. europaea*, grown in the presence of *N. winogradskyi* and *A. globiformis*, during the competition experiments at dilution rates of 0.004 (● and ■) and 0.01 (○ and □) h^{-1}. Enumerations were made according to the FA (○ and ●) and MPN (□ and ■) technique.

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 at 2 mM glucose in the reservoir, were 1:40 and 1:500 for D = 0.004 and 0.01 h^{-1}, respectively. If the deviations from the calculated line were only caused by nitrate-uptake by the heterotrophs, all measured numbers of *N. europaea* would be above the calculated ones. Therefore, it is proposed that the differences between the measured and calculated numbers of *N. europaea* were mainly caused by inaccuracies in the enumeration methods.

The decrease in numbers of *N. europaea* 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} at glucose concentrations in the reservoir above 2 mM. 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 more determined 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 (G.J. Both, Ph.D. thesis, Groningen, the Netherlands, 1990).

With respect to the decreased numbers of *N. europaea* and *N. winogradskyi,* 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 nitrite-oxidizers was no longer completely coupled to the decrease in numbers of the ammonium-oxidizers due to the amounts of nitrite formed. The amount of excreted organic compounds by *A. globiformis* can be seen in Fig. 5. The amounts of dissolved organic carbon above the critical C/N ratios increased as the glucose concentration 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. As glucose was detectable at none of the glucose concentrations, 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 addition of filtrate of

a mixed heterotrophic population to a batch culture of *Nitrobacter* sp. increased its activity. Also reduction of the lag period and an increased growth rate was observed after addition of 10 % of filtrate of a heterotrophic *Pseudomonas* sp. culture to continuous cultures of a *Nitrobacter* sp. (5). In addition, an increased release of attached *N. winogradskyi* cells from the vessel wall compared with attached *N. europaea* cells may be responsible for changing ratios between both 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 amount 37 % (44). The C-efficiencies of 36 % and 38 % found in this study for pure cultures of *A. globiformis*, growing on ammonium at D = 0.004 and 0.01 h^{-1}, respectively (Table 2), agreed well with this value. While 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. Assuming a carbon content of 50 %, the bacterial 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^+ (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 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, formed by *N. winogradskyi* (3). Their activity was inhibited for more than 80 % at a concentration of 10 μM sodium chlorite. 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_m values for ammonium oxidation of *N. europaea* in the study described here 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 by the FA technique were always higher than those obtained by 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 duplo of triplo 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 a top 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 having different C/N ratios.

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CHAPTER 4

EFFECTS OF GRAZING BY FLAGELLATES ON COMPETITION FOR AMMONIUM BETWEEN NITRIFYING AND HETEROTROPHIC BACTERIA IN CHEMOSTATS.

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SUMMARY

The enhanced mineralization of organic nitrogen by bacteriophagous protozoa is thought to favour the nitrification process in soils, in which nitrifying bacteria have to compete with heterotrophic bacteria for the available ammonium. To obtain more insight in this process, the influence of grazing by the bacteriovorous flagellate *Adriamonas peritocrescens* on the competition for limiting amounts of ammonium between the 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}. The ammonium concentration in the reservoir was maintained at 2 mM, whereas the glucose concentration was increased stepwise from 0 to 7 mM.

A. globiformis won the competition for limiting amounts of ammonium when the glucose concentration in the reservoirs increased, in agreement with previously described experiments in which the flagellates were not included. The numbers of nitrifying bacteria decreased as the numbers of heterotrophic bacteria rose with increasing glucose concentrations. Critical C/N ratios, i.e. ratios between glucose and ammonium in the reservoirs by which no nitrate was found in the culture vessels, of 12.5 and 10.5 were determined at D= 0.004 and 0.01 h^{-1}, respectively. Below these critical values, coexistence of the competing species was found. The numbers of nitrifying bacteria decreased more in the presence of flagellates than in their absence presumably by selective predation on the nitrifying bacteria, either in the liquid culture or on the glass wall of the culture vessels. Despite this, the rate of nitrate production did not decrease more in the presence of flagellates than in their absence. This demonstrates that no correlation has to be expected between numbers of nitrifying bacteria and their activity. Above the critical C/N ratios, low numbers of nitrifying bacteria were still found in the culture vessels, probably due to attachment of the nitrifying bacteria to the glass wall of the culture vessels. Like the numbers of heterotrophs, the numbers of flagellates increased when the glucose concentrations in the reservoirs increased. Numbers of 2 and 12 x 10^5 flagellates ml^{-1} were found at 7 mM glucose at D= 0.004 and 0.01 h^{-1}, respectively.

It was concluded that the critical C/N ratios were practically unaffected by the presence of protozoa. Although nitrate production rates were equal in the presence and absence of flagellates, the numbers of nitrifying bacteria decreased stronger in their presence. This indicates a higher activity per nitrifying cell in the presence of flagellates.

INTRODUCTION

Protozoa have been shown to affect N-transformation processes in soil by the enhancement of mineralization of nitrogen immobilized in bacterial biomass (3,15,39), although some other studies indicated that the microfauna had no significant effect on ammonification rates (1,2). Goldman et al. (13) found that when protozoa feed on bacteria, one-third of the nitrogen of the digested bacteria was converted into protozoan biomass, one-third consisting mainly of cell walls and organelles, was excreted as indigestible organic N, and one-third was excreted as ammonium. Enhancing the turnover rate of nitrogen in a system by protozoan grazing may stimulate the growth of N-limited heterotrophic bacteria and plants (10,12,26) or increase the nitrification rate (16).

In previous experiments with mixed cultures of *N. europaea* and *A. globiformis* in the presence of *N. winogradskyi* in dual energy limited chemostats, the nitrification process was inhibited by NH_4^+ assimilation by the more competitive heterotrophic bacteria above critical C/N ratios of 11.6 and 9.6 for D= 0.004 and 0.01 h^{-1}, respectively (44). Due to the competitive abilities of *A. globiformis*, the numbers of nitrifying bacteria as well as the concentrations of nitrate decreased as the glucose concentrations in the reservoirs increased. At the critical C/N ratios, *A. globiformis* consumed all the mineral nitrogen and glucose added to the culture vessels. Below these ratios, the heterotrophic bacteria were glucose limited, the surplus of ammonium was nitrified and coexistence of the competing species was found in steady state situations. Above these ratios, the heterotrophic bacteria were nitrogen limited and the nitrification process was inhibited.

In the experiment described in this paper, part of the organic nitrogen immobilized in the mixed bacterial population, was expected to be mineralized by the grazing activities of the protozoa. Below the critical C/N ratio, where the heterotrophic bacteria are carbon limited, the ammonium excreted by the flagellates was expected to be converted into nitrate by the nitrifying population. Above the critical C/N ratio, the mineralized ammonium was assumed to be used by the nitrogen limited heterotrophic bacteria. We expected that, because of the extra ammonium, the numbers of nitrifying bacteria would decrease less in the presence of protozoa than in their absence when glucose concentrations increased and that the critical C/N ratios would increase.

The aim of this study was to investigate the influence of grazing by the flagellate *A. peritocrescens* on the competition for limiting amounts of ammonium between the

ammonium-oxidizing species *N. europaea* and the heterotrophic species *A. globiformis* in the presence of *N. winogradskyi* in chemostats with increasing carbon to nitrogen ratios in the influent. This was done at two dilution rates.

MATERIALS AND METHODS

Organisms and culture conditions.

Nitrosomonas europaea ATCC 19718 and *Nitrobacter winogradskyi* ATCC 25391 were used as the nitrifying bacteria in the competition experiments. They probably belong to the dominant nitrifying bacteria in a calcareous pasture soil near Brummen (52°05' N, 06°09' E) in the Netherlands. Before inoculation in the chemostat they were grown in mixed batch cultures (44). *Arthrobacter globiformis* was used as the heterotrophic bacterium. It was isolated from the rhizosphere of Ribwort plantain (*Plantago lanceolata*), that was growing in a pot containing the pasture soil mentioned above and it was cultured in a medium containing ammonium sulphate and glucose (44). The flagellate *Adriamonas peritocrescens* was used as the bacterial grazer in this study. It was isolated from the pasture soil mentioned above. The isolation procedure, monoxenisation and culturing have been described (Verhagen et al., submitted for publication).

Competition experiments.

The experiments were performed in the presence of *N. winogradskyi* in order to prevent possible toxic effects of nitrite on the competing organisms.
The competition experiments were performed using Biostat M fermenters (B. Braun, Melsungen, FRG) with culture volumes of 1250 ml. The dilution rates studied were 0.004 and 0.01 h^{-1}. The compositions of the media have previously been described (44). The pH of the cultures was maintained at 7.5 with either 0.5 N NaOH or 0.1 N HCl. Temperature and oxygen concentration were maintained at 25°C and at least 60 % of air saturation, respectively. The cultures were stirred at 150 rev per min.

In a culture vessel, 1 l sterile mineral medium was inoculated with 250 ml of a four week-old mixed batch culture of *N. europaea* and *N. winogradskyi* containing 7.5 and 6.0 x 10^6 bacteria per ml, respectively. When the 2 mM ammonium present was totally converted into nitrate, the influent and effluent pumps were started. After analysis

of the steady state culture, 50 ml of a 3 day-old glucose-free culture of *A. globiformis*, pre-grown on 1 mM glucose and containing 3 x 10^7 cells per ml, was added. A new medium reservoir containing the same medium as before but supplemented with 1 mM glucose, was connected to the growth vessel. After analysis of the steady state culture, the vessel was inoculated with 10 ml of a monoxenical flagellate culture, grown on *A. globiformis* for 3 days and containing 2 x 10^6 flagellates per ml. A new steady state was reached at 1 mM glucose. Then, new medium reservoirs with increasing glucose concentrations were connected to the growth vessel, whereupon new steady states were awaited. At every steady state, samples were taken to determine ammonium, nitrite, nitrate, glucose, total and dissolved organic carbon concentrations and pH. The numbers of nitrifying bacteria were determined by MPN and FA counts, numbers of heterotrophic bacteria by FA and plate counts and numbers of flagellates by MPN and direct counts using a counting chamber. Samples of the medium reservoirs were taken to determine ammonium, glucose and pH. Enumerations of heterotrophic bacteria by plate counting on a rich agar medium were also used as tests for purity of the mixed cultures. Samples to analyze the steady state cultures were stored at -25°C.

Control experiments.

For comparison with the mixed bacterial cultures, control experiments were done in which *A. globiformis* was grown without nitrifying bacteria in the presence and absence of flagellates in media containing ammonium and glucose. The compositions of the media and the culture conditions were identical to those described above. The experiments were done at $D = 0.01\ h^{-1}$.

Most Probable Number enumerations (33).

For the enumerations of nitrifying bacteria and flagellates, twelve replicates were made per dilution. The dilution procedures, compositions of the media and culturing of the food bacterium *A. globiformis* have previously been described (44). The most probable numbers of nitrifying bacteria or flagellates were obtained from statistical tables, which were generated by a computer program (29).

Fluorescent Antibody enumerations (36).

Counts of nitrifying and heterotrophic bacteria were performed using antisera

obtained from immunized rabbits. The preparation of the antiserum and the staining procedure have previously been described (44).

Plate Counts.

Enumerations of heterotrophic bacteria were done by plating ten tenfold dilutions of culture samples in phosphate buffer (pH 7.0) on a rich agar medium. The dilution procedure, composition of the agar medium and incubation conditions have previously been described (44).

Counting Chamber enumerations.

Numbers of flagellates were determined using a counting chamber. A subsample (2.0 ml) from the culture vessel was fixed with Lugol's Iodine with a final concentration of 5 % (v/v). Fixed samples, in which the flagellates were stained by the free iodine, were stored at 4°C and counted within 2 days using an inverted microscope.

Analytical Methods.

Concentrations of ammonium, nitrite and nitrate were determined using a Technicon Traacs 800 autoanalyzer (Technicon Instr. Corp., Tarrytown, New York, USA) with a detection level of 0.01 mM for all three compounds. The critical C/N ratio for each dilution rate was determined by drawing the most probable line through the nitrate concentrations that were not zero (linear curve fit). The intersection with the X-axis was the critical C/N ratio.

Total and dissolved organic carbon were determined with a total organic carbon analyzer (O.I. Corp., College Station, USA) using potassium hydrogen phthalate as standard and potassium persulphate as oxidizing agent. Dissolved organic carbon was determined in the supernatant of the sample after centrifugation at 15,000 x g for 15 minutes. Net cell organic carbon was determined by subtracting the dissolved organic carbon from the total amount of organic carbon. The C/N ratio of the mixed communities was calculated by dividing the net cell organic carbon by the ammonium used.

Glucose concentrations were determined using a test combination for glucose (Boehringer Mannheim Diagnostica, Mannheim, FRG) based on the GOD-POD method (photometric determination of glucose with glucose oxidase and peroxidase). The detection level was 0.05 mM.

RESULTS

Mineral nitrogen concentrations.

When mineral medium without glucose was supplied, the 2 mM ammonium was completely converted into 2 mM nitrate by the nitrifying bacteria (Fig. 1).

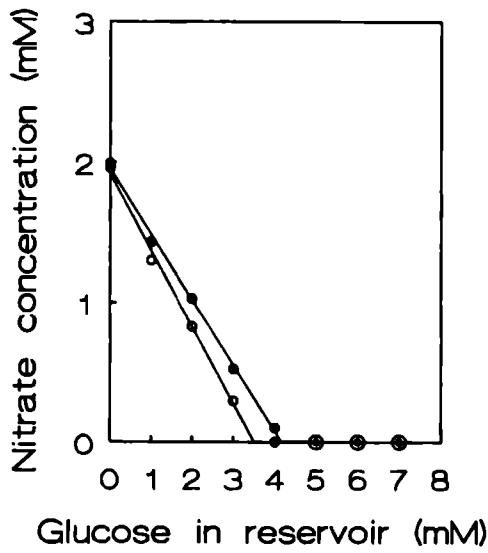

Fig. 1. Steady state concentrations of nitrate in the culture vessels containing *N. europaea*, *N. winogradskyi*, *A. globiformis* and the flagellate *A. peritocrescens*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (●) and 0.01 (○) h^{-1}.

Nitrate concentrations decreased linearly as the glucose concentrations increased and they were zero at critical C/N ratios of 12.5 and 10.5 for D= 0.004 and 0.01 h^{-1}, respectively. No nitrate was detected in the culture vessels above these ratios. The steady state ammonium and nitrite concentrations were below the detection level at both dilution rates at all glucose concentrations supplied.

In the control experiments without nitrifying bacteria, critical C/N ratios of 9.4 and 9.8 were found in the absence and presence of flagellates, respectively. Ammonium concentrations decreased linearly and they were zero above the critical C/N ratios.

Glucose concentrations.

The steady state glucose concentrations were below the detection level at both dilution rates at all glucose concentrations supplied, except for 6 and 7 mM glucose at D= 0.01 h^{-1} when 0.46 and 1.55 mM glucose were measured, respectively.

Numbers of *N. europaea*.

At both dilution rates, the numbers of *N. europaea* decreased as the glucose concentration increased (Fig. 2). At 1 Mm glucose and D= 0.004 h^{-1}, the FA number of *N. europaea* in the presence of flagellates was 21 % of the number in their absence (Fig. 2A). The numbers of ammonium oxidizers at this dilution rate decreased up to 5 mM glucose according to both enumeration methods. At higher glucose concentrations

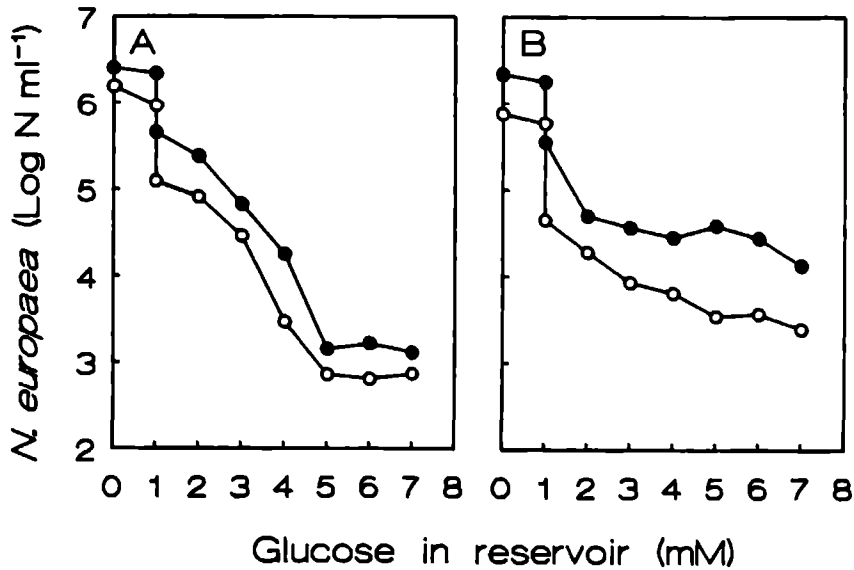

Fig. 2. Steady state numbers of *N. europaea* grown in the presence of *N. winogradskyi*, *A. globiformis* and the flagellate *A. peritocrescens*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. Enumerations were performed according to the FA (●) and the MPN (○) technique. No flagellates and heterotrophic bacteria were present at 0 mM glucose. The high and low numbers at 1 mM glucose are the numbers in the absence and presence of flagellates, respectively.

there was no further decrease in numbers. The FA number at 7 mM glucose was 0.05 % of the original number at 0 mM glucose. The MPN numbers were lower than the FA numbers at all glucose concentrations supplied. According to the observed differences between the FA and the MPN enumeration techniques, only 17 to 57 % of the *N. europaea* cells were or became active during the incubation time of the MPN enumerations at this dilution rate. At D= 0.01 h^{-1} and 1 mM glucose, the FA number of *N. europaea* in the presence of flagellates was 20 % of the number in their absence (Fig. 2B). The FA numbers of ammonium oxidizers at this dilution rate decreased mostly between 0 and 2 mM glucose, whereas only a slight decrease in numbers was observed above 2 mM glucose. At 7 mM glucose, the FA number of *N. europaea* was 0.6 % of the original number at 0 mM. The MPN numbers of *N. europaea* were lower than the FA numbers. According to the observed differences between the FA and the MPN numbers, only 9 to 38 % of the ammonium oxidizing cells were or became active during incubation of the MPN enumerations at this dilution rate.

Numbers of *N. winogradskyi.*

The numbers of *N. winogradskyi* decreased at both dilution rates as the glucose concentration increased (Fig. 3). The decreases in the FA numbers of *N. winogradskyi* were similar at both dilution rates.

At D= 0.004 h^{-1} and 1 mM glucose, the FA number of *N. winogradskyi* in the presence of flagellates was 30 % of the number in their absence (Fig. 3A). The FA numbers of nitrite oxidizers at this dilution rate gradually decreased up to 4 mM glucose. They did not change at glucose concentrations higher than 4 mM and at 7 mM glucose the FA number was 2 % of the original number at 0 mM. The FA and MPN numbers of nitrite oxidizers corresponded rather well up to 4 and 2 mM glucose at D= 0.004 and 0.01 h^{-1}, respectively, but at higher glucose concentrations the MPN numbers were lower than the FA numbers. The differences were probably due to inaccuracies in the enumeration techniques.

At D= 0.01 h^{-1} and 1 mM glucose, the FA number of *N. winogradskyi* in the presence of flagellates was 39 % of the number in their absence (Fig. 3B). The numbers of nitrite oxidizers at this dilution rate decreased up to 5 mM glucose. The numbers did not change at glucose concentrations higher than 5 mM and at 7 mM glucose the FA number was 2 % of the original number at 0 mM. Differences between the two counting techniques were probably due to inaccuracies in the enumeration methods.

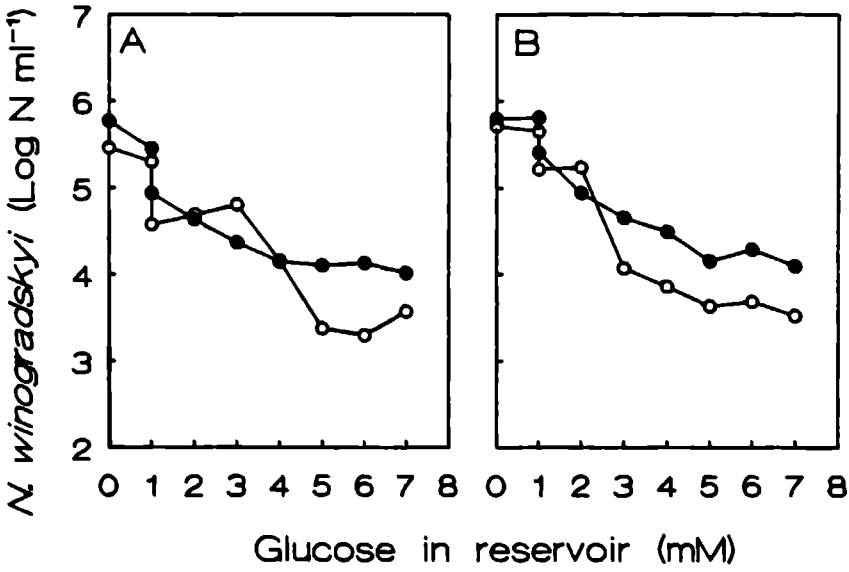

Fig. 3. Steady state numbers of *N. winogradskyi* grown in the presence of *N. europaea*, *A. globiformis* and the flagellate *A. peritocrescens*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. Enumerations were performed according to the FA (●) and the MPN (○) technique. No flagellates and heterotrophic bacteria were present at 0 mM glucose. The high and low numbers at 1 mM glucose are the numbers in the absence and presence of flagellates, repsectively.

Numbers of *A. globiformis*.

The numbers of *A. globiformis* increased gradually at both dilution rates as the glucose concentration increased (Fig. 4). At 1 mM glucose, the FA numbers of *A. globiformis* in the presence of flagellates were 1.3 and 1.5 times the numbers in their absence for D= 0.004 and 0.01 h^{-1}, respectively. The plate count numbers were lower than the FA numbers at both dilution rates and all glucose concentrations supplied. At D= 0.004 h^{-1}, the FA numbers of *A. globiformis* did not change between 6 and 7 mM glucose, probably due to the nitrogen limitation above the critical C/N ratio (Fig. 4A). The FA number of heterotrophic bacteria at 7 mM glucose was 5 times the number at 1 mM. The numbers of heterotrophic bacteria growing at D= 0.004 h^{-1} were always lower than those at D= 0.01 h^{-1}. At D= 0.01 h^{-1}, the numbers of *A. globiformis* did not increase at glucose concentrations higher than 5 mM (Fig. 4B). The FA number of

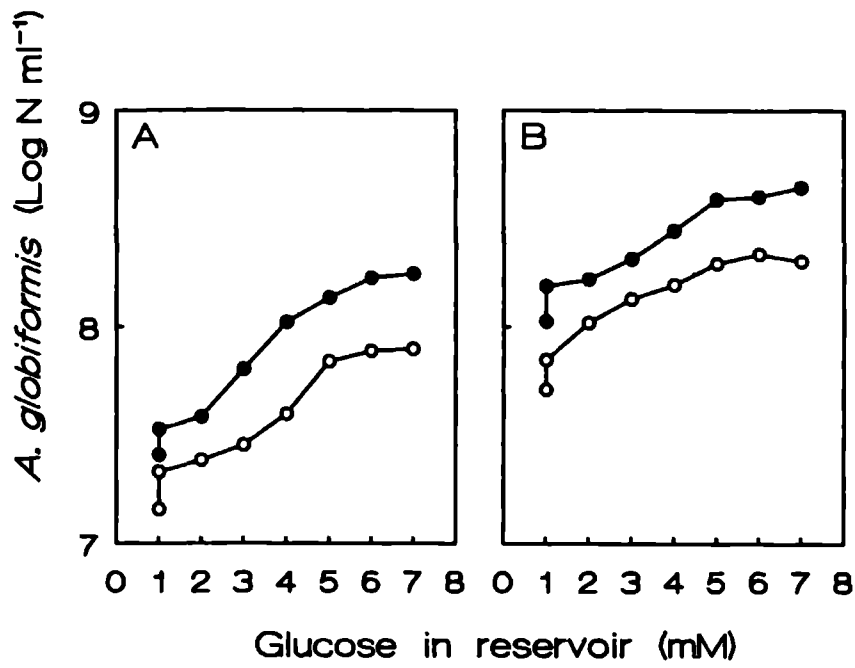

Fig. 4. Steady state numbers of *A. globiformis* grown in the presence of *N. europaea*, *N. winogradskyi* and the flagellate *A. peritocrescens*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. Enumerations were performed according to the FA (●) and the Plate Count (○) technique. The low and high numbers at 1 mM glucose are the numbers in the absence and presence of flagellates, respectively.

heterotrophic bacteria at 7 mM glucose was 3 times the number at 1 mM.

In the control experiments, the numbers of *A. globiformis* were comparable to those in the competition experiments. The numbers of heterotrophic bacteria in the presence of flagellates were 36-51 % of those in their absence.

Numbers of *A. peritocrescens*.

The numbers of flagellates increased at both dilution rates as the glucose concentration increased (Fig. 5). The MPN and direct counting numbers of flagellates corresponded well at both dilution rates. At D= 0.004 h^{-1}, the numbers of flagellates did not change between 6 and 7 mM glucose, in accordance with the numbers of bacteria (Fig. 5A). At 7 mM glucose the direct counting number was 6 times higher than the

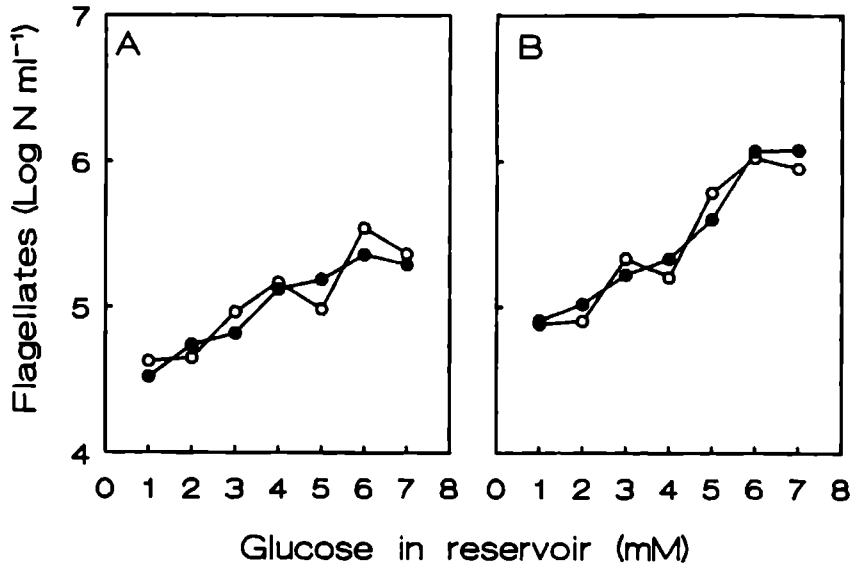

Fig. 5. Steady state numbers of the flagellate *A. peritocrescens* grown in the presence of *N. europaea*, *N. winogradskyi* and *A. globiformis*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. Enumerations were performed according to the Counting Chamber (●) and the MPN (○) technique.

number at 1 mM and amounted to 2 x 10^5 cells ml^{-1}. The numbers of flagellates up to 6 mM glucose increased more at D= 0.01 h^{-1} than at 0.004 h^{-1} (Fig. 5B). They did not increase at glucose concentrations higher than 6 mM. At 7 mM glucose the direct counting number was 15 times higher than the number at 1 mM and amounted to 1 x 10^6 flagellates ml^{-1}.

In the control experiments, the number of flagellates increased slightly as the glucose concentration increased and amounted to 3 x 10^5 flagellates ml^{-1} at 6 mM glucose.

Organic carbon.

Total as well as cell organic carbon concentrations increased as glucose concentrations increased at both dilution rates (Fig. 6). The total and cell organic carbon concentrations in the culture were lower at D= 0.004 h^{-1} than at 0.01 h^{-1}, similar to the numbers of heterotrophic bacteria. Total organic carbon concentrations increased at

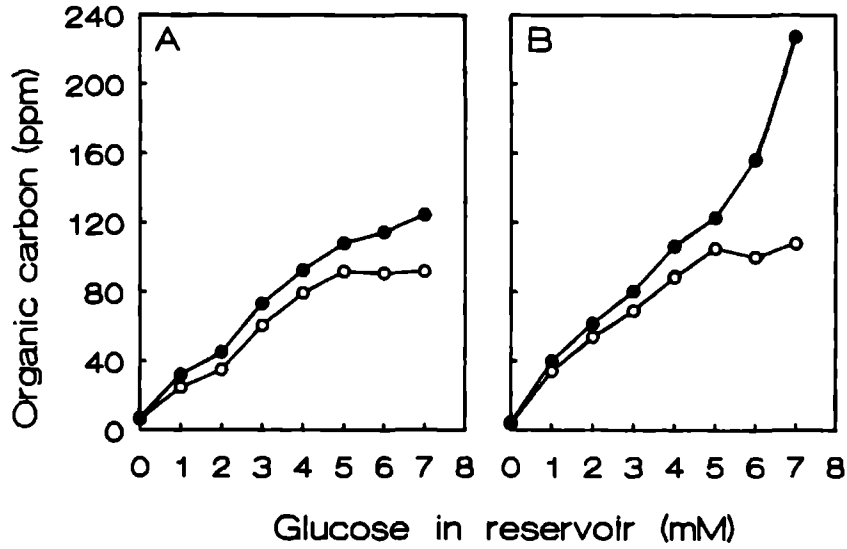

Fig. 6. Steady state concentrations of total organic carbon (●) and cell organic carbon (○) in the culture vessels containing *N. europaea*, *N. winogradskyi*, *A. globiformis* and the flagellate *A. peritocrescens*, with increasing glucose concentrations in the reservoir at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. No flagellates and heterotrophic bacteria were present at 0 mM glucose.

glucose concentrations higher than 5 mM, but the cell organic carbon concentrations did not change. At D= 0.004 and 0.01 h^{-1}, the maximal cell organic carbon concentrations were 90 and 105 mg C L^{-1}, respectively.

Below the critical C/N ratios, the mean C/N ratios of the mixed populations with flagellates growing at D= 0.004 and 0.01 h^{-1} were 3.46 and 3.49, respectively.

DISCUSSION

The heterotrophic *A. globiformis* won the competition for limiting amounts of ammonium in the mixed continuous cultures of this study. Nitrate concentrations in the culture vessels decreased linearly as the glucose to ammonium ratios in the reservoirs increased. Only the ammonium apparently not needed for assimilation processes in the heterotrophic bacteria, was converted into nitrate by the nitrifying community. Also in

other studies, heterotrophic bacteria were found to be more competitive for the available ammonium than the nitrifying bacteria (23,31,32,44). Critical C/N ratios were 12.5 and 10.5 at D= 0.004 and 0.01 h^{-1} in this study, respectively, whereas in similar experiments without flagellates they were 11.6 and 9.6, respectively (44). Hence, critical C/N ratios were 8 and 9 % higher in the presence of flagellates at D= 0.004 and 0.01 h^{-1}, respectively. However, we think that these increases are too small to be statistically significant. This is supported by the results from the control experiments, in which critical C/N ratios of 9.4 and 9.8 were found in the absence and presence of flagellates, respectively. This increase of 4 % is not statistically significant. The small increases in critical C/N ratios, if any, might be a consequence of the mineralization of bacterial nitrogen by the flagellates, as has often been reported for protozoa (10,12,15,26,39). Nitrifying and heterotrophic bacteria might profit by this ammonification below and above the critical C/N ratio, respectively.

Predator-prey relationships can have positive as well as negative effects on the prey organisms. On the one hand, bacterial numbers may decrease in the presence of protozoa by the consumption of bacteria, whereas on the other hand, bacterial numbers may increase by the higher rate of mineralization of nutrients in the presence of protozoa (3,10,12,26,39) or by the production of a growth factor by the protozoa (16). The ultimate effect of the addition of protozoa to a bacterial community can hardly be predicted, since the result depends on the experimental conditions such as the composition of the media and on the bacterial species used. The FA numbers of *N. europaea* at 1 mM glucose in the presence of flagellates were 21 and 20 % of those found in the absence of protozoa at D= 0.004 and 0.01 h^{-1}, respectively (Fig. 2). Similarly, the FA numbers of *N. winogradskyi* in the presence of protozoa were 30 and 39 % of those found in the absence of microfauna at D= 0.004 and 0.01 h^{-1}, respectively (Fig. 3). However, the FA numbers of *A. globiformis* at 1 mM glucose with protozoa were 1.3 and 1.5 times the numbers without flagellates at D= 0.004 and 0.01 h^{-1}, respectively (Fig. 4). Apparently, only the nitrifying bacteria decreased in numbers when the cultures were inoculated with flagellates, whereas the numbers of heterotrophic bacteria increased. This observation can be explained as follows:

the nitrifying bacteria were selectively grazed upon by the flagellates, although their numbers were lower than those of the heterotrophic bacteria. The ratios between the FA numbers of nitrifying and heterotrophic bacteria in the liquid at 1 mM glucose in the reservoir amounted to 1:2 and 1:7 in the absence of flagellates and 1:63 and 1:259 in their presence at D= 0.004 and 0.01 h^{-1}, respectively. The selective predation on nitrifying bacteria could be due to differences in cell size between the nitrifying and

heterotrophic bacteria. Nitrifying bacteria tend to be quite large in size due to the necessity of having large amounts of intracellular respiratory membranes. There is growing evidence for a strong selection pressure by bacteriovorous protozoa on larger compared to smaller bacteria (8,14). The selective predation of soil protozoa on mixed bacterial populations has been reported before (35,41,42). In addition, a part of the nitrifying bacteria was probably attached to the glass wall of the culture vessel as was discussed before (44). These attached cells were selectively grazed upon by the flagellates. The assumption of Verstraete (45), that because of their low densities nitrifying bacteria are unlikely to be intensively grazed upon by protozoa, does not apply here. If a layer of nitrifying bacteria is present on the glass wall of a vessel, the density of attached cells may be sufficiently high to be grazed upon by flagellates. The selective predation of attached bacteria has been reported by Caron (6), who examined four species of heterotrophic microflagellates for their ability to graze attached and unattached bacteria. Two species of microflagellates efficiently grazed unattached bacteria but showed little or no ability to graze attached or aggregated cells. In contrast, *Rhynchomonas nasuta* and *Bodo* sp. showed marked preferences for attached and aggregated bacteria and a limited ability to graze unattached cells, although the densities of unattached bacteria was 5-90 times those of attached ones. Sibbald and Albright (40) described that the microflagellate *Bodo* sp. selectively grazed aggregated bacteria, whereas the microflagellate *Paraphyromonas* sp. selectively grazed free-living bacteria.

The numbers of nitrifying bacteria below the critical C/N ratios were lower in the presence of flagellates than in their absence at both dilution rates (44). However, the nitrate productions were not dependent on the absence or presence of protozoa at both dilution rates. Thus, the activity per cell is higher in the presence of flagellates. This demonstrates that there has not to be a direct relation between the numbers and activities of nitrifying bacteria. This has been reported before in soil (4,5,46). The activity of nitrifying bacteria was reported before to be favoured by the presence of protozoa (16). Consequently, the assumption of a constant nitrification rate per cell can not be made for nitrifying bacteria. The result that flagellate grazing appeared to stimulate per cell rate of nitrification in *N. europaea* is supported by other studies, in which also stimulation of bacterial activities by protozoa was found. Sherr et al. (38) reported that grazing by bacteriovorous microflagellates enhanced the bacterial breakdown of the polysaccharide cell wall of *Peridimium* cells by heterotrophic bacteria in lake water. Stimulation of nitrogen fixation by *Azotobacter* in the presence of protozoa was reported by Nasir (28) and by Cutler and Bal (11).

Calculations were made of the numbers of *N. europaea* that were to be expected

on basis of the measured nitrate concentrations as described before (44). In these calculations the cell yields of *N. europaea* in combination with *N. winogradskyi* but without heterotrophic bacteria and flagellates were used. All measured numbers of *N. europaea* were lower than were expected on basis of the nitrate concentrations (Fig. 7).

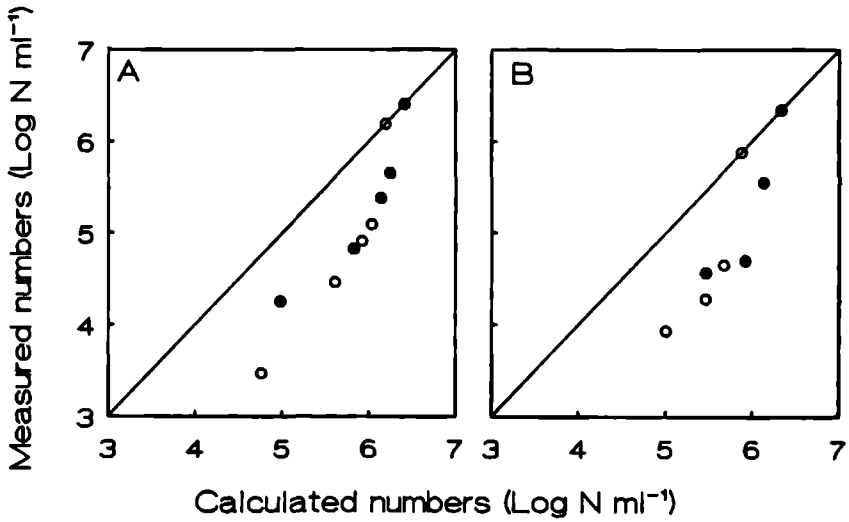

Fig. 7. Calculated against measured numbers of *N. europaea* grown in the presence of *N. winogradskyi*, *A. globiformis* and the flagellate *A. peritocrescens*, during the competition experiments at dilution rates of 0.004 (A) and 0.01 (B) h^{-1}. Enumerations were performed according to the FA (●) and MPN (○) technique.

Nitrate uptake by the heterotrophic bacteria can be excluded because then the measured numbers of nitrifying bacteria would be higher than could be expected on basis of the nitrate concentrations. Inaccuracies in the enumerations techniques can also be excluded because then the points would lie at both sides of the ideal line. Differences in bacterial C/N ratios of *A. globiformis* in pure and mixed cultures can also be excluded since bacterial C/N ratios of *A. globiformis* were almost similar in pure and mixed cultures (44). The most probable explanation for the fact that the numbers of nitrifying bacteria were lower than expected on basis of the measured nitrate concentrations is that the expected numbers of *N. europaea* were calculated using the cell yield in cultures without flagellates. Apparently, this cell yield per nitrate produced is too high in the presence of protozoa.

The outcomes of the experiments may be influenced by the possible inhibition of

N. europaea by either glucose, toxic products from glucose metabolism by *A. globiformis* or toxic organic excretion products from the flagellates. In previous experiments it was shown that glucose, up to a final concentration of 1 mM, and ammonium-free supernatant of an early stationary phase culture of *A. globiformis* did not inhibit or stimulate nitrate production (44). Also ammonium-free supernatant of a culture of flagellates, growing on *A. globiformis* did not inhibit nitrate production. Glucose and other organic compounds such as formate, acetate, butyrate, pyruvate, and glycerol were reported not to affect nitrate formation (24,25). Growth responses to individual amino acids or vitamins in log phase cultures of *N. europaea* were observed by Clark and Schmidt (9). Some amino acids and vitamins stimulated nitrite formation and protein synthesis by the ammonium oxidizers, whereas others were inhibitory. The inhibition of ammonium-oxidizing bacteria by organic compounds originating from plant roots is widely documented (27,30,43). These allelopathic effects ascribed to tannins and tannin derived compounds were of no importance in our chemostat experiments.

The mean C/N ratios of the mixed cultures of microflora and microfauna amounted to 3.46 and 3.49 at D= 0.004 and 0.01 h^{-1}, respectively. In previous experiments without flagellates, the mean bacterial C/N ratios were 3.46 and 3.48 in pure cultures of *A. globiformis* and 3.54 and 3.56 in mixed cultures of *A. globiformis*, *N. europaea* and *N. winogradskyi* (44). Apparently, the C/N ratio of a mixed culture of bacteria and protozoa is practically not affected by the presence or absence of flagellates, although for protozoa C/N ratios of 5-7 were reported (22). The relative low numbers and the small size of the flagellates were probably due to this observation. Assuming a carbon content of 50 % and C/N ratios of 3.46 and 3.49 for the mixed communities, the cultures had nitrogen contents of 14.5 and 14.3 % at D= 0.004 and 0.01 h^{-1}, respectively, illustrating the excess of nitrogen in relation to glucose available for the heterotrophic bacteria (32). In similar competition experiments without flagellates a nitrogen content of 14.4 % was found for both dilution rates (44).

At glucose concentrations higher than 5 mM, i.e. above the critical C/N ratios, total organic carbon concentrations increased as glucose concentrations increased at both dilution rates, but the cell organic carbon concentrations remained constant. This was probably due to the nitrogen limitation of the heterotrophic bacteria above the critical C/N ratios, which restricted the increases in cell biomass. As glucose was detectable at none of the glucose concentrations at D= 0.004 h^{-1}, it was supposed that between 4 and 5 mM glucose the surplus of glucose was taken up by *A. globiformis* and was probably converted into glycogen, whereas above 5 mM glucose the surplus of glucose was excreted in some organic form after uptake. At D= 0.01 h^{-1}, some glycogen was formed

in the cells between 4 and 5 mM glucose, but above 5 mM glucose in the reservoir, only a part of the surplus of glucose was taken up and excreted in some organic form, whereas the other part was not taken up and was measured as glucose (see Results). This explains the sharp increase in total organic carbon concentrations at D= 0.01 h^{-1}, whereas the net cell organic carbon concentrations remained constant above 5 mM glucose.

The FA numbers of *A. globiformis* at 1 mM glucose were 30 and 50 % higher in the presence of flagellates than in their absence at D= 0.004 and 0.01 h^{-1}, respectively (Fig. 4). These increases in numbers can not be explained by N mineralisation by the protozoa, because the heterotrophic bacteria are carbon limited below the critical C/N ratios. As the glucose concentrations in the reservoirs increased above 1 mM, the numbers of *A. globiformis* increased gradually at both dilution rates . However, these increases were rather small in relation to those without flagellates (44). At 5 mM glucose, the numbers of *A. globiformis* in the grazed systems amounted to only 28 and 15 % of those in the ungrazed ones at D= 0.004 and 0.01 h^{-1}, respectively. Protozoa were reported to have the ability to decrease bacterial numbers drastically (18,19), but the predator does not reduce them below a certain number (20). Sambanis and Frederickson (34) found that a bacterial density in the order of 10^4-10^7 cells per ml persisted in the presence of viable protozoa in chemostat cultures. In the experiments described here, protozoan numbers did not increase further, although still 10^7-10^8 heterotrophic bacteria per ml were present. This is likely not due to inedibility of the heterotrophic bacteria, because all *Arthrobacter* strains were found to be readily and completely eaten by soil protozoa (21). However, the numbers of the nitrifying bacteria, of which a part was probably attached to the glass wall of the culture vessel, were reduced to a lower level than those of the heterotrophic bacteria because they could be grazed upon from the glass wall where densities were probably higher than in the liquid culture or the flagellates could have a preference for attached bacteria (see above).

The numbers of flagellates were determined by the numbers of bacteria. The higher numbers of heterotrophic bacteria at D= 0.01 h^{-1} than at 0.004 h^{-1} gave higher numbers of flagellates at D= 0.01 h^{-1} at all glucose concentrations supplied. The numbers of flagellates at 7 mM glucose in the reservoir were 6-fold and 15-fold those at 1 mM glucose at D= 0.004 and 0.01 h^{-1}, respectively. This was probably due to the higher bacterial production in the culture vessel at a dilution rate of 0.01 h^{-1}. However, not only the numbers of flagellates were higher at D =0.01 h^{-1} but they increased also more at this dilution rate as glucose concentrations in the reservoirs increased. The highest increase in numbers of flagellates was observed between 4 and 6 mM glucose at D= 0.01 h^{-1}, whereas this strong increase in numbers was absent at D= 0.004 h^{-1}. The strong increase

in numbers at D= 0.01 h^{-1} agrees with a strong increase in the dissolved organic carbon concentrations at high glucose concentrations at this dilution rate (Fig. 6B), which was caused by the presence of glucose (see Results). Glucose was only present at D= 0.01 h^{-1} at high glucose concentrations. The strong increase in numbers of flagellates only at D= 0.01 h^{-1} may be explained by the possible usage of organic compounds in addition to bacteria by the flagellates. Uptake of glucose by the flagellates is not very likely, since Haas and Webb (17) reported no evidence for uptake of sugars or amino acids by marine bacteriovorous flagellates at substrate concentrations up to 0.75 mg L^{-1}, based on radioisotope assays. However, usage by the flagellates of high molecular weight polysaccharide molecules in addition to bacteria at high glucose concentrations in the present study might be an explanation for the observed increase in numbers. Sherr (37) reported that bacteriovorous flagellates could ingest, and grow at the expense of, high molecular weight dextran molecules, which were produced by bacteria. Above 6 mM glucose in the reservoir, the numbers of flagellates did not increase any more, probably because numbers of *N. europaea, N. winogradskyi* and *A. globiformis* did not change above 6 mM glucose.

The MPN numbers of *N. europaea* were lower than the FA numbers at all glucose concentrations supplied at both dilution rates (Fig. 2). This was also observed for *N. winogradskyi* at a number of glucose concentrations at both dilution rates and besides the differences in numbers, the MPN numbers of nitrite oxidizers fluctuated more than the FA numbers (Fig 3). These observations demonstrated that the used MPN technique was inadequate to enumerate nitrifying bacteria, even in these controlled laboratory experiments. In contrast to this, the MPN method for the enumeration of flagellates appeared to be adequate, since the MPN and direct counting numbers of *A. peritocrescens* corresponded well at all glucose concentrations supplied at both dilution rates (Fig. 5). Another study, carried out by Caron et al. (7), indicated that the densities of nanoflagellates determined in marine and freshwater samples by a MPN and a direct microscopical counting method, differed by as much as four orders of magnitude. The difference between the two studies may be due to an incomplete excystment of the protozoa in the marine and freshwater samples of Caron et al., whereas in the present study in chemostats, flagellate cells were actively growing. This explanation is supported by the result of Caron et al. (7), who found that in laboratory experiments, the MPN counts of flagellates constituted a much greater percentage of the direct counts during the exponential growth phase of the flagellates than during the stationary growth phase. The plate counts of *A. globiformis* were lower than the FA numbers at all glucose concentrations supplied at both dilution rates. The more or less constant differences

between the two enumeration techniques were probably due to the fact that the heterotrophic bacteria often occurred in double or triple coccoid forms, which were counted as single colony forming units in the plate counts. Because of this, the plate count technique is, in addition to its rather low precision, not adequate to estimate the numbers of *A. globiformis* in continuous cultures.

It may be concluded that *A. globiformis* was more competitive for limiting amounts of ammonium than *N. europaea*. The critical C/N ratios were practically unaffected by the presence of protozoa. However, the numbers of nitrifying bacteria decreased more in the presence of flagellates than in their absence presumably by selective predation on the nitrifying bacteria, either in the liquid culture or on the glass wall of the culture vessel. Although numbers of nitrifying bacteria were lower in the presence of flagellates, the nitrate productions were equal with and without flagellates. This means a higher activity per cell in the presence of protozoa.

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CHAPTER 5

COMPETITION FOR AMMONIUM BETWEEN NITRIFYING AND HETEROTROPHIC BACTERIA IN CONTINUOUSLY PERCOLATED SOIL COLUMNS.

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SUMMARY

Although 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 get more insight in these observations, the 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 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* won the competition 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 zero above 10 mM glucose in the reservoir, although still 10^7 nitrifying cells per gram dry soil were present. This demonstrated that there was no correlation between the numbers of nitrifying bacteria and their activity. The numbers and activities of *N. winogradskyi* decreased less than those of *N. europaea* in all parts of the soil columns, probably due to heterotrophic growth of the nitrite-oxidizing bacteria on organic substrates excreted by the heterotrophic bacteria or to nitrate reduction at reduced oxygen concentrations by the nitrite-oxidizing bacteria. Our conclusion was that the nitrifying bacteria lost the competition for ammonium in soil columns, but 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.

INTRODUCTION

In grassland soils rich in carbon, absence or low rates of nitrate production have often been reported (20,37,38). In these soils also the numbers and activities of the nitrifying bacteria were low compared to those in soils with other vegetations (9,31,32). A possible explanation for these observations has been given by Verhagen and Laanbroek (50). They showed that in experiments with mixed cultures of *N. europaea*, *N. wino-*

gradskyi, and *A. 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 in the medium, the nitrification process was inhibited and nitrate concentrations became zero.

In a system with *n* limiting substrates *n* different bacterial populations can at most 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 with two limiting substrates at low C/N ratios in the medium, whereas at high C/N ratios, where both populations were 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 wall of the culture vessels. Attached ammonium- and nitrite-oxidizing cells were reported to be more active than free-living ones (3,18,26). Hence, attached nitrifying bacteria survived in the chemostats in the presence of the heterotrophic cells and probably used small parts 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 is interesting to perform similar competition experiments in soil columns in which large parts of the nitrifying population are probably adsorbed to soil particles. Since adsorbed nitrifying cells have a greater competitive power than free-living cells, it is expected that more nitrifying bacteria will survive the suppression of the nitrifying process by the heterotrophic bacteria in relation to the dratic decreases in numbers of nitrifying bacteria in the chemostat experiments mentioned earlier.

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* in soil columns, continuously percolated with media with different carbon to nitrogen 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 0-30 cm layer of a calcareous grassland near Brummen (52°05' North, 06°09' East) in the Netherlands. The soil was sieved (2 mm) to remove roots and stones and was well mixed. It is a sandy soil containing 21.3 % coarse sand, 69.3 % fine sand, 4.1 % silt, and 5.3 % clay. The pH-H_2O, pH-KCl, calcium carbonate and organic carbon concentrations of 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.

Arthrobacter 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 procedure and culturing of the heterotrophic bacterium has previously been described (50). *N. europaea* ATCC 19718 and *N. winogradskyi* ATCC 25391 were used as the nitrifying bacteria in the soil column experiment. They probably belong to the dominant nitrifying bacteria in the grassland soil mentioned above, as was indicated by a positive reaction to specific antibodies against these bacteria in the most diluted positive tubes of a MPN enumeration. The culturing of a mixed nitrifying population before inoculation in the soil columns was similar as was described by Verhagen and Laanbroek (50).

Competition experiments.

The experiments were performed in the presence of *N. winogradskyi* in order to prevent possible toxic effects of nitrite on the competing organisms. The experiments were performed using 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 by a 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

in order to drip sterile medium with or without glucose continuously on top of the soil column. A small steel tube through the rubber stopper was connected to a conical flask and was used for inoculation with bacteria. It also served as an air exhaust during the experiment. 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 per min.

Each column was filled with 250 g of the soil described above and was subsequently percolated with demineralized water for 1-2 days until the soil was compacted and a soil column of 15 cm was formed. After sterilization by γ-ray irradiation (4.5 Mrad), the soil column was percolated with sterile demineralized water for two weeks to remove possible toxic compounds formed by the radiation. It was then percolated with mineral medium containing (per liter): $(NH_4)_2SO_4$, 0.33 g; KH_2PO_4, 0.1 g; $MgSO_4.7H_2O$, 40 mg; $CaCl_2$, 20 mg; NaCl, 0.5 g and trace elements solution, 1 ml. The composition of the trace elements solution has been described before (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 percolate, the soil column was inoculated with a mixture of 25 ml of a four week-old mixed batch culture of *N. europaea* and *N. winogradskyi*, containing 5 x 10^6 and 3 x 10^6 cells per ml, respectively, and 5 ml of a 3 day-old glucose-free culture of *A. globiformis*, pre-cultured on 1 mM glucose and containing 3 x 10^7 cells per ml. Percolation with mineral medium containing 5 mM ammonium was continued until 5 mM nitrate was found in the percolates. Then, new medium vessels were connected containing the mineral medium described above supplemented with 0.40, 0.79, 1.19, 1.59, 1.98, or 2.38 g glucose.H_2O per liter. Two columns were used for each glucose concentration and 4 columns received no glucose. Samples of the percolates were taken three times a week to determine ammonium, nitrite, nitrate, glucose and pH. After 10 weeks of percolation the soil columns were harvested.

At the end of the experiment, 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, 0-3 cm; layer B, 3-6 cm; layer R, 6-15 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 (0-3 cm) by FA counts. Mineral nitrogen concentrations and pH were determined in 1 M KCl- and in water-extracts, respectively. The organic carbon concentrations were determined. Samples of the medium reservoirs were taken to determine ammonium, glucose and pH.

Most Probable Number enumerations (42).

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

Fluorescent Antibody enumerations (51).

A sample of moist soil from the column (1.00 g) was mixed with 0.5 g glass beads, 2.8 g chelating resin (sodium form, dry mesh 50-100, Sigma, St. Louis) and 6.0 ml 0.1 % (w/v) cholic acid (sodium salt, Sigma, St. Louis). The mixture was shaken for 2 hours at 100 rev per min and was then allowed to stand for 1 minute. The supernatant was successively filtered over nylon filters with pore sizes of 20 and 5 μm. Using a syringe, 0.5 ml Percoll (Pharmacia, Uppsala, Sweden) was brought under 1.00 ml of the obtained filtrate (30). After centrifugation at 10,000 x g for 10 minutes in a Biofuge A table centrifuge, the upper 1.0 ml was filtered over a black polycarbonate membrane filter (Nuclepore Corp., Pleasanton) with a pore size of 0.2 μm (21). The staining procedure for the bacteria on the filter using antiserum prepared from blood from an immunized rabbit, has previously been described (50).

Determination of potential nitrifying activities (45).

The medium for the determination of the potential ammonium-oxidizing activity contained (per liter): $(NH_4)_2SO_4$, 0.33 g; K_2HPO_4, 0.14 g; KH_2PO_4, 27 mg and $NaClO_3$, 1.06 g and for the determination of the potential nitrite-oxidizing activity (per liter): $NaNO_2$, 6.9, 13.8, 20.7 or 34.5 mg; K_2HPO_4, 0.14 g and KH_2PO_4, 27 mg. The pH of both media was 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 unknown at which nitrite concentration maximum nitrite-oxidizing activities are reached and substrate inhibition of the nitrite-oxidizing bacteria might occur

at higher concentrations. Therefore, potential nitrite-oxidizing activities were determined using 4 different nitrite concentrations. The rates of nitrite formation and consumption were calculated with linear regression. In determining 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", using a computer program (12).

Samples (5.0 g) of a well-mixed layer were supplemented with 25 mg $CaCO_3$ and 12.5 ml of the medium described above. For the determination of the potential ammonium-oxidizing activity the nitrite oxidation was inhibited by 10 mM chlorate present in the medium (4). For the determination of the potential nitrite-oxidizing activity, 25 μl 1 % (w/v) nitrapyrin (Dow Chemical Comp., Michigan) in 96 % ethanol was added to inhibit ammonium oxidation (6,34). The mixtures were incubated at 25°C and shaken at 150 rev per min. Formation of nitrite in the potential ammonium oxidation determinations and consumption of nitrite in the potential nitrite oxidation measurements were followed for 6 hours. Samples to determine nitrite were taken every hour. After centrifugation of a sample at 15,000 x g in a Biofuge A table centrifuge for 5 minutes, 0.50 ml of the sample was mixed with 0.50 ml 2 M KCl solution. Samples were stored at 4°C and analyzed within 1 day.

Determinations of mineral nitrogen, pH and organic carbon.

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

Analytical methods.

Concentrations of ammonium, nitrite and nitrate were determined using a Technicon Traacs 800 autoanalyzer (Technicon Instr. Corp., N. Y.) with a detection level of 0.01 mM N for all three compounds. Glucose concentrations were determined with a test-combination for glucose (Boehringer Mannheim Diagnostica, Mannheim, Germany) based on the GOD-POD method (photometric determination of glucose with glucose oxidase and peroxidase). The detection level for glucose was 0.05 mM.

Dissolved oxygen concentrations were measured using an oxygen monitor (Strathkelvin Instr., Glasgow, UK) fitted with a micro-electrode.

RESULTS

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

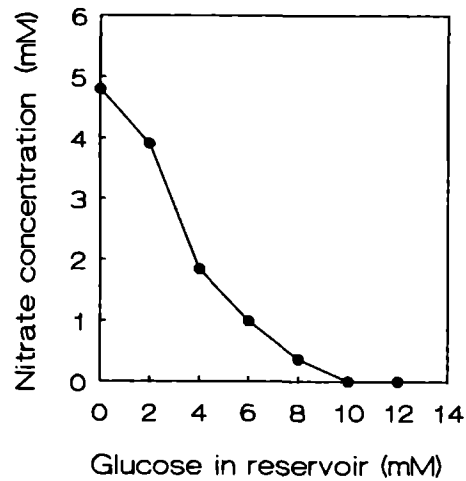

Fig. 1. Concentrations of nitrate in the percolates of the soil columns after percolation for 10 weeks with media containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C. Data represent the means of duplicate soil columns.

Ammonium concentrations in soil extracts of layer A were higher than those in 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

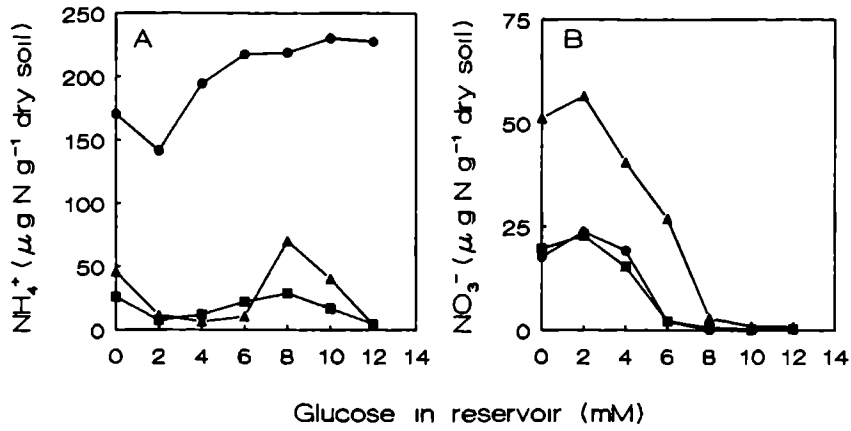

Fig. 2. Concentrations of ammonium (A) and nitrate (B) in 1 M KCl extracts (1:5) of the three layers of the soil columns percolated for 10 weeks with media containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C: ●:layer A,0-3 cm;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

above 2 mM glucose (Fig. 2B). Nitrate concentrations in layers A and R were equal and they were zero above 8 mM glucose. Nitrate concentrations in layer B were higher than those in layers A and R and they were zero above 10 mM glucose.

Glucose concentrations.

The glucose concentrations in the percolates were below the detection level during percolation at all glucose concentrations supplied.

Numbers and potential activities of *N. europaea*.

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. In all soil columns, the numbers of *N. europaea* in layers A and B were equal, whereas the numbers in layer R were 3-4 times lower than those in the upper layers.

The potential ammonium-oxidizing activities of *N. europaea* decreased with

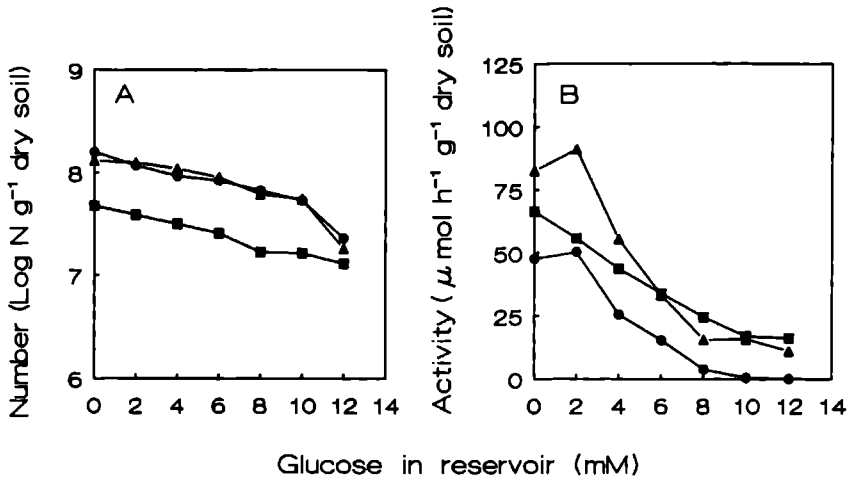

Fig. 3. Numbers (A) and potential ammonium-oxidizing activities (B) of *N. europaea* cells grown in the presence of *N. winogradskyi* and *A. globiformis*, in the three layers of the soil columns percolated for 10 weeks with media containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C: ●:layer A,0-3 cm; ▲:layer B,3-6 cm; ■:layer R,6-15 cm. Cells were enumerated with the MPN technique. Data represent the means of duplicate soil columns.

increasing glucose concentrations (Fig. 3B). The potential activities were lowest in layer A at all glucose concentrations supplied. Above 10 mM glucose, no activity of ammonium-oxidizing bacteria was found in this layer, although they 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. Above 2 mM glucose, activities in layer B decreased until they became constant above 8 mM glucose.

Numbers and potential activities of *N. winogradskyi*.

In all soil columns, numbers in layers B and R were equal, except for those at 12 mM glucose (Fig. 4A). Numbers in layer A were 2-4 times higher than those in the underlying layers. In layers A and B, numbers of nitrite-oxidizing bacteria were constant up to 10 mM glucose, but at 12 mM glucose, an increase in numbers was observed. At 10 mM glucose, the numbers in layers A and B had decreased to 28 and 55 % of those

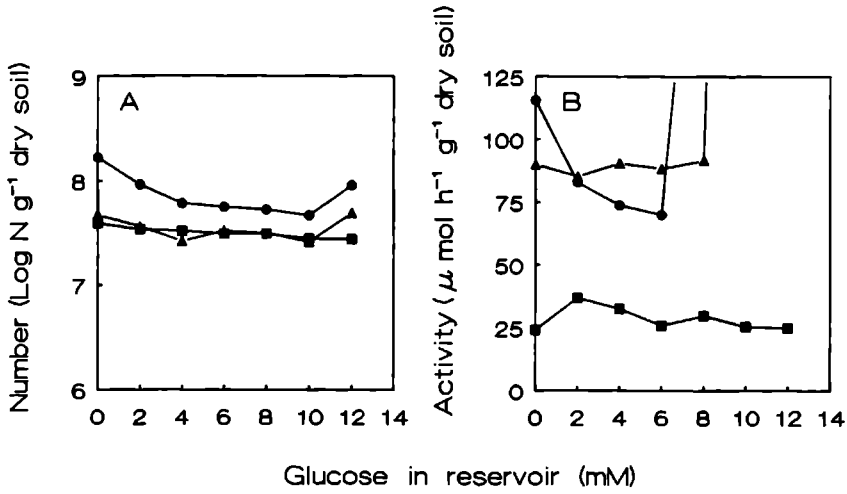

Fig. 4. Numbers (A) and potential nitrite-oxidizing activities (B) of *N. winogradskyi* cells grown in the presence of *N. europaea* and *A. globiformis*, in the three layers of the soil columns percolated for 10 weeks with media containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C: ●:layer A,0-3 cm; ▲:layer B,3-6 cm; ■:layer R,6-15 cm. Cells were enumerated with the MPN technique. Data represent the means of duplicate soil columns.

at 0 mM glucose, respectively. Numbers of *N. winogradskyi* in layer R were more or less constant at all glucose concentrations supplied. At 10 mM glucose, the number had decreased to 71 % of that at 0 mM glucose.

The potential nitrite-oxidizing activities of *N. winogradskyi* in the soil columns percolated with mineral medium were almost equally high in layers A and B (Fig. 4B). The potential activities in layer R were 4-5 times lower than the comparable activities in the upper layers. The potential activity in layer A decreased between 0 and 6 mM glucose, whereas above 6 mM glucose, a sharp increase in activity to 1400 μmol h^{-1} g^{-1} dry soil at 12 mM glucose was measured. In layer B, the activities were constant up to 8 mM glucose, but at higher glucose concentrations, a sharp increase to 725 μmol h^{-1} g^{-1} dry soil 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*.

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* were about 1, except for 12 mM glucose, where a low ratio was found due to the relatively sharp decrease and increase in numbers of

Table 1. Ratios between the numbers of *N. europaea* and *N. winogradskyi* grown in the presence of *A. globiformis*, in the three layers of the soil columns, which were percolated for 10 weeks with media 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)	layer		
	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

Data are means of duplicate soil columns.

ammonium- and nitrite-oxidizing bacteria, respectively (Fig. 4A). In layer B, the ratios were higher than those in layer A, due to the lower numbers of *N. winogradskyi* in layer B than in layer A (Fig. 4A), whereas the numbers of *N. europaea* were equal in both layers (Fig. 3A). Ratios in layer B amounted to 2-4 at all glucose concentrations, except for 12 mM glucose, where a low ratio was found for the same reason as in layer A. In layer R, the ratio decreased from 1.2 to 0.5 with increasing glucose concentrations, due to a steady decrease in numbers of *N. europaea*.

Ratios between the potential activities of *N. europaea* and *N. winogradskyi*.

Generally, potential activities of the nitrite-oxidizing bacteria in layers A and B

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

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 the soil columns, which were percolated for 10 weeks with media 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)	layer		
	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

ND, Not determined, due to unmeasurable potential nitrite-oxidizing activities (see text).
Data are means of duplicate soil columns.

Numbers of *A. globiformis*.

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 number of heterotrophic bacteria at 12 mM glucose was lower than that at 10 mM glucose.

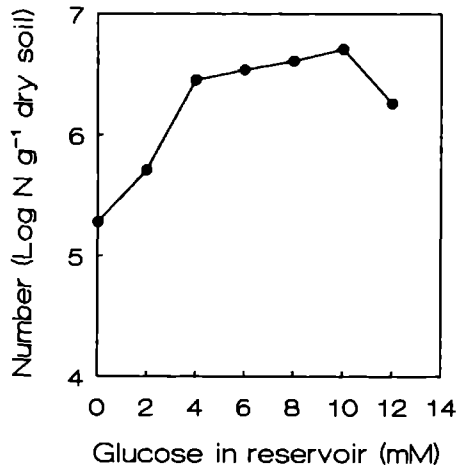

Fig. 5. Numbers of *A. globiformis* cells grown in the presence of *N. europaea* and *N. winogradskyi*, in layer A (0-3 cm) of the soil columns percolated for 10 weeks with media containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C.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

pH and organic carbon.

The pH in the percolates of the soil columns percolated with mineral medium for 10 weeks, amounted to 6.9, whereas the pH in the percolates of the soil columns percolated with media containing glucose, increased almost linearly with increasing glucose concentrations up to 8.2 at 12 mM glucose.

The pH in 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 pH 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 0.3-0.4 unit was observed between 4 and 6 mM glucose, but at higher glucose concentrations the pH of the suspensions of layers B and R were constant again.

The organic carbon concentrations of the soil columns percolated with mineral medium for 10 weeks, were equal for all layers and amounted to 4.2 % of dry soil (Fig. 6). In layer A, the organic carbon concentration rapidly increased between 0 and 2 mM glucose. Above 2 mM glucose, it was constant up to 8 mM glucose. At higher

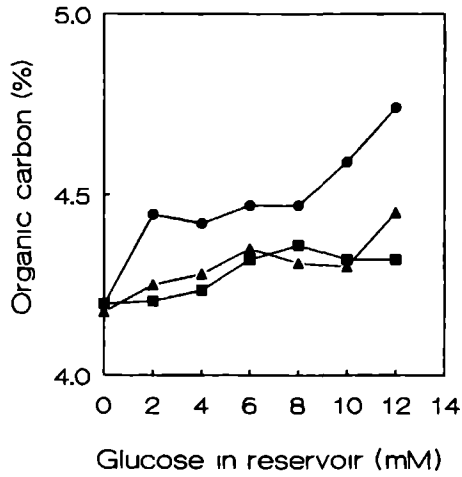

Fig. 6. Organic carbon concentrations in the three layers of the soil columns after percolation for 10 weeks with media containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes) and 20°C: ●:layer A,0-3 cm; ▲:layer B,3-6 cm; ■:layer R,6-15 cm. Data represent the means of duplicate soil columns.

glucose concentrations, the organic carbon concentration increased again. Organic carbon concentrations of layers B and R were equal at all glucose concentrations, except at 12 mM glucose, where a small increase in the organic carbon concentration of layer B was observed. From 0 to 6 mM glucose, the organic carbon concentrations of 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 column percolated with 10 mM glucose for 10 weeks, the dissolved oxygen concentration 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. This was the same at a depth of 7 cm. It was therefore assumed that below a depth of 2 cm, the dissolved oxygen concentration was constant at ca. 50 % of air saturation.

DISCUSSION

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

Soil columns have often been used to study the effects of environmental factors on the activity and survival of nitrifying bacteria (1,22,28,46). In the soil column experiment performed in this study, the competition for ammonium between nitrifying and heterotrophic bacteria takes place as long as the numbers of heterotrophic bacteria increase during the percolation time. When the numbers of *A. globiformis* have reached 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 experiment described here. *A. globiformis* won the competition for limiting amounts of ammonium as glucose concentrations increased in the medium. This was concluded on basis of the low potential ammonium-oxidizing activities at higher glucose concentrations. The decrease in nitrate concentrations in the percolates with increasing glucose concentrations in the reservoir could be due to smaller amounts of ammonium 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 since in similar competition experiments in chemostats it was shown that the heterotrophic bacteria did not use nitrate in addition to ammonium (50). At 10 and 12 mM glucose, nitrate concentrations in the percolates were zero and also the activities of the ammonium-oxidizing population in the uppermost 3 cm of the soil columns were zero at these glucose concentrations. This indicates that the heterotrophic bacteria completely

outcompeted the nitrifying bacteria and that the heterotrophic bacteria only used ammonium as N-source. It may be discussed 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 zero 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 is 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 substrate ammonium. Formation of thin channels in the soil, through which the medium containing glucose flowed, might have played a role in this. Incomplete nitrification in a continuous-flow nitrification column was reported before by Cox et al. (16).

The outcomes of the experiments may be 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 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 has stimulated or inhibited nitrate production in the experiment described here since 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 since the glucose concentrations in the percolates were always below the detection level. In addition, glucose and other organic compounds such as formate, acetate, pyruvate, and glycerol were 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 were ascribed to tannins and tannin derived compounds.

In this study, activity and survival of *N. europaea* cells, as indicated by the viable MPN counts, were differently affected 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 rate in the three layers of the soil columns as the glucose concentrations increased (Fig. 3A). In spite of 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 A, activities were even zero at 10 and 12 mM glucose, although still more than 10^7 ammonium-oxidizing bacteria per gram dry soil were present. This 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 a previous experiment, the 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 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 wall was a way to survive high C/N ratios. Activity determinations could not be done in that experiment. Comparison of the competition experiments performed in soil columns and in chemostats demonstrated clearly that adsorption of nitrifying bacteria to solid particles had apparently 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 might have survived as inactive, but viable cells. This may be a reason why there is no relationship between the numbers and activities of nitrifying bacteria.

Comparison of the decreases in numbers of *N. europaea* and *N. winogradskyi* showed a greater decrease for the ammonium-oxidizing than for the nitrite-oxidizing bacteria at higher glucose concentrations. The only substantial decrease in numbers of *N. winogradskyi* 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 given for this different behaviour of *N. europaea* and *N. winogradskyi*:

1. 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 numbers of *N. winogradskyi* was not coupled to the decrease in numbers of *N. europaea*. Since glucose was found in none of the percolates of the soil columns, it is proposed 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. This was observed before in similar competition experiments in chemostats (50). Some of these unidentified organic compounds might be used by *N. winogradskyi* for growth. Stimulation of nitrite-oxidizing bacteria by organic compounds in the environment has often been reported (10,11,12,13,47).
2. nitrate reduction by the nitrite-oxidizing bacteria using nitrate instead of oxygen for the oxidation of organic compounds, excreted by the heterotrophic bacteria. The dissolved

oxygen concentration below 2 cm from the top of the soil column, percolated with medium containing 10 mM glucose, was about 50 % of air saturation. Denitrification at reduced oxygen concentrations has been reported for various bacteria (40).

3. a higher death rate of the ammonium-oxidizing bacteria at higher glucose concentrations. This explanation is less likely since the smaller decrease in numbers of *N. winogradskyi* was also observed in competition experiments between *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 the potential nitrite-oxidizing activities were found (Fig. 4B). However, the fast disappearance of nitrite from the incubation media was apparently not due to higher potential activities of the nitrite-oxidizing bacteria. Heterotrophic bacteria, nitrogen limited at higher glucose concentrations, are assumed to be responsible for the fast disappearance of the small amounts of nitrite in the incubation media. In the potential ammonium-oxidizing activities, no sharp increases were observed. This was due to the fact that a surplus of ammonium was present in the determinations, through which ammonium usage by the heterotrophic bacteria did not interact with the potential ammonium-oxidizing activity determinations.

Nitrogen balances were calculated for the soil columns. For these calculations, in the first instance it was assumed that the increases in organic carbon measured in the layers of the soil columns were totally in the form of cell material (Fig. 6). The increases in organic carbon in the three layers of the soil columns were compared to 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 media containing 10 and 12 mM glucose, N-balances fit when bacterial C/N ratios of 9.1 and 12.1 are used, respectively. These appear to be realistic values for the C/N ratio of bacteria, since Rosswall (41) reported bacterial C/N ratios of 12.5 at N-starvation levels. However, if the increases in organic carbon measured in the layers of the soil columns were not totally in the form of cell material and only a part of the excreted organic carbon was taken up by *N. winogradskyi*, N-balances fit at lower bacterial C/N ratios than those mentioned above. For example, if only 50 % of the increases in organic carbon in the layers was in the form of cell material, N-balances fit when bacterial C/N ratios of 4.5 and 6.1 were assumed for 10 and 12 mM glucose, respectively. In the competition experiments in chemostats between the same bacterial species, a bacterial C/N ratio of 3.5 was found at low C/N ratios in the medium (50).

In determining 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 by the formation of chlorite by the nitrite oxidizer (4). Also *N. europaea* was found to be very sensitive to chlorite, whereas its activity was 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 oxidation rates. It is unknown how much chlorate was converted into chlorite in the incubation mixtures before complete inhibition of *N. winogradskyi* appeared and whether chlorite was excreted into the suspensions by the nitrite-oxidizing bacteria.
In determining the potential nitrite-oxidizing activity of a soil 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 on the soil used. In literature, inhibition rates between 69 and 97 % are reported for soils (14). Inhibition of a *Nitrosomonas* species by nitrapyrin was very effective (6). In soil suspensions of an ammonium-amended silt loam soil, an inhibition 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 of this study were determined.

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

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CHAPTER 6

EFFECTS OF GRAZING BY FLAGELLATES ON COMPETITION FOR AMMONIUM BETWEEN NITRIFYING AND HETEROTROPHIC BACTERIA IN SOIL COLUMNS.

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SUMMARY

The enhanced mineralization of immobilized nitrogen by bacteriophagous protozoa has been thought to favour the nitrification process in soils in which nitrifying bacteria have to compete with heterotrophic bacteria for the available ammonium. To obtain more insight into this process, the influence of grazing by the flagellate *Adriamonas peritocrescens* on the competition for ammonium between the chemolithotrophic species *Nitrosomonas europaea* and the heterotrophic species *Arthrobacter globiformis* in the presence of *Nitrobacter winogradskyi* was studied in soil columns, which were continuously percolated with media containing 5 mM ammonium and different amounts of glucose at a dilution rate of 0.007 h^{-1} (liquid volumes).

A. globiformis won the competition for ammonium, in agreement with the result of the experiment without flagellates. The grazing activities of the flagellates had two prominent effects on the competition between *N. europaea* and *A. globiformis*. Firstly, the distribution of ammonium over the profile of the soil columns was more uniform in the presence of flagellates than in their absence. In the absence of flagellates, relatively high amounts of ammonium accumulated in the upper layer (0-3 cm), whereas in the underlying layers the ammonium concentrations were low. In the presence of flagellates, however, considerable amounts of ammonium were found in the lower layers, whereas less ammonium accumulated in the upper layer. Secondly, the potential ammonium-oxidizing activities of *N. europaea* were stimulated in the presence of flagellates. The numbers of *N. europaea* at different glucose concentrations in the presence of flagellates were comparable to those in their absence. However, in the presence of flagellates the potential ammonium-oxidizing activities were 4-5 times higher than in their absence.

In conclusion, grazing by protozoa may result in a stimulation of growth and activity of nitrifying bacteria. It may lead to a higher production of nitrate in systems, in which the heterotrophic bacteria are limited by carbon.

INTRODUCTION

Soil N processes may be affected by the presence of bacteriophagous protozoa

(3,8). Grazing of protozoa on bacteria has often been reported to enhance the mineralization of bacterial nitrogen (6,11,13,27,32), although in some other studies no significant effects on ammonification rates by the grazing activities of soil microfauna were found (1,2). Enhanced nitrogen mineralization may either stimulate the growth of N-limited heterotrophic bacteria and plants (7,12,16,18) or increase the rate of nitrification in soils with low C/N ratios (14).

In competition experiments, conducted with mixed cultures of *N. europaea* and *A. globiformis* in the presence of *N. winogradskyi* but in the absence of flagellates in chemostats and continuously percolated soil columns, the nitrification process was shown to be inhibited by NH_4^+ assimilation by the more competitive heterotrophic bacteria (28,29). Should protozoa be introduced in similar experiments, the grazing activities of the flagellates are expected to result in the mineralization of part of the organic N immobilized in the mixed bacterial community. At low C/N ratios in the medium, the ammonium excreted by the protozoa will probably be converted into nitrate by the nitrifying community, as the heterotrophic bacteria are limited by carbon at low C/N ratios. As a result, the nitrate production at low C/N ratios is expected to be higher in the presence of flagellates than in their absence. At high C/N ratios in the medium, however, the mineralized ammonium excreted by the flagellates is expected to be used by the heterotrophic bacteria, which are nitrogen limited at high C/N ratios.

Except the increased mineralization of immobilized nitrogen, the stimulating effect of protozoa on the nitrification process may be due to an unknown growth factor, which is excreted by the protozoa in liquid cultures (14). In this paper, transportation of ammonium towards the nitrifying bacteria, which are possibly growing in immobile micro-colonies, or of nitrifying bacteria towards their substrate ammonium is also suggested as a possible mechanism.

The aim of this study was to investigate the influence of grazing by the bacteriophagous flagellate *Adriamonas peritocrescens* on the nitrate production in carbon or nitrogen limited mixed cultures of the chemolithotrophic species *Nitrosomonas europaea* and the heterotrophic species *Athrobacter globiformis* in the presence of *Nitrobacter winogradskyi* in non-saturated soil columns, which were continuously percolated with media containing 5 mM ammonium and different concentrations of glucose at a dilution rate of 0.007 h^{-1}, based on liquid volumes.

MATERIALS AND METHODS

Soil sampling.

Soil was collected from the top 0-30 cm layer of a grassland near Brummen (52°05' N, 06°09' E) in the Netherlands. The soil was sieved (2 mm) to remove roots and stones and was well mixed. It is a sandy soil containing 5.3 % clay; 4.1 % silt; 69.3 % fine sand, and 21.3 % coarse sand. The pH-H_2O of the sieved mixed soil was 7.8, the pH-KCl was 7.4, the content of calcium carbonate was 1.6 % and of organic matter 4.2 %. Soil was stored before use at 4°C for 2 weeks.

Organisms and culture conditions.

N. europaea ATCC 19718 and *N. winogradskyi* ATCC 25391 were used as the nitrifying bacteria in this study. They probably belong to the dominant nitrifying bacteria in the grassland soil described above, as was indicated by a positive reaction to specific antibodies against these bacteria in the most diluted positive tubes of a MPN enumeration. The culturing of a mixed nitrifying population before inoculation in the soil columns was identical as has been described previously (29).

A. globiformis was used as the heterotrophic bacterium. It was isolated from the rhizosphere of Ribwort plantain (*Plantago lanceolata*) growing in a pot containing the soil described above. The isolation procedure and culturing of the heterotrophic bacterium have been described previously (28).

The flagellate *A. peritocrescens* isolated from the grassland soil described above, was used as the bacterial grazer in this study. It was enriched on heterotrophic bacteria growing on 0.02 % (w/v) proteose peptone, isolated by a dilution method, and monoxenically grown on *A. globiformis*.

Competition experiments.

The experiments were performed in the presence of *N. winogradskyi* in order to prevent possible toxic effects of nitrite on the competing organisms. Each soil column contained 250 g soil described above and had an effective length of 15 cm. The construction and γ-sterilization of the soil percolation columns, the compositions of the

media and the performance of the experiment have previously been described (29). The inoculum contained 6.9 x 10^6 ammonium-oxidizing, 4.8 x 10^6 nitrite-oxidizing and 1.2 x 10^7 heterotrophic bacteria per ml (numbers determined by specific Fluorescent Antibody (FA) microscopy, see below) and its pH was 7.2. Percolation of the soil columns with mineral medium containing 5 mM ammonium was continued until 5 mM nitrate was found in the percolates. Then, the soil columns were inoculated with 10 ml of an actively growing culture of *A. peritocrescens*, containing 5.4 x 10^5 flagellates per ml (number determined with counting chamber). New medium vessels were connected containing the mineral medium described above supplemented with 0.40, 0.79, 1.19, 1.59, 1.98, or 2.38 g glucose.H_2O per litre. The glucose concentration in the medium for a soil column remained constant during the percolation period. Two columns were used for each glucose concentration and 4 columns received no glucose.

Samples of the percolates were taken three times a week to determine ammonium, nitrite, nitrate, glucose and pH. After 13 weeks of percolation the soil columns were harvested. At harvesting of the soil columns, 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, which was percolated for 13 weeks with medium containing 5 mM ammonium and 10 mM glucose. At harvesting, each column was divided into three parts: layer A, 0-3 cm; layer B, 3-6 cm; layer R, 6-15 cm. In each layer, 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 by FA counts and those of flagellates by MPN counts. Soil mineral nitrogen concentrations and pH were determined in 1 M KCl- and in water-extracts, respectively. The soil organic matter contents were also determined. Samples of the medium reservoirs were taken to determine ammonium, glucose and pH.

Most Probable Number enumerations (10,23).

The preparation of the soil suspensions, the dilution procedure and the composition of the media for the enumeration of nitrifying bacteria have previously been described (29). Twelve replicates were made per dilution. Tests for ammonium and nitrite oxidation at the end of the incubation period (9 weeks) were performed using 0.04 % (w/v) bromo cresol purple (pH-indicator) and the Griess Ilosvay reagents, respectively. Two suspensions per soil layer were made, which were counted separately.

To determine the number of flagellates, *A. globiformis* was used as the food bacterium in the incubations. A subsample (0.25 ml) of a soil suspension, 1:10 in 1 mM

phosphate buffer (pH 7.0), was diluted fourfold in sterile microtiter plates (Costar, Cambridge, UK) containing 0.75 ml of a culture of *A. globiformis* (pH 7.5) per well. Twelve replicates were made per dilution. Plates were incubated at 20°C for 12 days. Tests for flagellate growth were done using an inverted microscope. Two suspensions per soil layer were made, which were counted separately. The most probable numbers of nitrifying bacteria and flagellates were obtained from statistical tables that were generated by a computer program (19).

Fluorescent Antibody enumerations (25,31).

The extraction of the bacteria from the soil and the staining procedure on black polycarbonate membrane filters (Nuclepore Corp., Pleasanton, USA) with a 0.2 μm pore size (15) using antiserum prepared from blood from an immunized rabbit, have been described before (29).

Determination of the potential nitrifying activities (26).

The medium for the determinations contained (per litre): $(NH_4)_2SO_4$, 0.33 g; K_2HPO_4, 139 mg and KH_2PO_4, 27 mg and its pH was 7.5. No inhibitors were applied as sodiumchlorate was found to inhibit also partly ammonium oxidation, whereas N-serve did not completely inhibit ammonium oxidation (4).

Samples (10.0 g) of well-mixed soil were mixed with 50 mg $CaCO_3$ and 25 ml of the medium described above. The mixtures were incubated at 25°C and stirred at 150 rev per min. Formation of nitrite and nitrate was followed for 6 hours. Samples (1 ml) were taken every hour to determine the nitrite and nitrate concentrations. After centrifuging a sample at 15,000 x g in a Biofuge A table centrifuge for 5 minutes, 0.50 ml of the clear supernatant was mixed with 0.50 ml 2 M KCl solution. Samples were stored at 4°C and analyzed within a day. The potential nitrifying activities were determined in duplicate. Potential ammonium-oxidizing activities were calculated from the sum of the nitrite and nitrate formed. The rates of nitrite and nitrate formation were calculated with linear regression. Potential nitrite-oxidizing activities could be calculated from the formation of nitrate when accumulation of nitrite appeared.

Determinations of mineral nitrogen, pH and organic matter.

Mineral nitrogen concentrations were determined by stirring 2.5 g of moist soil

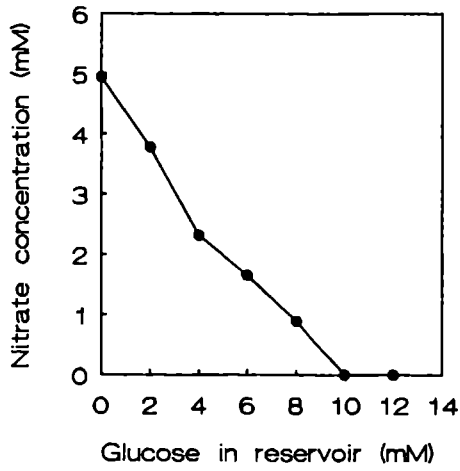

Fig. 1. Concentrations of nitrate in the percolates of the soil columns containing *N. europaea*, *N. winogradskyi*, *A. globiformis*, and the flagellate *A. peritocrescens*, after percolation for 13 weeks with media containing 5 mM ammonium and different concentrations of glucose at D= 0.007 h^{-1} (liquid volumes) and 20°C. Data represent the means of duplicate soil columns.

with 25 ml 1 M KCl solution. After 4 hours, samples was taken and centrifuged at 15,000 x g in a Biofuge A table centrifuge for 5 minutes. The supernatant was stored at 4°C and analyzed for mineral nitrogen compounds within a day. The pH-H_2O was determined by stirring 2.5 g of moist soil with 12.5 ml demineralized water for 2 hours. The organic matter contents were determined by the analysis of weight-losses after heating dry soil (5.00 g) at 550°C for 4 hours. All determinations were done in duplicate.

Analytical methods.

Concentrations of ammonium, nitrite, and nitrate were determined using a Technicon Traacs 800 autoanalyzer (Technicon Instr. Corp., New York, USA) with a detection level of 0.01 mM N for all three compounds. Glucose concentrations were determined with a test combination for glucose (Boehringer Mannheim Diagnostica, Mannheim, Germany) based on the GOD-POD method (photometric determination of glucose with glucose oxidase and peroxidase). The detection level for glucose was 0.05 mM. Dissolved oxygen concentrations were measured using an oxygen monitor (Strathkelvin Instr., Glasgow, UK) fitted with a micro-electrode.

RESULTS

Mineral nitrogen concentrations.

In the soil columns percolated with mineral medium, the 5 mM of ammonium was completely converted into 5 mM nitrate by the nitrifying community (Fig. 1). However, nitrate concentrations were lower at higher glucose concentrations and they were zero at 10 and 12 mM glucose. The ammonium and nitrite concentrations in the percolates were below the detection level (0.01 mM N) at all glucose concentrations supplied.

Ammonium concentrations in soil extracts decreased with increasing depth at all glucose concentrations supplied (Fig. 2A). Up to 8 mM glucose supplied, the only considerable decrease in ammonium concentration was observed in the upper layer between 0 and 2 mM glucose. Above 8 mM glucose, ammonium concentrations decreased slightly in all layers. Nitrate concentrations were lower in layer A than in layers B and R (Fig. 2B). They decreased in all three layers when the glucose concentration increased and above 1 mM glucose, they were zero in all three layers.

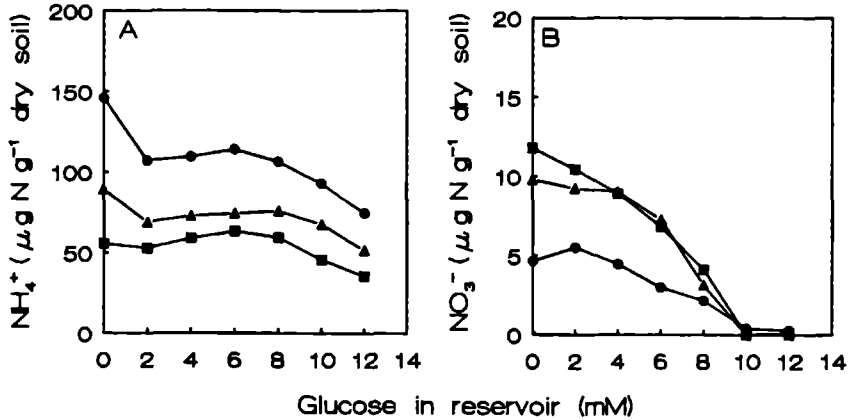

Fig. 2. Ammonium (A) and nitrate (B) concentrations in 1 M KCl extracts (1:5) of the three layers of the soil columns containing *N. europaea*, *N. winogradskyi*, *A. globiformis*, and the flagellate *A. peritocrescens*, after percolation for 13 weeks with media containing 5 mM ammonium and different glucose concentrations at D=0.007 h^{-1} (liquid volumes) and 20°C: ●:A,0-3 cm; ▲:B,3-6 cm; ■:R,6-15 cm. Data represent means of duplicate soil columns.

Glucose concentrations.

The glucose concentrations in the percolates were below the detection level (0.05 mM) during percolation at all glucose concentrations supplied. Glucose concentrations in extracts of the soil layers were not measured.

Numbers and potential ammonium-oxidizing activities of *N. europaea*.

The MPN-numbers of ammonium-oxidizing bacteria decreased as the glucose concentration increased in all three layers (Fig. 3A). The numbers of *N. europaea* also decreased with increasing depth at all glucose concentrations supplied. At 12 mM glucose, the numbers in layers A, B and R amounted to 8, 7 and 9 % of those found at 0 mM glucose, respectively.

The potential ammonium-oxidizing activities of *N. europaea* in the three layers decreased when glucose concentration increased (Fig. 3B). In agreement with the

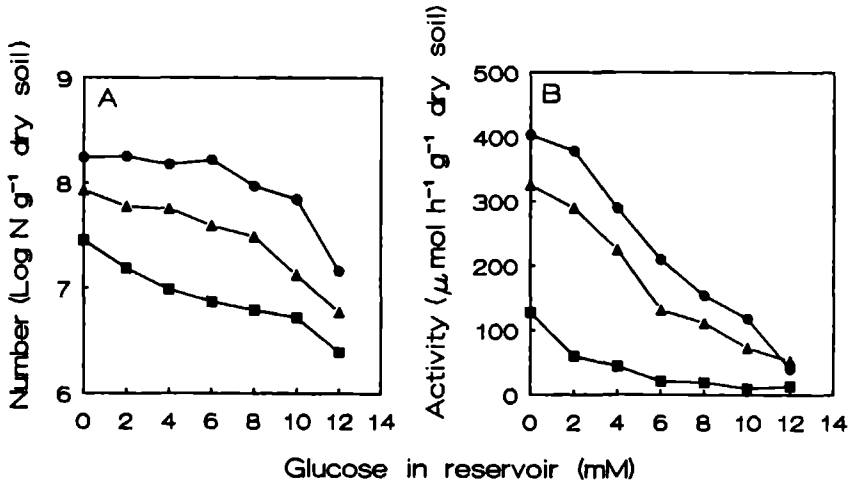

Fig. 3. Numbers (A) and potential ammonium-oxidizing activities (B) of *N. europaea* cells grown in the presence of *N. winogradskyi*, *A. globiformis*, and the flagellate *A. peritocrescens*, in the three layers of the soil columns after percolation for 13 weeks with media containing 5 mM ammonium and different concentrations of glucose at D= 0.007 h^{-1} (liquid volumes) and 20°C: ●:A,0-3 cm; ▲:B,3-6 cm; ■:R,6-15 cm. Cells were enumerated with the MPN technique. Data represent the means of duplicate soil columns.

numbers, the potential activities of *N. europaea* also decreased with increasing depth for all soil columns. At 12 mM glucose, the potential activities in layers A, B and R amounted to 10, 16 and 11 % of those observed at 0 mM glucose, respectively.

Numbers and potential nitrite-oxidizing activities of *N. winogradskyi*.

The MPN-numbers of nitrite-oxidizing bacteria decreased as the glucose concentration increased in all three layers (Fig. 4A). The numbers of *N. winogradskyi* also decreased with increasing depth, except for layers A and B above 8 mM glucose, where numbers were equal. At 12 mM glucose, the numbers in layers A, B and R amounted to 7, 28 and 27 % of those found at 0 mM glucose, respectively.

The potential nitrite-oxidizing activities decreased as the glucose concentration increased in all three layers (Fig. 4B). The potential activities also decreased with increasing depth, in agreement with the numbers of *N. winogradskyi* and the potential ammonium-oxidizing activities. At 12 mM glucose, the potential activities in layers A,B and R were to 11, 23 and 10 % of those observed at 0 mM glucose, respectively.

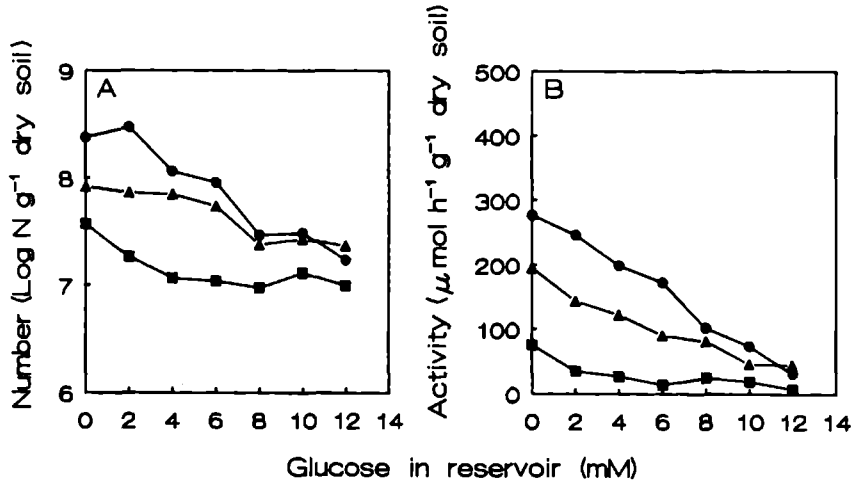

Fig. 4. Numbers (A) and potential nitrite-oxidizing activities (B) of *N. winogradskyi* cells grown in the presence of *N. europaea*, *A. globiformis*, and the flagellate *A. peritocrescens*, in the three layers of the soil columns after percolation for 13 weeks with media containing 5 mM ammonium and different concentrations of glucose at D= 0.007 h^{-1} (liquid volumes) and 20°C: ●:A,0-3 cm; ▲:B,3-6 cm; ■:R,6-15 cm. Cells were enumerated with the MPN technique. Data represent the means of duplicate soil columns.

Numbers of *A. globiformis*.

The FA-numbers of heterotrophic bacteria in layer A were higher than those in layers B and R for all glucose concentrations supplied (Fig. 5). In layers B and R the numbers of *A. globiformis* were more or less equal.

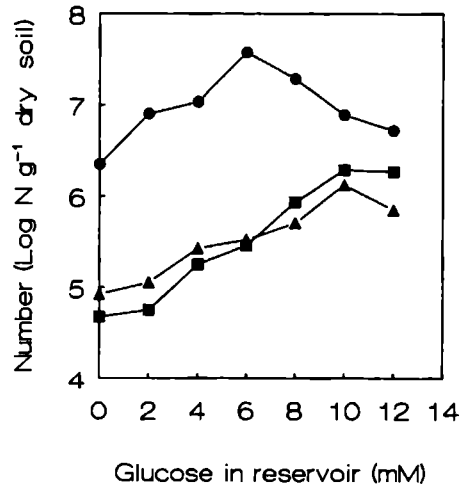

Fig. 5. Numbers of *A. globiformis* cells grown in the presence of *N. europaea*, *N. winogradskyi*, and the flagellate *A. peritocrescens*, in the three layers of the soil columns after percolation for 13 weeks with media containing 5 mM ammonium and different concentrations of glucose at D= 0.007 h^{-1} (liquid volumes) and 20°C: ●:A,0-3 cm; ▲:B,3-6 cm; ■:R,6-15 cm. Cells were enumerated with the FA technique. Data represent the means of duplicate soil columns.

In layer A, the numbers of heterotrophic bacteria increased up to 6 mM glucose. At that concentration, the number was 17 times higher than the one observed at 0 mM glucose. For an unknown reason, the numbers of *A. globiformis* decreased above 6 mM glucose. In layers B and R, the numbers of *A. globiformis* increased up to 10 mM glucose. At that concentration, the numbers were 16 and 41 times higher than those found at 0 mM glucose, respectively.

Numbers of *A. peritocrescens*.

In layer A, the MPN-numbers of flagellates increased as the glucose concentration increased until at 8 mM glucose, a maximum of 1.4 x 10^5 organisms per g dry soil was reached (Fig. 6). The numbers of flagellates found at concentrations of 8-12 mM glucose were 75 times higher than the one found at 0 mM. In layer B, numbers of *A. peritocrescens* increased up to 12 mM glucose. At that concentration, the number was 33 times higher than the one observed at 0 mM glucose. In layer R, the numbers increased sharply above 8 mM glucose and at 12 mM glucose, the number was 145 times higher than the one found at 0 mM glucose.

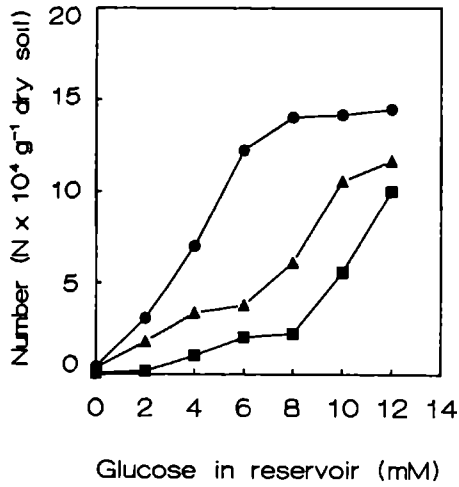

Fig. 6. Numbers of *A. peritocrescens* cells grown on *N. europaea*, *N. winogradskyi*, and *A. globiformis*, in the three layers of the soil columns after percolation for 13 weeks with media containing 5 mM ammonium and different concentrations of glucose at D= 0.007 h^{-1} (liquid volumes) and 20°C: ●:A,0-3 cm; ▲:B,3-6 cm; ■:R,6-15 cm. Cells were enumerated with the MPN technique. Data represent the means of duplicate soil columns.

pH and organic matter contents.

The mean pH in the percolates of the soil columns percolated with mineral medium, was 7.3 (data not shown). The pH of the percolates increased linearly as the glucose

concentration increased up to 8.1 at 12 mM glucose. In the soil suspensions, the pH-values in layer A were lower than those in layers B and R at all glucose concentrations supplied (data not shown). In the upper layer, the pH was 6.9 up to 4 mM glucose, but decreased to 6.4 between 4 and 12 mM glucose. The pH in soil suspensions of layers B and R was 7.2 at all glucose concentrations supplied.

Soil organic matter contents in the columns percolated with mineral medium were 4.0 % for all three layers (Fig. 7).

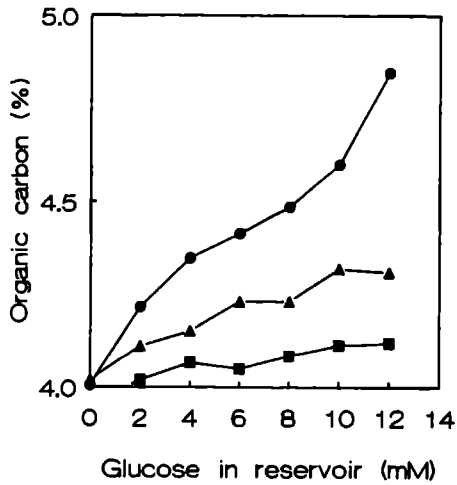

Fig. 7. Organic carbon contents in the three layers of the soil columns containing *N. europaea*, *N. winogradskyi*, *A. globiformis*, and the flagellate *A. peritocrescens*, after percolation for 13 weeks with media containing 5 mM ammonium and different concentrations of glucose at D= 0.007 h^{-1} (liquid volumes) and 20°C: ●:A,0-3 cm; ▲:B,3-6 cm; ■:R,6-15 cm. Data represent the means of duplicate soil columns.

Organic matter contents decreased with depth for all glucose concentrations supplied. In layer A, the organic matter content increased as the glucose concentration increased and was 4.8 % at 12 mM glucose. The largest increase in this layer was observed between 10 and 12 mM glucose, probably due to the presence of organic material excreted by the heterotrophic bacteria or to the presence of glucose that had not been taken up. In layers B and R, organic matter contents increased almost linearly as the glucose concentration in the reservoir increased.

Dissolved oxygen concentrations.

The dissolved oxygen concentrations in the uppermost 2 cm of the soil columns percolated with medium containing 5 mM ammonium and 10 mM glucose decreased linearly from 95 to 50-60 % of air saturation (data not shown). The dissolved oxygen concentrations between 2 and 4 cm and at 7 cm from the top of the soil columns were constant at 50-60 % of air saturation. Therefore, it was assumed that below a depth of 2 cm, the dissolved oxygen concentration was constant at 50-60 % of air saturation.

DISCUSSION

The presence of flagellates might have had different effects on the fate of ammonium in the soil columns at low and high glucose concentrations. At low glucose concentrations, where the heterotrophic bacteria are limited by carbon, the nitrifying bacteria apparently profited from the N-mineralizing activities of the flagellates and consequently, the nitrate concentrations in the percolates were higher in the presence of flagellates than in their absence (29), although the differences were probably not significant. At high glucose concentrations, however, the N-limited heterotrophic bacteria immobilized all the attainable ammonium, including the part mineralized by the protozoa. In general, nitrate production is expected to be stimulated by the grazing activities only in carbon-limited situations, whereas in nitrogen limited situations, where nitrate productions are generally low or absent, no direct effect of grazing on the production of nitrate can be expected.

The presence of flagellates in the soil columns apparently had no effect on the ultimate outcome of the competition for ammonium between the nitrification and the immobilization process. In the presence as well as in the absence of flagellates, the heterotrophic *A. globiformis* won the competition for available ammonium. However, the grazing activities of the flagellates in this study had two marked effects on the fate of ammonium in the soil columns. The first one is related to the distribution of ammonium over the profiles of the soil columns. In the absence as well as in the presence of flagellates, ammonium was found in KCl-extracts of all soil columns after 10 or 13 weeks of percolation. In the absence of flagellates, relatively high amounts of ammonium accumulated in the upper layer (0-3 cm), whereas in the underlying layers the ammonium

concentrations were low (29). We assumed that this KCl-extractable ammonium was not available for or attainable to the heterotrophic and nitrifying bacteria. It was suggested that the relatively high ammonium concentrations were due to a local separation of the ammonium and the bacteria, which were possibly growing in microcolonies. Incomplete nitrification in a continuous-flow nitrification column has previously been reported by Cox et al. (9). In the presence of flagellates, the distribution of ammonium over the profiles was more uniform than in their absence. The ammonium concentrations decreased with increasing depth and in layers B and R considerable amounts of ammonium were found, whereas less ammonium accumulated in the upper layer. The mechanism for the more uniform distribution of ammonium in the presence of flagellates in not clear. Possibly, transportation of medium containing ammonium towards microcolonies of nitrifying bacteria, which are more or less fixed at regular places in soil, has occurred by the movement of the flagellates.

A more uniform distribution of ammonium or nitrifying bacteria over the soil column may have lead to the second prominent effect of the flagellates, which is related to the potential ammonium-oxidizing activities of *N. europaea* in the layers of the soil columns. The numbers of ammonium-oxidizing bacteria were comparable in the presence and absence of flagellates (29). Also the decreases in the numbers of ammonium-oxidizing bacteria with increasing glucose concentrations were comparable, since in the presence of flagellates the MPN numbers of *N. europaea* at 12 mM glucose were about 10 % of those at 0 mM glucose, whereas in the absence of flagellates the MPN numbers at 12 mM glucose in layers A, B, and R amounted to 15, 14, and 27 % of those at 0 mM glucose, respectively (29). In spite of this, the potential ammonium-oxidizing activities were 4-5 times higher in the presence of flagellates than in their absence. The specific activities of the ammonium-oxidizing bacteria in the presence and absence of flagellates are given in Table 1. At all glucose concentrations supplied and in all three soil layers, the specific activities of *N. europaea* cells were higher in the presence of flagellates than in their absence. In spite of the predation pressure, the activity of the nitrifying bacteria was apparently stimulated in the presence of protozoa. Stimulation of the activity of nitrifying bacteria in the presence of bacteriophagous protozoa was reported before by Griffiths (14), who found that the higher nitrifying activity in the presence of protozoa was not due to the extra amount of mineralized ammonium available to the nitrifying community but he suggested that it was due to an unknown growth factor excreted by the flagellates. The stimulation of nitrification in the more heterogenous soil could also be explained by an activity of the flagellates which increases the availability of that part of the ammonium, which is inaccessible to the nitrifying bacteria in the absence of protozoa.

Table 1. Specific activities (in pMol NH_4^+ per cell per hour) of *N. europaea* grown in the presence of *N. winogradskyi* and *A. globiformis*, and in the absence (-F) (27) and presence (+F) of the flagellate *A. peritocrescens*, in the three layers of the soil columns after percolation for 13 weeks with media containing 5 mM ammonium and different concentrations of glucose at D= 0.007 h^{-1} (liquid volumes) and 20°C.

Glucose in reservoir (mM)	layer A		layer B		layer R	
	-F	+F	-F	+F	-F	+F
0	0.30	2.31	0.63	3.83	1.39	4.47
2	0.43	2.11	0.73	4.86	1.43	3.88
4	0.28	1.93	0.51	3.97	1.37	4.55
6	0.18	1.27	0.37	3.38	1.32	2.80
8	0.06	1.65	0.25	3.61	1.44	3.10
10	0.01	1.70	0.28	5.49	1.02	1.89
12	0	2.76	0.60	8.87	1.24	5.47

Layer A: 0-3 cm; layer B: 3-6 cm; layer R: 6-15 cm. Cells were enumerated with the MPN technique and activities were determined by potential ammonium-oxidizing activity measurements.

The transportation of medium containing ammonium towards the nitrifying bacteria mentioned above, might have played role in this.

As mentioned above, the heterotrophic *A. globiformis* won the competition for available ammonium, in agreement with similar soil column experiments without flagellates (29). This was concluded from the strong decreases in the potential ammonium-oxidizing activities (Fig.3A). At 12 mM glucose, the potential activities of *N. europaea* in the different soil layers had decreased to 10-16 % of those found at 0 mM glucose. This indicates that the heterotrophic bacteria almost completely outcompeted the nitrifying bacteria at high glucose concentrations. The competition for ammonium between nitrifying and heterotrophic bacteria in soil columns takes place as long as there is a build up of a heterotrophic population during the percolation period (28). Also in other studies heterotrophic bacteria were found to be more competitive for ammonium than nitrifying bacteria (17,21,22,28). An alternative explanation for the results presented here is that the nitrification process was inhibited by glucose or organic compounds originating from the heterotrophic bacteria or flagellates. However, these inhibitory

effects can be excluded here, since in separate experiments, it was found that neither glucose or supernatant of a culture of *A. globiformis* containing products of glucose metabolism, affected the ammonium oxidation rate by *N. europaea* (28). Addition of supernatant of a culture of flagellates to a batch culture of actively nitrifying bacteria, which contained a surplus of ammonium, was found not to inhibit nitrification.

As mentioned above, comparable numbers of ammonium oxidizers were found in the soil columns in the presence and absence of flagellates at all glucose concentrations supplied. These results do not agree with those from similar competition experiments in continuous cultures, where the numbers of *N. europaea* in the presence of flagellates were at most only 10 % of those in the absence of protozoa at all glucose concentrations supplied. The difference between the two model systems may be explained by assuming that the growth of nitrifying bacteria appeared in so-called microniches, as was already suggested above. A part of these microniches might have been inaccessible to the flagellates, giving the nitrifying bacteria protection against predation. The role of microniches in protecting bacteria against predation in soil has previously been demonstrated by Postma et al. (20).

The numbers of flagellates corresponded well with the numbers of heterotrophic bacteria, although the majority of cells in the soil were nitrifying bacteria. For example, the ratios between the numbers of the two nitrifying species and *A. globiformis* at 2 mM glucose in layer A amounted to 231:1 in the absence of flagellates and 22:1 in their presence. This difference in ratio was due to the numbers of heterotrophic bacteria, which in the presence of protozoa were one order of magnitude higher than in their absence, whereas the numbers of nitrifying bacteria were more or less equal at similar glucose concentrations. Although extra heterotrophic cells were added to the soil columns together with the inoculum of flagellates, a reason for the higher numbers of *A. globiformis* in the presence of protozoa can not be given. Increases in bacterial numbers in the presence of bacteriophagous protozoa have been reported before (5). This result does not agree with the one obtained in similar competition experiments in continuous cultures, in which the numbers of heterotrophic and nitrifying bacteria in the presence of protozoa were one order of magnitude lower than in their absence (28,30). An explanation for this difference between the two model systems can not be given. Possibly, growth in microniches, which were partly inaccessible to the protozoa, may again have given some protection against predation or selective predation resulting in formation of smaller cells may have increased the numbers of the heterotrophic bacteria. There was no further increase in the numbers of flagellates above 8 mM glucose, in agreement with the numbers of heterotrophic bacteria. Apparently, the protozoa did not reduce the bacterial numbers below a certain

level. Again, protection from predation by growth in inaccessible microniches might have played a role in this. This may not be the only reason, as Sambanis and Frederickson (24) found that in batch as well as in homogeneous continuous cultures 10^4-10^7 viable bacteria persisted in the presence of protozoa. Insufficient numbers of ingested bacteria may restrict further growth of the flagellates.

The present experiment in soil columns gives insight into the basic relationships between nitrifying bacteria, heterotrophic bacteria and protozoa. *A. globiformis* was again more competitive for limiting amounts of available ammonium than *N. europaea*. At low glucose concentrations, the nitrifying bacteria benefit from the nitrogen mineralized by the flagellates and consequently small increases in the nitrate concentrations of the percolates were observed. Such experiments are indispensable to understand the relationships between the functional groups in more complicated systems, such as natural soils covered by vegetation. The results obtained in a model system with plants will be presented in a subsequent paper.

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CHAPTER 7

COMPETITION FOR AMMONIUM BETWEEN NITRIFYING BACTERIA AND PLANT ROOTS IN SOIL IN POTS; EFFECTS OF GRAZING BY FLAGELLATES AND FERTILIZATION.

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SUMMARY

Grazing by protozoa and fertilization with ammonium has been thought to favour the nitrification process in soils, in which nitrifying bacteria have to compete with plant roots for the available ammonium. Considering this, the influence of grazing by the soil flagellate *Adriamonas peritocrescens* and of NH_4^+fertilization on the competition for ammonium between the chemolithotrophic species *Nitrosomonas europaea* and roots of *Plantago lanceolata* was studied in pots with γ-sterilized soil in the presence of *Nitrobacter winogradskyi*.

N. europaea was the weaker competitor in this study. In all planted pots, no significant effects of grazing and fertilization on the nitrification process were found, as roots of *P. lanceolata* were more competitive for ammonium than *N. europaea*. The potential nitrifying activities and the mineral nitrogen concentrations in the presence of a plant were almost zero, whereas the numbers of nitrifying bacteria were 200 times lower in the presence of plants than without them. The involvement of allelochemicals originating from plant roots is discussed and appeared to be unlikely. In the unplanted pots, higher amounts of nitrate and higher potential ammonium-oxidizing activities were found in the presence of flagellates and NH_4^+fertilizer than in their absence, although ammonium was present in all unplanted pots. A combination of the two factors favoured the nitrification process even more. Numbers of *N. europaea* and *N. winogradskyi* were not significantly different under the four treatments without plants. However, those of the former species agreed well with the observed potential ammonium-oxidizing activities. The mechanism behind the stimulation of the nitrification process and the availability of ammonium in the pots to the plant roots and the nitrifying bacteria is discussed.

It is concluded that roots of *P. lanceolata* are more competitive for ammonium than *N. europaea* cells. Grazing by flagellates and NH_4^+fertilization favour the nitrification process only in the absence of plant roots, whereas a combination of the two treatments had an additive effect.

INTRODUCTION

In the rhizosphere, plant roots, nitrifying and heterotrophic bacteria compete for ammonium that becomes available from the mineralization-immobilization turnover in the

soil or from fertilizer. In unfertilized grassland soils, numbers and activities of nitrifying bacteria are low and plants do not contain nitrate or the inducible enzyme nitrate reductase (3,11,20,21,30,31). Three theories were advanced to explain this suppression of the nitrification process in the rhizosphere of plants. The first theory is related to differences in affinity for ammonium of the competing organisms, as was reported by Rosswall (33). However, it may be argued that the Km values obtained in homogeneously mixed culture solutions, do not apply to the soil. In the second theory, the exudation of toxic compounds by the plant roots was suggested (2,22,23,29). These allelochemicals such as phenols and terpenes would be produced particularly by species from climax vegetations. In laboratory studies, it has been shown that these compounds indeed may exert a inhibiting effect on the nitrification process. However, under field conditions, the accumulation of these compounds to levels toxic for the nitrifying organisms has not been conclusively demonstrated. In addition, ammonium oxidation by chemolithotrophic bacteria was not significantly retarded by the addition of phenolic acids even at concentrations that greatly exceeded their concentrations in soil (19). The third theory is related to the spatial separation of the NH_4^+ source and the competing organisms (28). Plant roots have the ability to exploit the soil throughout by the formation of new roots, whereas the nitrifying bacteria are more or less immobile by adsorption to soil particles and subsequent growth in micro-colonies.

Grazing by protozoa may have a stimulating effect on the nitrification process. Some authors ascribed this to an increased mineralization of immobilized nitrogen (6,7,9,12,37,45), whereas Griffiths (13) reported this to be due to an unknown growth factor in liquid cultures. Verhagen et al. (41,43) suggested that also the transportation of ammonium to the nitrifying bacteria, which are possibly growing in micro-colonies, by the movement of the flagellates may play a role.

To obtain more insight into the competition for ammonium between plant roots, heterotrophic and nitrifying bacteria, and the effects of grazing by protozoa, a series of model experiments of increasing complexity was carried out involving representative species of each functional group. In continuous culture experiments the competition for limiting amounts of ammonium between *N. europaea* (in the presence of *N. winogradskyi)* and *Arthorbacter globiformis* was studied in the presence and absence of a flagellate species, both latter species isolated from the rhizosphere of *P. lanceolata* (40,42). In both experiments, *A. globiformis* was found to be the more competitive organism. Grazing by the flagellate resulted in a higher nitrate production per nitrifying cell. In similar experiments with continuously percolated, non-saturated soil columns, the heterotrophic bacteria were also more competitive for ammonium than the nitrifying

bacteria and the activity of the ammonium-oxidizing bacteria was stimulated in the presence of flagellates (41,43).

In the still more complex experiment described here, the competition for ammonium between *N. europaea* (together with *N. winogradskyi*) and plant roots was studied in pots with sterilized soil, planted with axenic Ribwort plantain (*P. lanceolata*). The effects of ammonium fertilization and of grazing by the soil flagellate *A. peritocrescens* were also investigated. Data on the effect of *A. globiformis*, which was also added, are not presented here, since inconsistent results were obtained. These were either due to a low recovery of cells in the FA enumerations or to failure of the heterotrophic bacterium to establish itself in the soil and rhizosphere under the experimental conditions.

MATERIALS AND METHODS

Soil sampling.

Soil was collected from the top 0-30 cm layer of a pasture near Brummen (52°05' N, 06°09' E) in the Netherlands. The soil was sieved (2 mm) and well mixed. The soil contains 21.3 % coarse sand, 69.3 % fine sand, 4.1 % silt, and 5.3 % clay. The pH-H_2O of the sieved mixed soil was 7.8, the pH-KCl was 7.3, the content of calcium carbonate was 1.6 % and of organic matter 5.0 %. Soil was stored before use at 4°C for 1 week.

Organisms and culture conditions.

The experiments were conducted using *N. europaea* ATCC 19718 and *N. winogradskyi* ATCC 25391 as nitrifying bacteria. They probably belong to the dominant nitrifying bacteria in the grassland soil described above (40). The culturing of a mixed nitrifying population in batch cultures before inoculation in the pots was identical as has been described previously (41).

A. globiformis was used as the heterotrophic bacterium. It was isolated from the rhizosphere of Ribwort plantain (*Plantago lancolata*) growing in a pot containing the soil described above. The isolation procedure and culturing of the heterotrophic bacterium have been described previously (40).

The flagellate *A. peritocrescens*, that was also isolated from the grassland soil described above, was used as the bacterial grazer. It was enriched in a soil suspension on heterotrophic bacteria growing on 0.02 % (w/v) proteose peptone, isolated by a dilution method, and grown monoxenically using *A. globiformis* as a prey organism.

Ribwort plantain (*P. lanceolata*) was used as the plant in this study. It was chosen because much information on its nitrogen uptake, metabolism, and habitat characteristics was available. To obtain sterile seedlings, 1 year-old seeds of *P. lanceolata* (hayfield-type) were treated with a 1 % (w/v) sodium hypochlorite solution for 10 minutes, after they were scoured with 96 % (v/v) ethanol for 1 minute. They were thoroughly rinsed with sterile water and, to check for bacterial infections, placed on an agar medium, containing (per litre): "Lab-Lemco" broth (Oxoid), 1.0 g; yeast extract (Difco), 2.0 g; tryptose (Oxoid), 5.0 g; NaCl, 5.0 g and glucose.H_2O, 1.0 g. The final pH of the agar medium was 7.5. Seeds were turned after 2 days and, to check for fungal infections, they were transferred after another 2 days to a malt agar medium containing (per litre): malt extract (Oxoid), 8.0 g and mycological peptone (Oxoid), 3.0 g. Seeds were turned after 2 days and non-infected seeds were transferred after another 2 days to water agar in order to germinate. Test plates for bacterial and fungal infections were incubated for another 2 weeks at 20°C to confirm sterility.

Competition experiments.

Black PVC pots with soil were incubated under 8 different treatments: —, G, F, GF, P, PG, PF and PGF in which G= grazing by flagellates, F= fertilization and P= plant (*P. lanceolata*). Each treatment was conducted in five-fold. The effects of grazing, fertilization and presence of a plant were studied by analyzing the soil compartment at the end of the experiment and by determining weights and composition of the plant material.

Each pot was filled with 610 g of moist soil. The soil moisture content (w/w) was 24 % (60 % WHC). Water was supplied by means of a PVC tube with lengthwise incisions, at both ends closed by a stopper and filled with fine gravel, which was inserted into the soil to facilitate an even distribution of water and nutrient solution. At the upper side, the soil was covered by a layer of small polyethylene pellets ("Stamylan", DSM, Geleen, the Netherlands) and aluminium foil folded double. After the pots with soil were sterilized by γ-ray irradiation (4.5 Mrad), half the pots were planted with one of the sterile seedlings. Pots were incubated in a growth cabinet with a day/night adjustment of 20/15° C for 14/10 hours per day. The light intensity was 236 $\mu E.s^{-1}.m^{-2}$ and the

humidity was 70 % of air saturation. After two weeks, all pots were inoculated by means of a syringe with 1 ml of a concentrated mixture of nitrifying and heterotrophic bacteria (pH 7.5) containing 5 x 10^7 ammonium-oxidizing, 2.6 x 10^7 nitrite-oxidizing and 2.5 x 10^9 heterotrophic bacteria per ml, *i.e.* 1.1 x 10^5, 5.6 x 10^4 and 5.4 x 10^6 cells per g of dry soil, respectively. Then, the pots were watered till the moisture content was about 30 % in order to ensure an even distribution of bacteria throughout the soil. One week after inoculation, half the planted and unplanted pots were inoculated with 2 ml of an active culture of flagellates, grown monoxenically on *A. globiformis* and containing 1 x 10^6 flagellates per ml, *i.e.* 4.3 x 10^3 per g of dry soil. Again the pots were given extra water. Half the planted and unplanted pots both with and without grazers were fertilized weekly. In the first week, fertilization was (mg per pot): $(NH_4)H_2PO_4$, 2.66; $(NH_4)_2SO_4$, 5.35; KCl, 5.18 and $MgSO_4.7H_2O$, 2.85. Amounts of nutrients were increased weekly by a factor of 1.37. In total 320 mg N was given to each fertilized pot. Fertilization of the unplanted pots was done by addition of a 5-fold concentrated nutrient solution in order to prevent high soil moisture contents. Pots were watered daily with sterile demineralized water. A trace element solution (1.0 ml), with a composition as described before (40), was added to 1 litre of water every two weeks in order to prevent exhaustion of minerals in the soil.

The pots were harvested after 14 weeks. When a plant was harvested, the soil was removed from the roots and fresh and dry weights of roots and shoots were determined. In addition, total and mineral nitrogen concentrations in the dried ground plant material were measured. Soil from a pot was mixed well. Numbers of nitrifying bacteria and of flagellates were determined by MPN counts. In addition, the potential ammonium- and nitrite-oxidizing activities were determined by incubation of soil suspensions under optimal conditions. Numbers of *A. globiformis* were established using specific antibody fluorescence microscopy. Soil mineral nitrogen concentrations, pH, and soil organic matter contents were determined in 1 M KCl-, in water-suspensions and by heating, respectively.

Most Probable Number enumerations (8,34).

The enumerations of nitrifying bacteria, the preparation of the soil suspensions, the dilution procedure and the compositions of the media have been described previously (41). Twelve replicates were made per dilution. At the end of an incubation period of 9 weeks, tests for ammonium and nitrite oxidation were performed using 0.04 % (w/v) bromo cresol purple (pH indicator) and the Griess Ilosvay reagents, respectively. Two

suspensions per pot were made, which were counted separately.
For the enumerations of flagellates, *A. globiformis* was used as the food bacterium. The preparation of the soil suspensions, the dilution procedure and the culturing of the prey organism have been described before (43). Twelve replicates were made per dilution. After incubation for 12 days at 20°C, wells were tested for flagellate growth using an inverted microscope. Numbers of nitrifying bacteria and flagellates were obtained from statistical tables that were generated by a computer program (27).

Fluorescent Antibody enumerations (35,44).

The extraction of the heterotrophic bacteria from the soil by stirring a soil suspension with chelating resin, cholic acid, and glass beads and the staining procedure for the bacteria on black polycarbonate membrane filters (Nuclepore Corp., Pleasanton, USA) with a pore size of 0.2 μm (14) using antiserum prepared from blood from an immunized rabbit, have been described before (41).

Potential Nitrifying Activities (36).

The medium for the determinations contained (per litre): $(NH_4)_2SO_4$, 132 mg; K_2HPO_4, 139 mg and KH_2PO_4, 27 mg, pH 7.5. No inhibitors were applied as sodiumchlorate was found to inhibit ammonium oxidation partly and N-serve did not completely inhibit ammonium oxidation (4).

Samples (40.0 g) of well-mixed soil were supplemented with 0.2 g $CaCO_3$ and 100 ml medium described above. The mixtures were incubated at 25°C and stirred at 150 rev per min. Formation of nitrite and nitrate was followed for 6 hours. Samples were taken every hour to determine nitrite and nitrate concentrations. After a sample was centrifuged at 15,000 x g in a Biofuge A table centrifuge for 5 minutes, 0.50 ml of the clear solution was mixed with 0.50 ml 2 M KCl solution. Samples were stored at 4°C and analyzed within 1 day. The potential nitrifying activities were determined in duplicate. Potential ammonium-oxidizing activities were calculated from the sum of the nitrite and nitrate formed. Potential nitrite-oxidizing activities were calculated from the nitrate formed and this could only be done when accumulation of nitrite appeared.

Soil mineral nitrogen, pH and soil organic carbon.

Soil mineral nitrogen concentrations were determined by stirring 10.0 g of moist

soil with 50 ml 1 M KCl solution at 100 rev per min for 4 hours.
The pH-H_2O of the soil was determined after 10.0 g of moist soil was stirred with 25 ml demineralized water at 100 rev per min for 2 hours.
The soil organic carbon content was determined from the weight-loss after heating a sample of 5.0 g of dry soil at 550°C for 4 hours.

Analytical Methods.

Mineral nitrogen concentrations in 1 M KCl extracts were determined using a Technicon Traacs 800 autoanalyzer (Technicon Instr. Corp., New York, USA).
Total and free nitrogen contents of the dried ground plant material were determined according to Novozamsky (25,26).
Organic nitrogen contents were estimated by subtracting free NO_3^- and NH_4^+ from the total nitrogen content.

Statistics.

The results were analyzed by analysis of variance over the three experimental factors, *i.e.* presence of a plant, presence of protozoa, and fertilization.
Differences between the treatment means were tested at the 5 per cent level using Tukey's test.

RESULTS

Mineral nitrogen concentrations.

The mean ammonium, nitrite, and nitrate concentrations are given in Table 1. In all pots without plants, ammonium was present. Considerable effects of grazing on the amounts of ammonium and nitrate were observed, irrespective whether fertilization was applied or not. However, the total amounts of mineral nitrogen were not affected by grazing. The amounts of nitrate in the fertilized pots were higher than in the unfertilized ones.
In all pots with plants, hardly any mineral nitrogen was detected and thus, no significant effects of grazing or fertilization could be observed.

Table 1. Mineral nitrogen concentrations in pots with sterilized soil containing *N. europaea* and *N. winogradskyi*, after incubation for 15 weeks at 20/15°C for 14/10 h per day. The soil moisture content was 24 % (60 % WHC) and the light intensity 236 $\mu E\ s^{-1}\ m^{-2}$.

Treatment	NH_4^+ ($\mu g\ N\ g^{-1}$)	NO_2^- ($\mu g\ N\ g^{-1}$)	NO_3^- ($\mu g\ N\ g^{-1}$)	Total mineral N ($\mu g\ N\ g^{-1}$)
—	75.6c*	0.3a	56.3b	132.2b
G	48.6b	0.7a	75.9c	125.2b
F	366.5e	0.3a	85.4d	452.2c
GF	311.5d	0.6a	142.8e	454.9c
P	0.8a	0	0.3a	1.1a
PG	0.7a	0	0.2a	0.9a
PF	1.4a	0	0.3a	1.7a
PGF	1.0a	0	0.3a	1.3a

G= grazers (flagellates); F= fertilizer (NH_4^+); P= plant (*P. lanceolata*).
Concentrations are expressed in μg N per gram dry soil.
Values are means of 5 replicates.
* Values within each column followed by a different letter are significantly different ($P < 0.05$) according to Tukey's test.

Numbers and potential ammonium-oxidizing activities of *N. europaea*.

Relatively high MPN-numbers of *N. europaea* were found in the unplanted pots, but due to the inaccuracy of the MPN method, the differences between the four treatments were not significant (Table 2). Nevertheless, the results suggest that the numbers of ammonium-oxidizing bacteria increased as a consequence of the addition of flagellates and fertilizer. The potential ammonium-oxidizing activities in the unplanted pots increased in the same sequence as did the numbers. Due to a higher accuracy of the latter method, these differences, which were of the same order of magnitude as those between the MPN-numbers, were significant.

The numbers of *N. europaea* in the planted pots were 150-280 times lower than those in the unplanted ones. Again, differences between the four treatments were not significant. The potential ammonium-oxidizing activities in the planted pots were not significantly different under the four treatments and they were almost zero, although still about 1×10^6 ammonium-oxidizing bacteria per gram dry soil were present.

Table 2. Numbers and potential ammonium-oxidizing activities of *N. europaea* added to pots with sterilized soil, in the presence of *N. winogradskyi* after incubation for 15 weeks at 20/15°C for 14/10 h per day. The soil moisture content was 24 % (60 % WHC) and the light intensity 236 μE s^{-1} m^{-2}.

Treatment	Numbers of *N. europaea* (x 10^6 g^{-1} dry soil)	Activities of *N. europaea* (μmol h^{-1} g^{-1} dry soil)
—	88b*	132b
G	175b	189c
F	249b	180c
GF	334b	325d
P	6.2a	3.3a
PG	1.0a	0.8a
PF	0.9a	0.4a
PGF	1.5a	0

G= grazers (flagellates); F= fertilizer (NH_4^+); P= plant (*P. lanceolata*).
Numbers of *N. europaea* were determined by Most Probable Number (MPN) counts.
Values are means of 5 replicates.
* Values within each column followed by a different letter are significantly different ($P < 0.05$) according to Tukey's test.

Numbers and potential nitrite-oxidizing activities of *N. winogradskyi*.

In the unplanted soil, the MPN-numbers of *N. winogradskyi* were relatively high and they were 200-1400 times higher than in the planted soil (Table 3). Differences between treatments again were not significant. The differences between the potential nitrite-oxidizing activities in the unplanted pots increased significantly in the same sequence as the ammonium-oxidizing activities. Significant effects of the addition of flagellates and of fertilizer were observed, and the combination of these two factors showed a strong positive interaction.

In the planted pots, again no significant effects of fertilization and addition of flagellates were observed. No potential activities could be established as no nitrite accumulation occurred during the determination of the potential nitrifying activities (see Methods).

Table 3. Numbers and potential nitrite-oxidizing activities of *N. winogradskyi* added to pots with sterilized soil, in the presence of *N. europaea* after incubation for 15 weeks at 20/15°C for 20/15 h per day. The soil moisture content was 24 % (60 % WHC) and the light intensity 236 μE s^{-1} m^{-2}.

Treatment	Numbers of *N. winogradskyi* (x 10^6 g^{-1} dry soil)	Activities of *N. winogradskyi* (μmol h^{-1} g^{-1} dry soil)
—	422b*	99a
G	678b	140b
F	485b	150b
GF	896b	282c
P	2.6a	n.d.
PG	0.9a	n.d.
PF	2.5a	n.d.
PGF	4.4a	n.d.

G= grazers (flagellates); F= fertilizer (NH_4^+); P= plant (*P. lanceolata*); n.d. = not determined. Numbers of *N. winogradskyi* were determined by Most Probable Number (MPN) counts. Values are means of 5 replicates.

* Values within each column followed by a different letter are significantly different ($P < 0.05$) according to Tukey's test.

Numbers of *A. globiformis*.

In all pots, the FA-numbers of heterotrophic bacteria were relatively low (data not shown). They were not significantly different under the treatments and ranged from 0.4 to 1.0 x 10^6 cells per gram dry soil, except in the unplanted fertilized pots with flagellates (treatment GF), in which the number was significantly higher and amounted to 3.8 x 10^6 cells per gram dry soil.

Numbers of *A. peritocrescens*.

The MPN-numbers of flagellates in the pots without plants and in the unfertilized pots with plants were similar and amounted to about 1 x 10^5 organisms per gram dry soil,

Table 4. Numbers of the flagellate *A. peritocrescens* added to pots with sterilized soil, in the presence of *N. europaea* and *N. winogradskyi* after incubation for 15 weeks at 20/15°C for 14/10 h per day. The soil moisture content was 24 % (60 % WHC) and the light intensity 236 $\mu E\ s^{-1}\ m^{-2}$.

Treatment	Numbers of flagellates (x 10^5 g^{-1} dry soil)	
	unplanted soil	planted soil
—	—	—
G	1.1b*	0.9b
F	—	—
GF	1.0b	0.1a

G= grazers (flagellates); F= fertilizer (NH_4^+).
Numbers of flagellates were determined by Most Probable Number (MPN) counts.
Values are means of 5 replicates.
* Values within the table followed by a different letter are significantly different ($P<0.05$) according to Tukey's test.

which means that they had proliferated in the soil (Table 4). Only a combination of plant and fertilization had a significant, negative effect on the numbers of flagellates. In this treatment, only 14,000 protozoa per gram dry soil were present.

Weights and nitrogen contents of *P. lanceolata*.

Only fertilization had a significant positive effect on the fresh and dry weights of the shoots of *P. lanceolata* (data not shown). No significant effects on the total and organic nitrogen contents of the roots and shoots of the plants under the four treatments were observed.

pH and soil organic matter contents.

In all pots, the pH was significantly lowered by fertilization (data not shown). In the planted pots, this was probably due to uptake of NH_4^+ by the plant roots, whereas in the unplanted pots this was probably due to nitrification. No significant effects of grazing on pH were detected. No significant differences in the soil organic matter contents under the eight treatments were observed.

DISCUSSION

The present experiments, carried out to investigate the competition for ammonium between nitrifying and heterotrophic bacteria and plant roots, are the most complex stage in a series of model experiments, in which this competition was studied in continuous cultures (40,42) and in soil columns (41,43). The competition for ammonium between plant roots, nitrifying and heterotrophic bacteria has been previously studied by others, but their results were not conclusive (16,32,33,46). Jansson (16) considered higher plants as weak competitors for ammonium and placed their ability compared with other organisms in the following order: heterotrophic microflora > nitrifying bacteria > higher plants. He based his finding, that plant roots were the weakest competitors for ammonium, on planted pot experiments, in which excess organic carbon completely immobilized the mineral nitrogen. Rosswall (33) on the other hand based his findings on the measured Km values of 14, 20-271, and 571-1286 μM N for ammonium uptake by heterotrophic bacteria, plant roots, and nitrifying bacteria, respectively, and concluded that nitrifying bacteria were the weakest competitors for ammonium. Jansson (16) did not take into account that plant roots are known to interfere with the nitrogen mineralization-immobilization turnover by the heterotrophic bacteria in soil. It can be questioned whether the Km-values obtained by Rosswall in homogeneously mixed culture solutions apply to the soil, where diffusion and mass flow are the operating forces. Differences in critical concentrations, below which no substrate is taken up, are possibly more important. The divergent opinions certainly have to do with the heterogeneity of soil ecosystems, where micro-sites with a shortage of nitrogen alternate with micro-sites that have an excess of nitrogen. For that reason, in the present study the competition for ammonium was investigated in model experiments under more standardized conditions.

In the experiments, pure cultures of *N. europaea*, *N. winogradskyi*, *A. globiformis* and a flagellate species growing monoxenically on the *Arthrobacter* strain, were added to axenic plants grown in γ-sterilized soil. At the end of the experiments, the pots were controlled for infections. Apart form the added strains, no other protozoa were found and heterotrophic bacteria other than *A. globiformis* were low in numbers. However, in all pots the yields of the FA-counts of *A. globiformis*, that in continuous culture studies gave the same numbers as direct microscopical counts, were unexpectedly low and did not exceed the number of added cells. They remained two orders of magnitude below those of both nitrifying strains. This could have been due to failure of *A. globiformis* to

establish itself in the bulk soil and rhizosphere under the experimental conditions. This is not very likely, as the species is a well-known soil organism. An alternative explanation could be that the numbers of heterotrophic bacteria have been underestimated by insufficient extraction of the bacteria from the soil particles or by a low recovery of the soil-grown cells in the staining procedure of the FA enumerations. For that reason, the competition for ammonium between *A. globiformis*, plant roots and nitrifying bacteria is not considered here. Here, the discussion will be focused on the effects of flagellates on nitrification and on the competition for ammonium between nitrifying bacteria and plant roots.

Effects of flagellates.

In the unplanted pots, higher amounts of nitrate and higher potential ammonium- and nitrite-oxidizing activities were found in the presence of flagellates and fertilizer than in their absence, whereas a combination of the two factors gave an additive effect (Tables 1,2,3). Numbers of *N. europaea* and *N. winogradskyi* were not significantly different in the unplanted pots, but those of the former species agreed well with its potential ammonium-oxidizing activities. The effect of the flagellate here can not be ascribed to an enhanced mineralization of immobilized nitrogen by the grazing activities of protozoa as has often been reported (1,5,15,17,18). In the present experiment no effect of the flagellate on the total amounts of mineral nitrogen was observed, *i.e.* no additional mineralization took place. Moreover, ammonium was still present in the 1 M KCl-extracts of the soil under all treatments. Nevertheless, nitrification was higher in the fertilized soil. It can be questioned, however, whether the ammonium fraction was completely available to the nitrifying bacteria. Jansson (16) found that a part of the ammonium apparently not available to the nitrifying population, could be extracted with a 1 M KCl solution. Smit and Woldendorp (38) found no correlation between the quantities of ammonium and numbers of nitrifying bacteria in the rhizosphere of *P. lanceolata* plants. They ascribed this to the fact that at micro-spots where nitrifying bacteria had developed, the ammonium ions would have been exhausted, whereas at other spots ammonium would have accumulated by mineralization, without nitrifying bacteria being present. Mobile protozoa may aid in distributing ammonium or nitrifying bacteria through the soil, as was suggested before by Verhagen et al (43). Griffiths (13) reported that in liquid cultures nitrification increased in the presence of bacteriophagous protozoa. However, increasing the concentration of NH_4^+ from 1 to 10 μg N l^{-1} in the absence of protozoa did not increase nitrification, indicating that the enhanced nitrification was due

to an unknown effect of protozoa rather than the excretion of NH_4^+. Production of some organic compound by the protozoa was suggested as a mechanism for enhancing nitrifying activity (13). Finally, in addition to an increased NH_4^+ availability, higher nitrate production rates per nitrifying cell were found in continuous culture and soil column experiments when flagellates were present compared to when they were absent (40,41,42,43). In the continuous culture experiments, the numbers of nitrifying bacteria were reduced one order of magnitude by the flagellates (40,42), but in the soil column and the present pot experiments, where the specific activities of the nitrifying cells were likewise stimulated, the numbers did not change, possibly due to protection against predation by growth in microniches. Therefore, it is concluded that the effects of the grazing flagellate on the nitrifying bacteria are complex. At one hand there are negative effects such as predation, that may, however, be compensated by increasing the amounts of available ammonium per cell. On the other hand, there are positive effects, such as a better distribution of ammonium or nitrifying bacteria through the soil and a higher rate of ammonium oxidation in the presence of protozoa.

Competition for ammonium between nitrifying bacteria and plant roots.

From the data in Tables 2 and 3 it appeared that the numbers and activities of *N. europaea* and *N. winogradskyi* were considerably reduced in the presence of a plant. As the maximum relative growth rate of *P. lanceolata* is 1.85 per week (10) and the exponentially increased ammonium addition rate was only 1.37 per week, the plants grew sub-maximal and from the absence of mineral nitrogen compounds in all planted pots (Table 1), it could be deduced that the plants were probably ammonium limited during the growth period. Although it is evident that *N. europaea* lost the competition for limiting amounts of ammonium, the actual mechanism is unknown. One possible explanation is that the inhibition of the nitrification process is due to allelochemicals originating from the roots of *P. lanceolata*. However, involvement of allelochemicals is not very likely, since the following three facts may be argued against it. 1. In the MPN-counts of nitrifying bacteria no inhibition of ammonium oxidation was found in the least-diluted soil suspensions. 2. In axenic non-ammonium limited model systems of *P. lanceolata* with mixed cultures of *N. europaea*, two *Nitrobacter* species and a *Pseudomonas* species, which enabled the examination of the spatial distribution around plant roots on a microscopic scale, a positive interaction of *N. europaea* with the plant roots was found (4). The stimulation of *N. europaea* in the rhizosphere was probably due to the presence of the ammonifying *Pseudomonas* species. 3. Newman (24) extracted

carbon compounds from *P. lanceolata*, which were toxic to germinating seeds but upon repeated addition of these extracts to the soil, this effect disappeared completely. Apparently, the appropriate microorganisms, which rapidly decomposed the toxic compounds, had been enriched. It has been reported that under field conditions excess ammonium is nitrified in the rhizosphere of *P. lanceolata* (39). This indicates that accumulation of allelochemicals will not occur in natural soils. Under uniform environmental conditions, a close relationship was observed between the numbers of nitrifying bacteria in the rhizosphere and the inducible enzyme nitrate reductase in the shoots of *P. lanceolata* (38). However, in the present pot experiment the heterotrophic bacterium did not colonize the rhizosphere and bulk soil, which opens the possibility of accumulation of organic compounds originating from plant roots.

In other situations and with other plant species the suppression of nitrification has often been reported (2,22,23,29). The results are not always convincing as they are based on experiments, in which plant extracts were added only once to the soil and not repeatedly as was done in the experiments of Newman (24), whereas the accumulation of toxic compounds in the soil to toxic levels was not demonstrated. Moreover, conclusive experiments on inhibition of nitrification by plants have to be carried out with non-limiting amounts of ammonium in order to eliminate competition by mechanisms other than allelochemicals.

The series of model experiments in continuous cultures, soil columns and pots with plants clearly showed that *N. europaea* in the competition for ammonium with *A. globiformis* and *P. lanceolata* is the weaker one. This result is in line with the function of nitrate as a sink for surplus of nitrogen. At the moment, we are not able to suggest a physiological mechanism to explain this weak competitive ability of nitrifying bacteria.

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CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

COMPETITION FOR AMMONIUM

In all model systems in this study, *Nitrosomonas europaea* finally lost the competition for ammonium from the heterotrophic species *Arthrobacter globiformis* as well as from roots of *Plantago lanceolata*. In case of competition between nitrifying and heterotrophic bacteria, the numbers of nitrifying bacteria strongly decreased as the glucose concentrations increased but they were not zero at the critical glucose concentration (chemostats), or numbers of nitrifying bacteria were almost constant when glucose concentrations increased but the potential ammonium-oxidizing activities strongly decreased (soil columns). Based on this observations, it is concluded that the actual mechanism of competition is mechanism III (see Chapter I; General Introduction). Apparently, *A. globiformis* had a higher specific affinity (Vmax/Km) for ammonium than *N. europaea* in the chemostats, but *N. europaea* cells could maintain themselves in the system by growth on the wall of the culture vessels. In comparing the numbers, nitrate productions and potential ammonium-oxidizing activities of *N. europaea* in the chemostats and the soil columns, it is concluded that no correlation exists between the measured numbers of nitrifying bacteria and their potential nitrifying activities, as was found before by other authors (2,3,4).

Some differences between the chemostats and soil columns were found. Firstly, in the chemostats all ammonium available for nitrification was converted into nitrate by *N. europaea* and *N. winogradskyi*, but in the soil columns nitrification was incomplete and a remarkable accumulation of ammonium occurred mainly in the upper layer (0-3 cm), probably due to a spatial separation between immobile (microcolonies of) *N. europaea* cells and their substrate ammonium. These high ammonium concentrations in the upper layer probably were toxic to the ammonium-oxidizing bacteria, which resulted in the highest potential ammonium-oxidizing activities in the underlying layer (3-6 cm). Ammonium was also found in the deeper layers of the soil columns, but in lower concentrations than in the upper layer. Secondly, in the chemostat experiments steady state situations were achieved, but in the soil column experiments these could not be reached. In the theoretical steady state situation of a soil column, glucose and ammonium are completely used for maintenance by the heterotrophic and nitrifying populations, respectively. To examine this idea, the soil column experiment should be repeated, but a time series of harvesting the soil columns should be included.

In case of competition for ammonium between *N. europaea* and roots of *P. lanceo-*

lata in the pot experiment containing γ-sterilized soil and pure cultures of microorganisms and axenic plants, the roots took up all ammonium present and the numbers of *N. europaea* cells were about two orders of magnitude lower than those in the pots without plants. The actual mechanism of competition could not be established but the higher competitive ability of *P. lanceolata* can be due to a better capacity of exploiting the soil with its growing roots, especially when growing in pots, as compared to immobile (microcolonies of) nitrifying bacteria. In line with the results of the soil column experiments, nitrification in the soil in the pots was incomplete, which was probably due to a spatial separation of immobile bacteria and their substrate. The competitive order between roots of *P. lanceolata* and the heterotrophic species *A. globiformis* could not be established in this experiment, as this common soil bacterial species did not establish in the γ-sterilized soil in pots for some unknown reason, even not in the fertilized pots without plants. In further research, attention should be paid to the reason for this phenomenon.

The involvement of allelochemicals in the pot experiment is unlikely, since 1) ammonium oxidation was not inhibited in the least-diluted soil suspensions of the MPN-enumerations of nitrifying bacteria, 2) a positive interaction of *N. europaea* with roots of *P. lanceolata* was found in axenic non-ammonium limited model systems of *P. lanceolata* with mixed cultures of *N. europaea*, two *Nitrobacter* species and a *Pseudomonas* species (5), and 3) a close relationship was observed between the MPN-numbers of nitrifying bacteria in the rhizosphere and the inducible enzyme nitrate reductase in the shoots of *P. lanceolata* (19).

In conclusion, the nitrification process in the rhizosphere of natural grasslands, with its high concentrations of carbonaceous compounds, can indeed be inhibited by 1) the immobilization process by heterotrophic bacteria under conditions of limitation by available ammonium, and 2) probably also by uptake of ammonium by roots of grassland plants. However, the repression of the nitrification process depends among other factors on the amount of available nitrogen and carbon present in soil and on the size, composition and spatial distribution of the bacterial communities. Nitrification is more probably to occur in highly fertilized grassland soils, in which the C/N ratio is lowered by the addition of nitrogen, than in natural grassland soils with its general excess of carbonaceous compounds. However, in actively nitrifying soils, nitrification can be incomplete and ammonium may be found, due to the spatial separation of (microcolonies of) nitrifying bacteria and their substrate. In contrast to the soil column system, the mixing of bacteria and ammonium in normal soils might be better, for example by tillage of arable soils.

EFFECTS OF PROTOZOA

The addition of flagellates to the bacterial communities competing for ammonium in the model systems was expected to exert a positive effect on the nitrification process due to the increased mineralization of organic nitrogen. This would result in an increased nitrate production, higher numbers of nitrifying bacteria and higher critical glucose concentrations in the chemostats experiments as compared to identical experiments without protozoa. Shortly, it would lead to a higher competitive ability of the nitrifying bacteria. However, the mineralizing effects by the protozoa appeared to be surprisingly small in the chemostat experiments, since it was found that the critical glucose concentrations and nitrate productions in the presence of flagellates were not significantly different from those in the absence of protozoa. The numbers of nitrifying bacteria in the presence of flagellates were even much lower in stead of higher than in the absence of protozoa. Consequently, the production of nitrate per nitrifying cell must have increased in the presence of protozoa, possibly by protozoan excretion of an organic compound stimulating the nitrifying activity (10).

Also in the soil column experiments, the N-mineralizing effects on nitrification were neglectible but in contrast to the chemostat experiments, the addition of protozoa had no effect on the numbers of nitrifying bacteria in the soil columns, possibly by protection of nitrifying bacteria in micro-niches of the soil. Also the nitrate concentrations in the effluents were almost equal in the presence and absence of flagellates. The potential ammonium-oxidizing activities, however, strongly increased in the presence of flagellates. In line with the chemostat experiments, this again could be due to excretion of a growth factor by the flagellates, but in the soil columns also to a better distribution through the soil of medium containing ammonium for the nitrifying bacteria. In the grazed columns, the rate of ammonium accumulation in the upper layer was lower than in the columns without flagellates and the ammonium was more evenly distributed over the soil layers.

In line with the chemostat and soil column systems, the flagellates had no effects on the mineralization of organic nitrogen in the pot experiment, since the total amount of mineral nitrogen did not increase in the grazed soils. But, in contrast to the former two model systems, the actual nitrification rate was stimulated, as a greater part of the mineral nitrogen present in the unplanted pots was in the form of nitrate when flagellates

were present. In line with the soil columns, the potential ammonium-oxidizing activities were strongly stimulated by the grazing activities of the flagellates, possibly due to a growth factor excreted by the flagellates or to the homogenizing effect on the nitrifying bacteria and ammonium by the protozoa mentioned above. Fertilization with ammonium also had a stimulating effect on the nitrification process, whereas combination of flagellates and fertilizer had an additional effect. The latter also suggests that ammonium, although present, limits the rate of nitrification.

Future research should focus on the following subjects:

1. Attention should be paid to the reason for the absence of increased N-mineralization in the presence of protozoa, which has been found by so many authors (1,7,8,9,12,18,20). Simplified experiments involving only nitrifying bacteria and protozoa in the absence of heterotrophic bacteria should be performed in order to investigate the nett effect of protozoa on nitrifying organisms.
2. Efforts should be put into the elucidation of the actual mechanism of protozoan stimulation of the nitrification process in liquid cultures as well as in soil model systems.
3. The grazing effects on nitrifying bacteria by flagellates other than *Adriamonas peritocrescens* and by other protozoa such as amoebae and ciliates should be investigated as well as the effects of grazing by bacteriovorous nematodes, which are present in lower numbers in soil but have ten times lower growth efficiencies, and hence a larger positive effect on N-mineralization.

These future experiments are important, as the ecological consequences of the grazing activities of protozoa could lead to a higher nitrate production rate in non N-limited soils, which are found all over in the Netherlands. In these soils, the nitrification process is not lowered by immobilization of ammonium by heterotrophic bacteria or by ammonium uptake by roots.

The higher nitrate production in the presence of protozoa may substantially contribute to environmental problems such as acidification of soils and subsequent reduced viability of trees (6,13,14), toxification of ground and drinking water by leaching of nitrate in agricultural areas as well as in many acid forest soils (11,15,17), and destruction of the stratospheric ozone layer, since nitrate production is accompanied by production of nitric and nitrous oxide (16).

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SUMMARY

The absence of nitrification in natural soils can be explained in two ways:

1. Allelopathic inhibition of the nitrification process by organic compounds originating from plant material.
2. Repression of the nitrification process by immobilization of ammonium by heterotrophic bacteria or by ammonium uptake by grassland plant roots.

Both processes are dealt with in **Chapter 1**. As allelopathic inhibition of nitrification is unlikely to occur in grassland soils, this thesis deals with the second explanation: the competition for limiting amounts of ammonium between nitrifying bacteria on one side and heterotrophic bacteria or plant roots on the other side. To obtain more insight in the competition for ammonium between nitrifying and heterotrophic bacteria and plant roots, a series of model experiments of increasing complexity was performed in model systems involving representative species of each functional group. As protozoa may have a stimulating effect on the nitrification process, by increased mineralization of organic nitrogen or by excretion of growth factors (Chapter 1), these organisms were also included in the experiments. Of the bacterial species used, the chemolithotrophic ammonium-oxidizing species *Nitrosomonas europaea* and the nitrite-oxidizing species *Nitrobacter winogradskyi* are common nitrifying microorganisms in the rhizosphere of the plant species used, i.e., *Plantago lanceolata* (Ribwort plantain), whereas the heterotrophic species used, i.e., *Arthrobacter globiformis*, and the novel flagellate species used, i.e., *Adriamonas peritocrescens*, were isolated from the rhizosphere of this plant species. The experiments were carried out in the following model systems: energy-limited continuous cultures, continuously percolated soil columns, and pots with or without plants.

In **Chapter 2**, the new free-living soil flagellate species *A. peritocrescens* is described, which has been studied by light microscopy as well as by scanning and transmission electron microscopy. The cell is elongate, 8 μm long and 4 μm wide, and has two anteriorly subapically inserted subequal flagella. A mouth is situated in the antero-ventral part of the cell. It has three flagellar roots: the major and minor mouth root run posteriorly to the right under the ventral cell membrane and the third root runs anteriorly and ends in a electron dense polar strip in the antero-dorsal part of the cell,

which gives rise to a corset of microtubules lying under the dorsal surface. Mitochondria have tubular cristae. The flagellate has minimum doubling times of 2-3 hours in dense, pure cultures of *A. globiformis*. The new genus is placed *incertae sedis* in the kingdom Protista.

In **Chapter 3**, the competition for limiting amounts of ammonium between *N. europaea* and *A. globiformis* in energy-limited continuous cultures at dilution rates of 0.004 and 0.01 h^{-1} is described. *A. globiformis* won the competition for ammonium, as was concluded from the decreases in nitrate concentrations and numbers of nitrifying bacteria when glucose concentrations in the inlet medium increased. Critical carbon to nitrogen ratios, at which the heterotrophic bacteria consume all mineral nitrogen present in the system for assimilation processes, of 11.6 and 9.6 were determined for the dilution rates of 0.004 and 0.01 h^{-1}, respectively. Below these critical C/N values, coexistence of the competing species was found in steady state situations, as *A. globiformis* and *N. europaea* were limited by glucose and ammonium, respectively. Above the critical C/N values, the nitrifying bacteria were not fully outcompeted by the heterotrophic bacteria, although both species were limited by ammonium. The appearance of nitrifying bacteria above the critical C/N values was ascribed to attachment to the glass wall of the culture vessels, from which a continuous release of nitrifying cells took place. Heterotrophic growth of *N. winogradskyi* on organic compounds excreted by the heterotrophic bacteria was advanced to explain the lower decrease in numbers of *N. winogradskyi* than those of *N. europaea*.

The effects of addition of the flagellate species *A. peritocrescens* to the mixture of microorganisms competing for ammonium are described in **Chapter 4**. The effect of grazing by protozoa on the critical C/N values was surprisingly small. Critical C/N ratios of 12.5 and 10.5 were found for the dilution rates of 0.004 and 0.01 h^{-1}, respectively, which values were not significantly different from those without flagellates (Chapter 3). As expected, the numbers of heterotrophic bacteria in the presence of flagellates increased less than in their absence when glucose concentrations in the inlet medium increased, which was ascribed to protozoan consumption of bacteria. Against the expectations, the numbers of nitrifying bacteria decreased more in the presence of flagellates than in their absence, presumably by selective predation on the nitrifying bacteria, either in the liquid culture or on the glass wall of the culture vessels. In spite of the lower numbers, the nitrate production rates were equal in the presence and absence of flagellates. This indicated that the activity per nitrifying cell in the presence of flagellates was higher than

in their absence. The fact that a constant nitrification rate per cell cannot be assumed for nitrifying bacteria makes enumerations of nitrifying bacteria in determining nitrate production rates useless, as no correlation has to be expected between the counted numbers of nitrifying bacteria and the measured nitrifying activity. Identical to the systems without flagellates, low numbers of nitrifying bacteria were still found in the continuous cultures above the critical C/N values, probably again due to growth on the glass wall of the culture vessels.

In **Chapter 5**, the competition for ammonium between *N. europaea* and *A. globiformis* in continuously percolated soil columns is described. As in the chemostat experiments, *A. globiformis* won the competition for ammonium, as was concluded from the decreases in the potential ammonium-oxidizing activities when glucose concentrations in the inlet medium increased. The numbers of nitrifying cells were lower at higher glucose concentrations and the potential and actual ammonium-oxidizing activities in the uppermost 3 cm of the soil columns were zero above a C/N ratio of 12 in the medium, although still 10^7 nitrifying cells per gram dry soil were present. This demonstrated that, as in the chemostat experiments, no correlation exists between the numbers of nitrifying bacteria and their activity. The nitrifying bacteria survived the high C/N values as viable, but inactive cells. Not all ammonium was nitrified in the soil columns. This was ascribed to a spatial separation between the immobile (microcolonies of) nitrifying bacteria and their substrate ammonium. Accumulation of ammonium at places in the soil matrix beyond the reach of the bacteria was observed in the upper layers (0-3 cm) of the soil columns. These high ammonium concentrations inhibited the nitrification process in that layer resulting in the highest potential ammonium-oxidizing activities in the underlying layer (3-6 cm). As in the chemostat experiments, the numbers of *N. winogradskyi* decreased less than those of *N. europaea* when glucose concentrations increased, which was probably due to heterotrophic growth of the nitrite-oxidizing bacteria on organic substrates or to denitrification at reduced oxygen concentrations.

The effects of addition of the flagellate species *A. peritocrescens* to the bacterial mixtures in the soil columns are described in **Chapter 6**. Two marked effects on the distribution of ammonium over the nitrification and immobilization process were observed. 1. The distribution of ammonium over the profiles of the soil columns in the presence of flagellates was more uniform than in their absence. In the absence of flagellates, relatively high amounts of ammonium accumulated in the upper layer (0-3 cm) (Chapter 5), whereas in the underlying layers the ammonium concentrations were low.

In the presence of flagellates, however, considerable amounts of ammonium were found in the lower layers, whereas less ammonium accumulated in the upper layer. 2. The potential ammonium-oxidizing activities of *N. europaea* were strongly stimulated in the presence of flagellates. Although the numbers of nitrifying bacteria in the presence of flagellates were comparable to those in their absence, the potential ammonium-oxidizing activities in the presence of flagellates were about 4-5 times higher than those in their absence. Grazing by protozoa may lead to a higher net nitrate production in systems, in which heterotrophic bacteria are limited by carbon.

In **Chapter 7**, the competition for ammonium between *N. europaea*, *A. globiformis*, and roots of *P. lanceolata* as well as the effects of grazing by the flagellate *A. peritocrescens* and of fertilization with ammonium on this competition were studied in pots with γ-sterilized soil. For an unknown reason, *A. globiformis* did not establish in the pots. The roots of *P. lanceolata* won the competition for ammonium from *N. europaea*. In the presence of plants, the potential nitrifying activities as well as the mineral nitrogen concentrations were almost zero and the numbers of nitrifying bacteria were 200 times lower than those in the absence of plants. No statistically significant effects of grazing and fertilization on the nitrification process were observed in the presence of plants. The involvement of allelochemicals in this competition was unlikely. In the absence of plants, grazing by flagellates as well as fertilization with ammonium had significant positive effects on the amounts of nitrate in the pots and on the potential ammonium-oxidizing activities. Combination of grazing and fertilization showed an additive effect. The mechanism behind the protozoan stimulation of the nitrification process has to be elucidated.

SAMENVATTING

Het ontbreken van nitrificatie in natuurlijke bodems kan op twee manieren worden verklaard:

1. Allelopathische inhibitie van het nitrificatieproces door organische verbindingen, die afkomstig zijn van plant materiaal.
2. Onderdrukking van het nitrificatieproces door immobilisatie van ammonium door hetero trofe bacteriën of door de opname van ammonium door wortels van grasland planten.

Beide processen zijn behandeld in **Hoofdstuk 1**. Omdat het onwaarschijnlijk is dat er allelopathische inhibitie van de nitrificatie optreedt in grasland bodems, concentreert dit proefschrift zich op de tweede verklaring: de concurrentie om limiterende hoeveelheden ammonium tussen nitrificerende bacteriën aan de ene zijde en heterotrofe bacteriën of plantewortels aan de andere zijde. Om meer inzicht te verkrijgen in de concurrentie om ammonium tussen nitrificerende en heterotrofe bacteriën en plantewortels, werden een serie modelexperimenten met oplopende complexiteit uitgevoerd in modelsystemen waarbij representatieve soorten van elke functionele groep waren betrokken. Omdat protozoën een stimulerend effect op het nitrificatieproces kunnen hebben, door verhoogde mineralisatie van organisch stikstof of door de uitscheiding van groeistoffen (Hoofdstuk 1), werden deze organismen bij de experimenten betrokken. Van de bij de experimenten gebruikte bacteriesoorten zijn de chemolithotrofe ammonium-oxyderende soort *Nitrosomonas europaea* en de nitriet-oxyderende soort *Nitrobacter winogradskyi* algemeen voorkomende nitrificerende microorganismen in de rhizosfeer van de gebruikte plantesoort, *Plantago lanceolata* (Smalle Weegbree), terwijl de gebruikte heterotrofe bacteriesoort, *Arthrobacter globiformis*, en de gebruikte nieuwe flagellaatsoort, *Adriamonas peritocrescens*, werden geïsoleerd uit de rhizosfeer van deze plantesoort. De experimenten werden uitgevoerd in de volgende modelsystemen: dubbel energie-gelimiteerde continu cultures, continu gepercoleerde bodemkolommen en potten met en zonder plant.

De nieuwe vrij-levende bodemflagellaat *A. peritocrescens* is in **Hoofdstuk 2** beschreven en werd zowel met lichtmicroscopie als met scanning en transmissie elektronen-microscopie bestudeerd. De cel is langgerekt, 8 μm lang en 4 μm breed, en

heeft twee anterieel subapicaal geinserteerde subgelijke flagellen. Een mond is gesitueerd in het anterieel-ventrale deel van de cel. De cel heeft drie flagellaire bundels van microtubuli: de grote en kleine mond bundels lopen posterieel aan de rechterzijde onder het ventrale celmembraan en de derde bundel loopt anterieel, eindigt in een elektronendichte poolstreep in het anterieel-dorsale deel van de cel en is de bron van een net van microtubuli, die liggen onder het dorsale celmembraan. Mitochondriën hebben tubulaire cristae. De flagellaat heeft minimale verdubbelingstijden van 2 tot 3 uur in dichte reincultures van *A. globiformis*. Het nieuwe genus wordt *incertae sedis* geplaatst binnen het Rijk der Protisten.

De concurrentie om limiterende hoeveelheden ammonium tussen *N. europaea* en *A. globiformis* in energie-gelimiteerde continu cultures bij verdunningssnelheden van 0.004 en 0.01 per uur is beschreven in **Hoofdstuk 3**. *A. globiformis* won de concurrentie om het ammonium, zoals werd geconcludeerd uit de afnamen in nitraat-concentraties en aantallen nitrificerende bacteriën wanneer de glucose-concentraties in het invoermedium toenamen. De kritische koolstof:stikstof verhoudingen, waarbij de heterotrofe bacteriën alle aanwezige minerale stikstof gebruikten voor assimilatie-processen, bedroegen 11.6 en 9.6 voor de verdunningssnelheden van respectievelijk 0.004 en 0.01 per uur. Coëxistentie van de concurrerende bacteriesoorten werd gevonden beneden deze kritische koolstof:stikstof verhoudingen, omdat *A. globiformis* en *N. europaea* werden gelimiteerd door respectievelijk glucose en ammonium. De nitrificerende bacteriën werden niet volledig weggeconcurreerd door de heterotrofe bacteriën boven deze kritische koolstof:stikstof verhoudingen, hoewel beide bacteriesoorten door ammonium werden gelimiteerd. De aanwezigheid van nitrificerende bacteriën boven de kritische koolstof:stikstof verhoudingen werd toegeschreven aan hechting aan de glaswand van de reactorvaten, waarvandaan continu nitrificerende cellen loskwamen. Heterotrofe groei van *N. winogradskyi* op organische verbindingen uitgescheiden door de heterotrofe bacteriën werd voorgesteld ter verklaring van de lagere afnamen in de aantallen van *N. winogradskyi* dan van *N. europaea*.

De effecten van toevoeging van de flagellaat *A. peritocrescens* aan het mengsel van microorganismen, die concurreren om ammonium, is beschreven in **Hoofdstuk 4**. Het effect van het grazen door protozoën op de kritische koolstof:stikstof verhoudingen bleek verrassend klein. Kritische koolstof:stikstof verhoudingen van 12.5 en 10.5 werden gevonden voor de verdunningssnelheden van respectievelijk 0.004 en 0.01 per uur. Deze waarden zijn niet significant verschillend van degenen in afwezigheid van flagellaten

(Hoofdstuk 3). Zoals verwacht namen de aantallen heterotrofe bacteriën in aanwezigheid van flagellaten minder toe dan in hun afwezigheid bij oplopende glucose-concentraties in het invoermedium. Dit werd aan consumptie van bacteriën door de protozoën toegeschreven. Tegen de verwachtingen in namen de aantallen nitrificerende bacteriën in aanwezigheid van de flagellaten meer af dan in hun afwezigheid, waarschijnlijk door selectieve predatie van de nitrificerende bacteriën, of in de vloeistof of op de glaswand van het reactorvat. Ondanks deze lagere aantallen waren de nitraatproducties gelijk in aan- en afwezigheid van flagellaten. Dit duidde erop dat de activiteit per nitrificerende cel hoger was in aanwezigheid van flagellaten dan in hun afwezigheid. Het feit dat er niet een constante nitrificatie-snelheid kan worden verondersteld voor nitrificerende bacteriën maakt het tellen van deze bacteriën bij de bepaling van nitraat produktie snelheden overbodig, omdat er geen correlatie kan worden verwacht tussen de getelde aantallen nitrificerende bacteriën en de gemeten nitrificatie activiteit. Zoals in de chemostaten zonder flagellaten werden er lage aantallen nitrificerende bacteriën gevonden in de continu cultures boven de kritische koolstof:stikstof verhoudingen. Dit werd waarschijnlijk opnieuw veroorzaakt door groei op de glaswand van de reactorvaten.

De concurrentie om ammonium tussen *N. europaea* en *A. globiformis* in continu gepercoleerde bodemkolommen is beschreven in **Hoofdstuk 5**. Evenals in de chemostaat experimenten won *A. globiformis* de concurrentie om ammonium, zoals werd geconcludeerd uit de afnamen in de potentiële ammonium-oxydatie activiteiten bij oplopende glucose concentraties in het invoermedium. De aantallen nitrificerende bacteriën waren lager bij hogere glucose concentraties en de potentiële ammonium-oxydatie activiteiten in de bovenste 3 cm van de bodemkolommen waren nul boven een koolstof:stikstof verhouding van 12 in het medium, ondanks dat er nog steeds 10^7 nitrificerende cellen per gram droge grond aanwezig waren. Dit toonde aan dat er, evenals in de chemostaat experimenten, geen correlatie bestaat tussen de aantallen nitrificerende bacteriën en hun activiteit. De nitrificerende bacteriën overleefden de hoge koolstof:stikstof waarden in het medium als levensvatbare, maar inactieve cellen. Het ammonium werd niet volledig genitrificeerd in de bodemkolommen. Dit werd toegeschreven aan een ruimtelijke scheiding tussen de immobiele (micro-kolonies van) nitrificerende bacteriën en hun substraat ammonium. Accumulatie van ammonium werd waargenomen in de bovenste laag (0 tot 3 cm) van de bodemkolommen. Deze hoge ammonium concentraties remden het nitrificatieproces in deze laag, hetgeen resulteerde in de hoogste potentiële ammonium-oxyderende activiteiten in de onderliggende laag (3 tot 6 cm). Evenals in de chemostaat experimenten namen de aantallen van *N.*

winogradskyi minder af dan deze van *N. europaea* bij oplopende glucose concentraties. Dit werd waarschijnlijk veroorzaakt door heterotrofe groei van de nitriet-oxydeerders op organische substraten of door denitrificatie bij gereduceerde zuurstof concentraties.

De effecten van toevoeging van de flagellaat *A. peritocrescens* aan de bacteriemengsels in de bodemkolommen zijn beschreven in **Hoofdstuk 6**. Twee opvallende effecten op de verdeling van ammonium over het nitrificatie- en immobilisatie-proces werden waargenomen. 1. De verdeling van ammonium over het profiel van de bodemkolommen was in aanwezigheid van flagellaten gelijkmatiger dan in hun afwezigheid. In afwezigheid van flagellaten accumuleerde relatief veel ammonium in de bovenste laag (0 tot 3 cm) (Hoofdstuk 5), terwijl de ammonium concentraties in de onderliggende lagen laag waren. Maar, in aanwezigheid van flagellaten werden aanzienlijke hoeveelheden ammonium in de lagere lagen gevonden, terwijl er minder ammonium accumuleerde in de bovenste laag. 2. De potentiële ammonium-oxyderende activiteiten van *N. europaea* werden sterk gestimuleerd in de aanwezigheid van flagellaten. Ondanks dat de aantallen nitrificerende bacteriën in de aanwezigheid van flagellaten vergelijkbaar waren met deze in hun afwezigheid, waren de potentiële ammonium-oxyderende activiteiten in aanwezigheid van protozoën 4 tot 5 maal hoger dan in hun afwezigheid. Het begrazen door protozoën kan leiden tot een hogere netto produktie van nitraat in systemen, waarin de heterotrofe bacteriën worden gelimiteerd door koolstof.

De concurrentie om ammonium tussen *N. europaea* en wortels van *P. lanceolata* evenals de effecten van begrazing door de flagellaat *A. peritocrescens* en van bemesting met ammonium op deze concurrentie werden bestudeerd in potten met γ-gesteriliseerde grond en deze werden beschreven in **Hoofdstuk 7**. De wortels van *P. lanceolata* wonnen de concurrentie om ammonium. Statistisch significante effecten van begrazing en bemesting op het nitrificatieproces werden niet gevonden. In aanwezigheid van een plant waren de potentiële nitrificerende activiteiten evenals de minerale stikstof concentraties bijna nul en waren de aantallen nitrificerende bacteriën 200 maal lager dan deze in afwezigheid van een plant. De betrokkenheid van allelopathische verbindingen in deze concurrentie was onwaarschijnlijk. In afwezigheid van een plant hadden zowel begrazing door flagellaten als bemesting met ammonium een significant positief effect op de hoeveelheden nitraat en op de potentiële ammonium-oxyderende activiteiten. Combinatie van begrazing en bemesting had een elkaar versterkend effect. Het mechanisme achter de stimulatie van het nitrificatieproces door protozoën dient nog te worden opgehelderd.

NAWOORD

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Frank.

CURRICULUM VITAE

Frank Verhagen werd geboren op 22 oktober 1961 te Berlicum. In 1980 werd het diploma Gymnasium ß behaald aan het R.K. Gymnasium Bernrode te Heeswijk-Dinther. In hetzelfde jaar begon hij met de studie Scheikunde aan de Katholieke Universiteit Nijmegen. Het kandidaatsexamen (S2) en doctoraal examen scheikunde werden afgelegd in respectievelijk december 1983 en december 1986. De doctoraal fase van zijn studie bestond uit:

* het hoofdvak Chemische Microbiologie (Prof.Dr.Ir. G.D. Vogels), waarin hij microörganismen afkomstig uit de pens van herkauwers bestudeerde in zowel continu als batch cultures.
* het hoofdvak Organische Chemie (Prof.Dr. R.J.F. Nivard), waarin hij werkte aan de synthese van analoga van de anti-tumor verbinding Adriamycine.
* het caputcollege Moleculaire Symmetrie.

In december 1986 werd hij door de Koninklijke Nederlandse Akademie van Wetenschappen aangesteld op de afdeling Bodembiologie van het Instituut voor Oecologisch Onderzoek (thans Centrum voor Terrestrische Oecologie van het Nederlands Instituut voor Oecologisch Onderzoek) te Heteren voor een vierjarig promotie-onderzoek naar:

* de competitieve verhoudingen tussen nitrificerende en heterotrofe bacteriën en plantewortels in de concurrentie om ammonium.
* de invloed van bacterie-etende protozoën op de competitieve sterkte van nitrificerende bacteriën.

De resultaten van deze onderzoeken zijn beschreven in dit proefschrift. Het schrijven van het proefschrift combineerde hij met de noodgedwongen medeverantwoordelijkheid voor de ouderlijke boerderij. In augustus en september 1991 verbleef hij op de Department of Zoology, University of Bristol, England voor het in Hoofdstuk 2 gepresenteerde onderzoek naar de niet eerder beschreven bodemflagellaat *Adriamonas peritocrescens*.

Naar alle waarschijnlijkheid zal hij begin 1993 starten als post-doc op voornoemd instituut met een door de EEG gesubsidieerd project betreffende onderzoek naar denitrificerende microörganismen in de rhizosfeer van degenererende rietvelden.