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Activity of Chemolithotrophic Nitrifying Bacteria under Stress in Natural Soils

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1. Introduction

Nitrification is an important process in the biogeochemical cycle of nitrogen, linking its reduced and oxidized parts. Since the conversion of ammonium to nitrate has a great impact on the environment, such as weathering of soils, production of greenhouse gases, and eutrophication of surface and ground waters (Van Breemen and Van Dijk, 1988), it is important to know the characteristics of the responsible organisms. Although many organotrophic microorganisms are able to produce oxidized nitrogenous compounds such as nitrite and nitrate (Focht and Verstraete, 1977; Killham, 1987; Kuenen and Robertson, 1987), chemolithotrophic nitrifying bacteria are considered to be the most important group producing these compounds from ammonia. A contribution to nitrate production by organotrophic microorganisms has only been observed in some acid coniferous forest soils (Schimel *et al.*, 1984; Killham, 1986, 1990). According to *Bergey's Manual of Systematic Bacteriology* (Watson *et al.*, 1989), the family of nitrifying bacteria is a diverse group of rods, vibrios, cocci, and spirilla, all having the ability to utilize ammonia or nitrite as a major source of energy and carbon dioxide as the chief source of carbon. With the exception of *Nitrobacter* species, all others are obligate chemolithotrophs, but some can grow mixotrophically on a mixture of CO_2 and small organic compounds. All strains are aerobic, but some may proliferate at low oxygen concentrations. Some *Nitrobacter* species might even grow at the expense of nitrate reduction in the absence of oxygen. Another important characteristic, especially of the chemolithotrophic

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ammonia-oxidizing bacteria that have been isolated, is their sensitivity to low pH. The limited metabolic abilities of the chemolithotrophic nitrifying microorganisms will obviously restrict the number of suitable habitats. Nitrifying bacteria are commonly found in oxic environments with a neutral or slightly alkaline pH where ammonium is produced from organic matter by mineralization. However, chemolithotrophic ammonia-oxidizing bacteria are also observed in extreme environments such as acid tea soils (Walker and Wickramsinghe, 1979), sandstones of historic monuments (Meincke *et al.*, 1989; Spieck *et al.*, 1992), oceanic oxygen minimum zones (Ward, 1986), and the anaerobic hypolimnion of domestic wastewater reservoirs (Abeliovich, 1987).

Although sharing a specific and unique ability, i.e., the oxidation of ammonia without the necessity of organic compounds, the chemolithotrophic ammonia-oxidizing bacteria represent a phylogenetically diverse group of microorganisms belonging to different subdivisions of the Proteobacteria (Woese *et al.*, 1984, 1985). Notwithstanding their highly different morphologies, *Nitrosospira, Nitrosolobus,* and *Nitrosovibrio* seem to be closely related phylogenetically on the basis of 16s rRNS analysis (Head *et al.*, 1993). According to Macdonald (1986), *Nitrosolobus,* not the well-known *Nitrosomonas,* seems to be the most common ammonia-oxidizing genus in soils. In a stream receiving geothermal inputs of ammonium, *Nitrosospira* strains outnumbered the *Nitrosomonas* strains (Cooper, 1983). In spite of this, most experiments have been done with the latter genus. Hence, care must be taken with extrapolating ecophysiological knowledge of *Nitrosomonas* species to the *in situ* behavior of the whole ammonia-oxidizing community. With respect to the oxidation of nitrite by chemolithotrophic bacteria, the well-known and best-studied *Nitrobacter* is still regarded as the most dominant genus in soil.

The activity of chemolithotrophic nitrifying bacteria in natural soils characterized by conditions that might be regarded as marginal for these bacteria, i.e., acid heathland and forest soils as well as oxygen or ammonium-limited grassland soils, has been studied intensively by our department. The results of these studies will be discussed from an ecological perspective. Recently, the physiological characteristics of the chemolithotrophic nitrifying bacteria have been reviewed by Prosser (1989) and by Bock, Harms, Koops, and co-workers (Bock and Koops, 1992; Bock *et al.*, 1992; Koops and Möller, 1992). In a former paper, we related the activity of nitrifying bacteria to the nitrogen nutrition of plants in natural ecosystems (Woldendorp and Laanbroek, 1989).

2. Nitrification at Low pH

2.1. Pure Culture Studies

In *Nitrosomonas europaea,* ammonia rather than ammonium appears to be the real substrate of ammonia mono-oxygenase, which is the first enzyme in

volved in its chemolithotrophic energy generation (Suzuki *et al.*, 1974). In addition, bacterial membranes are permeable to ammonia but not to ammonium (Kleiner, 1985). However, this latter aspect might be of less importance when the ammonia mono-oxygenase is situated at the outside of the bacterial cell membrane as is hypothesized by Bock *et al.* (1992). The obligate use of ammonia and not of ammonium may explain why the activity of the chemolithotrophic ammonia-oxidizing bacteria is restricted to circumneutral conditions, where ammonia is more stable than under acid conditions ($pK_{NH3/NH4OH} = 9.25$). The oxidation of hydroxylamine, the product of the enzyme ammonia mono-oxygenase, appeared to be less acid-sensitive in *N. europaea* strain ATCC 19178 compared to that of ammonia oxidation (Frijlink *et al.*, 1992a). Even at pH 5.0, a proton gradient across the cell membrane was produced during the oxidation of hydroxylamine, which led to ATP synthesis and energy-dependent transport of amino acids. The fact that hydroxylamine and not ammonia was oxidized at pH 5.0 suggests that ammonia hydroxylation is the predominant acid-sensitive process in *N. europaea*. Since *Nitrosomonas* cells failed to grow on hydroxylamine (Frijlink *et al.*, 1992a), as was also observed by others (compare Bock *et al.*, 1992), growth of these strictly chemolithotrophic organisms in acid environments is restricted to ammonia as a substrate. Notwithstanding the problem of growth on ammonia at low pH, ammonia-oxidizing bacteria have been isolated from acid soils (Allison and Prosser, 1991; De Boer *et al.*, 1989a; Hankinson and Schmidt, 1984; Martikainen and Nurmiaho-Lassila, 1985; Walker and Wickramasinghe, 1979). All of these strains turned out to be acid-sensitive. Therefore, ammonia oxidation by this type of acid-sensitive chemolithotrophic bacteria in acid soils is supposed to be restricted to microhabitats of circumneutral pH. In contrast, acid-tolerant or even acidophilic nitrite-oxidizing *Nitrobacter* strains have been isolated from acid soils (De Boer *et al.*, 1989a; Hankinson and Schmidt, 1988). Hence, the oxidation of ammonia in acid soils seems to be limited by the characteristics of the ammonia-oxidizing bacteria and accumulation of nitrite is hardly to be expected due to the acid-tolerance of the nitrite-oxidizing cells.

Besides the occurrence of nitrification in microhabitats of circumneutral pH, ammonia oxidation at low pH might also be stimulated by other factors such as the presence of surfaces or the hydrolysis of urea. A strain of *N. europaea,* which had a pH minimum of 7.0 for growth in liquid batch culture, was able to produce nitrite at a pH of 6.0 when attached to particles in continuous flow sand columns (Allison and Prosser, 1993). A mixed culture of *Nitrosospira* and *Nitrobacter,* both isolated from a fertilized, acid heathland soil, was only able to oxidize ammonia to nitrate at neutral pH (De Boer and Laanbroek, 1989). Nitrification by this mixed culture stopped at pH 5.5, unless urea was added. In the presence of urea, nitrate formation from ammonia was possible at a constantly low pH of 4.5 (Fig. 1). However, ammonia oxidation stopped as soon as urea was depleted. At a pH maintained at 5.0, the production of nitrate continued for another week after the urea was exhausted. Hence, the ratio between ammonium

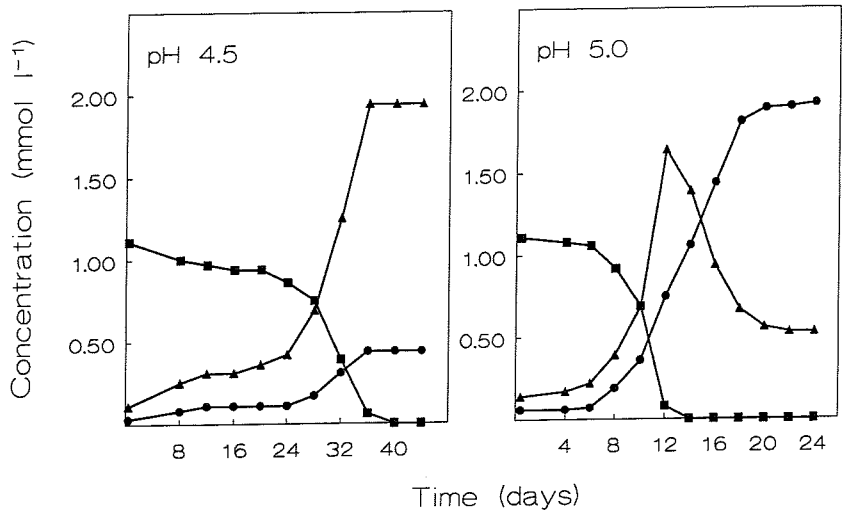

Figure 1. Changes in concentrations of urea (squares), ammonium (triangles), and nitrate (circles) during the incubation of mixed cultures of *Nitrosospira* strain AHB1 and *Nitrobacter* strain NHB1, at pH 4.5 and pH 5.0. Both strains had been isolated from a fertilized heathland soil. (After De Boer and Laanbroek, 1989.)

and nitrate produced at the end of the incubation period was dependent on the ambient pH.

The mechanism by which urea activates the cells is not yet understood. In contrast to ammonium, urea may provide the membrane-bound enzyme ammonia mono-oxygenase with substrate at a low pH. The enzyme urease in *N. europaea* is assumed to be located inside the bacterial cells (W. De Boer, personal communication). The ammonia produced by this enzyme might diffuse across the bacterial membrane where it is (partly) captured by the periplasmatic enzyme ammonia mono-oxygenase. The occurrence of chemodenitrification of nitrite at low pH as described by Van Cleemput and Baert (1984) could be excluded by the complete recovery of added urea in the form of ammonium and nitrate. The results with the mixed culture of the isolated *Nitrosospira* and *Nitrobacter* strains also underscored the acid-tolerant nature of the isolated nitrite-oxidizing strain. The ability to hydrolyse urea is quite common among the chemolithotrophic ammonia-oxidizing bacteria (Koops and Möller, 1992). Most of the *Nitrosomonas* and *Nitrosospira* strains isolated from acid soils in Scotland, appeared to be also ureolytic as well as acid-sensitive (Allison and Prosser, 1991).

2.2. Nitrification in Acid Heathland and Forest Soils

In Dutch acid heathland and forest soils characterized by atmospheric nitro-

apparently chemolithotrophic as was indicated by inhibition experiments with acetylene (De Boer *et al.*, 1988, 1990, 1992, 1993; Martikainen and De Boer, 1993; Stams *et al.*, 1990; Tietema *et al.*, 1992; Troelstra *et al.*, 1990). Two types of chemolithotrophic nitrification could be distinguished in these soils: An acid-sensitive type, as defined by the absence of nitrate production at initial pH values below 6.0, and an acid-tolerant type, characterized by ammonium oxidation that started at pH values as low as pH 4.0 (De Boer *et al.*, 1989b, 1990). Enumerations of acid-tolerant ammonia-oxidizing bacteria by a most probable number (MPN) procedure, using mineral medium with ammonium as the sole energy substrate, only yielded numbers at pH 4.5 or 6.0, but not at pH 7.5. The acid-sensitive, but not the acid-tolerant type, could be stimulated by increasing the pH from 4.0 to 6.0 (De Boer *et al.*, 1989b). Nitrification in suspensions of degrading needles or the litter layer from a Douglas fir soil at pH 6.0 was several times higher than at pH 4.0, whereas pH had a variable effect on nitrification in suspensions of the fermentation layers (Fig. 2) (De Boer *et al.*, 1992). No effect on nitrification was observed in suspensions of the humus layers. In accordance with this result, MPN enumerations of (acid-sensitive) ammonia-oxidizing bacteria at pH 7.5 yielded larger numbers in the litter layer. This indicated a niche differentiation between acid-sensitive and acid-tolerant bacteria in the different forest soil layers due to environmental conditions. During active nitrate produc-

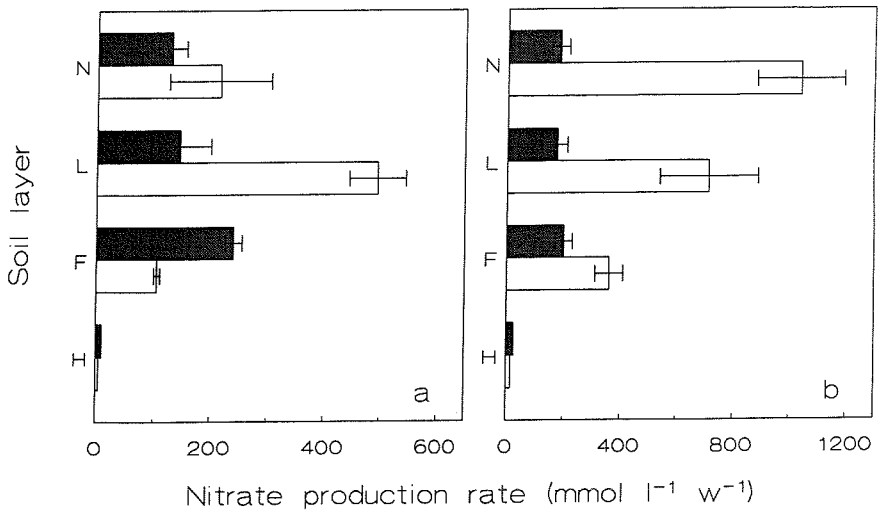

Figure 2. Nitrification rates in suspensions of overground degrading needles (N) and different soil layers of a Douglas fir forest soil: litter layer (L), fermentation layer (F), humus layer (H). Samples collected in (a) May 1989 and (b) January 1990 were incubated for 2 weeks at 20 °C and pH4 (open bars) or pH6 (filled bars). No suspensions of the mineral layer were incubated in January 1990. Data represent the mean of two replicates. (Data from De Boer *et al.*, 1992.)

tion in suspensions of heathland humus at constant pH 4.0, numbers of nitrite-oxidizing bacteria increased, whereas the MPN numbers of the ammonium-oxidizing cells determined at pH 6.0 remained at the same low level (De Boer *et al.*, 1989b).

In accordance with the observations in Dutch ammonium-saturated forest soils, a different behavior of nitrifying bacteria with respect to pH could be observed on a pine forest site in Finland receiving high ammonium deposition rates (Martikainen *et al.*, 1993): high nitrification potentials in the litter layer at pH 6.0 compared to pH 4.0, but no pH effect in the organic and upper mineral layers. In aerobic suspensions of the upper 5 cm of the soil profile of a drained, Finnish mire, the nitrification rate was higher at pH 6.0 than at pH 4.0 (Lang *et al.*, 1993). However, deeper in the soil profile (5–25 cm), nitrification was greater at pH 4.0 than at pH 6.0. The effect of acetylene inhibition on nitrification was also dependent on the sampling layer as well as on pH. In the upper layer, nitrification was almost completely inhibited at pH 6.0, but practically not at pH 4.0. In the deeper layers, acetylene inhibited nitrification at pH 4.0 but not at pH 6.0. Hence, it could be concluded that in each layer the most dominant type of nitrification was autotrophic.

Several attempts to isolate the bacteria responsible for ammonia oxidation at pH 4.0 failed. Experiments with suspensions of the organic layer of a Douglas fir forest soil showed that filtration of the suspension stopped chemolithotrophic nitrification at pH 4.0 but not at pH 6.0 (De Boer *et al.*, 1991). This suggests that the acid-tolerant ammonia oxidation was dependent on the presence of particles or aggregates. From electron microscopy, it appeared that small aggregates consisted of *Nitrosospira*-like cells embedded in an unknown biopolymer. In the larger aggregates, *Nitrobacter*-like bacteria, characterized by their peripheral cytomembrane systems, could be recognized at the outer side of the aggregates. The function of aggregate formation is not yet known. It is obvious that it does not protect the cells against low pH, since the pH in the aggregates will be even lower than in the surrounding medium due to the proton production by the ammonia-oxidizing bacteria. Protection of the cells against oxygen or nitric acid and concomitantly nitric oxide was not found (W. De Boer, personal communication). Cells of an acid-sensitive *Nitrosospira* strain isolated from acid heathland soil immobilized in alginate were also able to nitrify at a fixed pH value of 4.0 (De Boer *et al.*, 1995). Daily fluctuations between pH 6.0 and 4.0 of an actively nitrifying mixed culture of the acid-sensitive *Nitrosospira* strain and an acid-tolerant *Nitrobacter* strain induced adaptation of single cells to be active at constant pH 4.0. Continuation of ammonia oxidation by these adapted cells after subculturing in fresh medium at pH 4.0 was dependent on the degree of dilution, since a high density of cells was apparently needed for a successful ammonia oxidation at low pH. These latter results suggest that acid-tolerant ammonium-oxidizing bacteria might be in fact adapted acid-sensitive cells.

3. Nitrification at Low Oxygen Concentrations

3.1. Pure Culture Studies

The first enzyme of the machinery involved in the oxidation of ammonia to nitrite by *N. europaea,* the copper-containing ammonia mono-oxygenase, is dependent on oxygen (Wood, 1987). Hence, chemolithotrophic nitrification cannot occur in the absence of oxygen. Oxygen is also the preferred electron acceptor for reoxidation of reduced compounds produced in excess during the oxidation of hydroxylamine, which is the product of the enzyme ammonia mono-oxygenase. However, during ammonia oxidation by *Nitrosomonas* strains at low oxygen tensions, nitrite itself might function as an alternative electron acceptor, with nitric oxide, nitrous oxide, or dinitrogen gas as end products (Bock *et al.*, 1992; Poth, 1986; Poth and Focht, 1985). Recently, it was shown by Anderson *et al.* (1993) that the size of nitrous and nitric oxide production by *N. europaea* was even more dependent on the presence of relatively high nitrite concentrations than on that of low partial oxygen concentrations. This suggests that nitrite and oxygen competed for electrons produced during the oxidation of hydroxylamine. Nitric oxide production by a *Nitrosomonas* strain isolated from sewage was stimulated by addition of organic materials (Poth, 1986). A slow consumption of pyruvate by *N. europaea* could be observed during anaerobic incubation in the presence of nitrite (Stüven *et al.*, 1992). Under these oxygen-limited conditions, nitrite was apparently reduced to nitric oxide and nitrous oxide. However, no increase in cell protein was observed. The statement of Abeliovich and Vonshak (1992) that *N. europaea* is able to grow anaerobically, using pyruvate as an electron donor and nitrite as an electron acceptor, is even more remarkable. Nitrite consumption by this organism was stimulated by the addition of ammonium. However, the role of ammonium with respect to anaerobic growth remained obscure. In addition to the production of nitric oxide by reduction of nitrite, this gas might also be formed directly during the oxidation of hydroxylamine (Hooper and Terry, 1979). The production of nitric oxide by *Nitrosomonas* strains was independent of the oxygen tension applied, whereas the production of nitrous oxide increased with decreasing pO_2 (Anderson and Levine, 1986). The ratio of nitric and nitrous oxide production during mixotrophic growth of *N. europaea* in a batch culture increased with increasing nitrite concentration (Stüven *et al.*, 1992). During growth of *N. europaea* on a mixture of ammonia and formate, hydroxylamine is released into the medium, where it subsequently reduces nitrite to nitric oxide and nitrous oxide by chemodenitrification. The production of nitric oxide and nitrous oxide at reduced oxygen tensions is not restricted to *Nitrosomonas* strains, but was also observed with *Nitrosolobus, Nitrosospira,* and *Nitrosococcus* isolates (Goreau *et al.*, 1980), as well as with a *Nitrosovibrio* strain (Stüven *et al.*, 1992).

Similar to the ammonia oxidation, oxygen is the preferred electron acceptor during the oxidation of nitrite by chemolithotrophic nitrite-oxidizing bacteria. In contrast to the ammonia-oxidizing bacteria, nitrite-oxidizing bacteria are not dependent on a mono-oxygenase for generation of energy. However, anaerobic growth of nitrite-oxidizing bacteria on nitrite with nitrate as an alternative electron acceptor is not feasible. Anaerobic growth on nitric oxide, which is an alternative inorganic growth substrate for *Nitrobacter winogradskyi* under oxic conditions (compare Bock *et al.*, 1992), has not yet been demonstrated. In anoxic environments, in the presence of simple organic compounds, *Nitrobacter* strains are able to grow anaerobically by reduction of nitrate to nitrite, ammonia, and nitrogen gases, in particular, nitrous oxide (Freitag *et al.*, 1987; Bock *et al.*, 1988). Nitrite-oxidizing cells of *Nitrobacter vulgaris* possess a nitrite reductase that generates nitric oxide (Ahlers *et al.*, 1990). This enzyme might have a function in NADH synthesis in *Nitrobacter* species at low nitrite concentrations (Freitag and Bock, 1990). Nitric oxide was also produced when nitrite-oxidizing cells of *Nitrobacter hamburgensis* grew mixotrophically at low oxygen tensions (Bock *et al.*, 1992). However, the *in situ* production of nitric oxide by nitrite-oxidizing bacteria in (semi)oxic environments is questionable. The production of this gas from corroding building stones was apparently caused by ammonia-oxidizing bacteria and not by *Nitrobacter* cells (Baumgärtner *et al.*, 1991). The same observation has been made with respect to nitrous oxide production during pure culture experiments (Goreau *et al.*, 1980).

In conclusion, the ammonia- as well as the nitrite-oxidizing bacteria can apparently cope with oxygen-limited conditions by using nitrite or nitrate as electron acceptors alternative to oxygen. However, due to the strict dependency on oxygen of the mono-oxygenase, chemolithotrophic oxidation of ammonia to nitrite cannot occur under strictly anoxic conditions. It seems unlikely that ammonia-oxidizing strains other than *Nitrosomonas* could oxidize ammonia without a monooxygenase.

3.2. Nitrification in Oxygen-Limited, Waterlogged Grassland Soils

In soils rich in organic material such as grassland soils, anoxic conditions are rapidly established after waterlogging due to a decreased oxygen diffusion in combination with oxygen consumption by soil organisms and plant roots (Ponnamperuma, 1984). According to the strict dependency on oxygen of the ammonia mono-oxygenase, as previously discussed, ammonia oxidation will stop in these waterlogged soils. However, radial oxygen loss from aerenchymatous plant roots creates a small oxidized layer around the roots in an otherwise anoxic environment (Laan *et al.*, 1989a). This radial oxygen loss might favor oxidation processes in the rhizosphere (Armstrong, 1964; Sand-Jensen *et al.*, 1982). One such oxidation process may be chemolithotrophic nitrification. The production of

nitrate in the oxidized rhizosphere of waterlogged plants is indicated by the presence of nitrate in the root zone of the perennial macrophyte *Littorella uniflora* (Christensen and Sørensen, 1986), the induction of the enzyme nitrate reductase in bog and rice plants (Blacquière, 1986; Uhel *et al.*, 1989), increased numbers of chemolithotrophic ammonia- and nitrite-oxidizing bacteria in the root zone of the bog plant *Glyceria maxima* compared to the reduced bulk soil (Fig. 3) (Both *et al.*, 1992a), and the preservation of potential nitrification activities in the waterlogged root zone of *Rumex palustris* (Engelaar *et al.*, in press). As only ammonium was supplied as inorganic nitrogen source, the high nitrate reductase activity measured in the leaves of the flooding-resistant, aerenchymatous plant species *R. palustris* indicated that there was also nitrate formation in its root zone. Nitrate consumption by *Rumex* species is well reflected by the level of nitrate reductase (Langelaan and Troelstra, 1992). Under waterlogged conditions, low nitrate reductase levels and low potential nitrification activities were observed in the leaves and root zones, respectively, of *Rumex thyrsiflorus*, which is also a *Rumex* species not resistant to flooding. In contrast to flooding-resistant *Rumex* species, *R. thyrsiflorus* produces hardly any new roots with aerenchyma

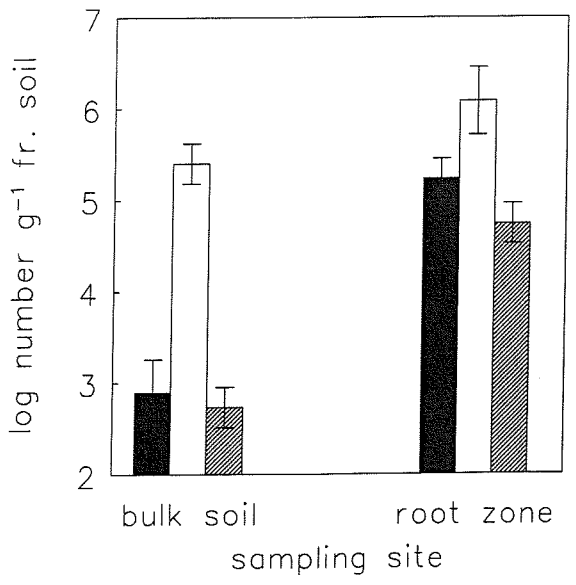

Figure 3. Most probable numbers of ammonium-oxidizing bacteria (filled bars) and nitrite-oxidizing bacteria enumerated in soil samples from out- and inside the root zone of *Glyceria maxima* in waterlogged soils. Numbers of nitrite-oxidizing bacteria were determined at 0.05 mM (open bars) and 5.0 mM (hatched bars), respectively. Bars indicate 95% confidence levels. (After Both *et al.*, 1992a.)

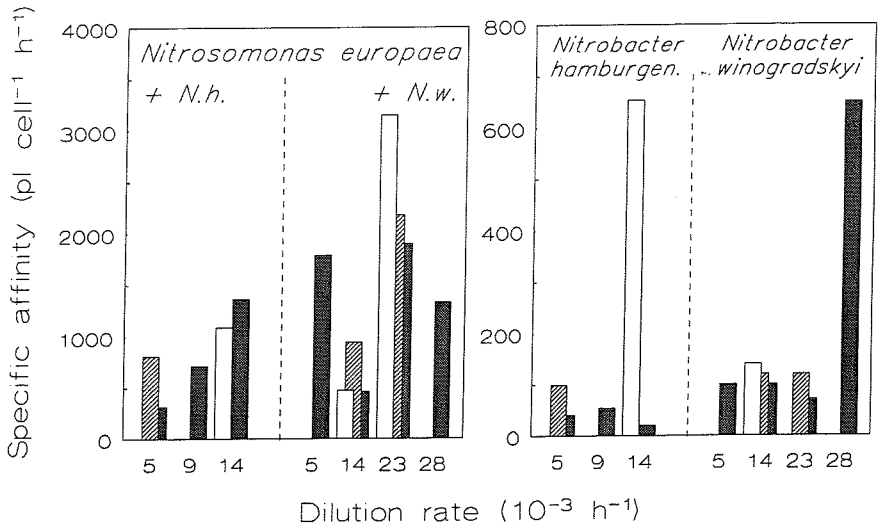

Figure 4. Specific affinities for oxygen consumption determined in mixed, steady-state cultures of *Nitrosomonas europaea* and either *Nitrobacter winogradskyi* (N.w.) or *Nitrobacter hamburgensis* (N.h.) grown in chemostats at 25 °C, pH 7.5, different dilution rates, and oxygen concentrations: 80% (filled bars), 10% (hatched bars), and 0% (open bars) oxygen. Notice the difference in scale used for the ammonia- and nitrite-oxidizing strains. (Modified after Laanbroek and Gerards, 1993; Laanbroek *et al.*, 1994.)

after flooding, and the radial oxygen release was consequently much lower (Laan *et al.*, 1989a,b). A prerequisite for the occurrence of ammonia oxidation in the root zone of flooded *R. palustris* is a continuous supply of sufficient ammonium (Engelaar *et al.*, 1991); otherwise, nitrification will be repressed by ammonium-assimilating organotrophic bacteria and plant roots, as will be discussed in Section 4.

The fact that increased numbers of chemolithotrophic nitrifying bacteria or at least nitrifying activity can be observed in the root zone of waterlogged plants indicates that these bacteria are able to consume a part of the oxygen released by the roots. The affinity of the chemolithotrophic ammonium-oxidizing bacteria for oxygen is mentioned to be much lower than the affinity of organotrophic bacteria for this electron acceptor (Prosser, 1989). However, half-saturation constants for oxygen found for *N. europaea* in mixed continuous cultures with either *N. hamburgensis* or *N. winogradskyi* (Fig. 4) were of the same magnitude as the K_m value for oxygen consumption measured with the organotrophic *Pseudomonas chlororaphis* (Laanbroek and Gerards, 1993; Laanbroek *et al.*, 1994; Bodelier and Laanbroek, unpublished results). In the small oxidized layer around plant roots, the outcome of a competition for oxygen will be determined by the differences in specific affinities of the competing organisms for oxygen. These differ-

ences are indicated by the ratio between the maximum oxygen consumption rate of the cells and the half-saturation constant for oxygen (Button, 1985). The average specific affinity for oxygen of the ammonia-oxidizing bacteria obtained with mixed continuous cultures of *N. europaea* and either *N. hamburgensis* or *N. winogradskyi* grown at different oxygen concentrations (Laanbroek *et al.*, 1994; Laanbroek and Gerards, 1993) are several orders of magnitude lower than those mentioned for organotrophic bacteria (Button, 1985). The fact that chemolithotrophic nitrification still occurs in the oxidized root zone of waterlogged plants suggests that the radial oxygen loss by the plants is proportionally larger than the release of usable carbon compounds. Hence, the organotrophic bacteria in the oxidized root zone appear to be carbon limited. Nitrogen limitation of organotrophic bacteria in the presence of actively ammonia-oxidizing cells is not very likely as is discussed in the next section.

3.3. Competition for Oxygen between Ammonia- and Nitrite-Oxidizing Bacteria

When at least a part of the available oxygen in the root zone is captured by the nitrifying bacteria, complete oxidation of ammonia to nitrate without accumulation of nitrite will be determined by the relative affinities of the ammonia- and nitrite-oxidizing bacteria for oxygen. When the oxygen supply was limited, nitrite accumulated in a mixed continuous culture of *N. europaea* and *N. winogradskyi* and cell numbers of the latter declined. This observation was supported by the specific affinities for oxygen measured for these species (Laanbroek and Gerards, 1993). The ammonia-oxidizing population had a greater specific affinity for oxygen than the nitrite-oxidizing bacteria (Fig. 4). Since the cell numbers of both species were of the same magnitude, most of the limiting amount of oxygen was used by the ammonium-oxidizing species. However, in a mixed continuous culture of *N. europaea* and mixotrophically growing *N. hamburgensis,* no nitrite accumulation was observed under oxygen-limited conditions, whereas the concentration of ammonium in the culture not used by the ammonia-oxidizing bacteria gradually increased and their numbers concomitantly decreased (Laanbroek *et al.*, 1994). Under conditions of limited oxygen supply, the specific affinities of the nitrite-oxidizing *N. hamburgensis* and the ammonia-oxidizing *N. europaea* were comparable and two to three times greater than the specific affinity of the nitrite-oxidizing *N. winogradskyi* under the same growing conditions (Fig. 4). Under conditions of sufficient oxygen supply, the specific affinities of *N. hamburgensis* for oxygen were much less than those of *N. europaea* and even less than those of *N. winogradskyi.* Cessation of the oxygen supply resulted in an immediate accumulation of nitrite in the mixed cultures of *N. europaea* and either *N. hamburgensis* or *N. winogradskyi* that had been growing at 80% oxygen saturation. The disadvantage of the low specific affinity

of *N. hamburgensis* for oxygen under conditions of sufficient oxygen supply was not compensated for by its larger population size by means of mixotrophic growth on nitrite plus pyruvate. On the basis of its relatively great specific affinity under oxygen-limited conditions, it was hypothesized that in contrast to the more oxic *N. winogradskyi, N. hamburgensis* is adapted to growth in habitats that are constantly oxygen-limited (Both *et al.*, 1992c; Laanbroek *et al.*, 1994). If so, the similar numbers of *N. winogradskyi* and *N. hamburgensis* in peat bog soils, as has been observed by Laanbroek and Gerards (unpublished results) using specific antibody fluorescence enumerations, indicate that these waterlogged soils contain oxic microsites possibly in the rhizosphere of aerenchymatous plants.

An alternative explanation of nitrite accumulation by mixed cultures of *N. europaea* and *Nitrobacter* species growing in the presence of organic compounds was presented by Stüven *et al.* (1992). These authors observed the accumulation of hydroxylamine during mixotrophic growth of *N. europaea* and *N. winogradskyi* or *N. vulgaris* on ammonium and pyruvate. A concomitant oxidation of ammonia and pyruvate by *N. europaea* may result in an overproduction of hydroxylamine, which may subsequently be released in the medium. Due to the extreme sensitivity of *Nitrobacter* species to this first intermediate of ammonia oxidation (Castignetti and Gunner, 1982), nitrite oxidation was apparently inhibited. The difference between *N. winogradskyi* and *N. hamburgensis* with respect to nitrite accumulation as observed in mixed oxygen-limited cultures (Laanbroek *et al.*, 1994; Laanbroek and Gerards, 1993) might then be explained by the different abilities for mixotrophic growth of these nitrite-oxidizing species. In contrast to the mixotrophic *N. hamburgensis,* the more chemolithotrophic *N. winogradskyi* would leave more organic compounds to be oxidized for the ammonia-oxidizing *N. europaea.* No pyruvate could be measured in mixed cultures of *N. europaea* and *N. hamburgensis,* whereas in mixed cultures of *N. europaea* and *N. winogradskyi* only a fraction of the pyruvate was consumed by the organisms (Laanbroek and Gerards, 1993; Laanbroek *et al.*, 1994).

4. Nitrification at Low Ammonium Concentrations

4.1. Pure Culture Studies

Half saturation concentrations for ammonia oxidation of *Nitrosomonas* cells are relatively high compared to *in situ* concentrations of ammonium (Prosser, 1989). There is a discrepancy between the measured low affinities of pure cultures of chemolithotrophic bacteria for ammonia and the observation that nitrification proceeds in oceanic waters at immeasurably low substrate concentrations. According to Ward (1986), this can be explained by at least two hypotheses: (1) pure cultures of bacteria, which constitute at least the major part

of the natural population as indicated by immunofluorescent techniques, have lost the ability to nitrify at environmental low ammonia concentrations after isolation, and (2) ammonia-oxidizing bacteria possess complex enzyme systems capable of utilizing ammonia at concentration ranges of several orders of magnitude. Only the less sensitive system would then be measured under laboratory conditions. The K_m values for ammonium oxidation observed for *N. europaea* grown in mixed culture with either *N. hamburgensis* or *N. winogradskyi*, i.e., 0.4–3.6 mM, were relatively independent of growth rate and oxygen concentration (Laanbroek *et al.*, 1994; Laanbroek and Gerards, 1993). The specific affinities of *N. europaea* for ammonia determined in these mixed culture experiments were several orders of magnitude lower than those mentioned for organotrophic bacteria, algae, or marine phytoplankton (Button, 1985).

Competition experiments in continuous cultures between *N. europaea* and the organotrophic *Arthrobacter globiformis*, performed in the presence of *N. winogradskyi* to prevent nitrite accumulation reaching toxic levels, showed that the chemolithotrophic bacteria were the weakest competitors for ammonium (Verhagen and Laanbroek, 1991). *Nitrosomonas europaea* could only oxidize the ammonium not used by the organotrophic bacteria (Fig. 5). However, when the organotrophic bacteria were both carbon- and nitrogen-limited, the nitrifying

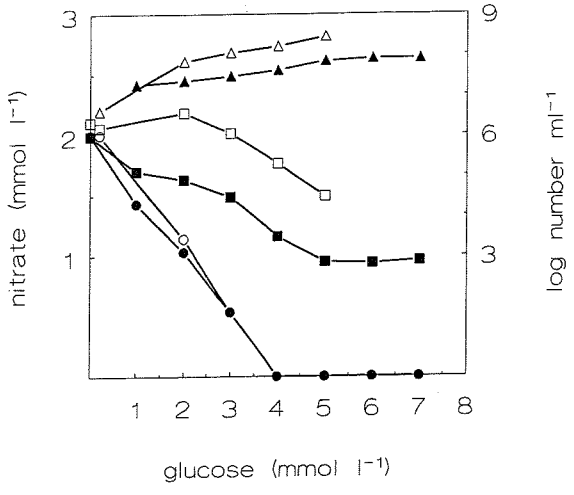

Figure 5. Concentrations of nitrate (circles), numbers of the ammonium-oxidizing bacterium *Nitrosomonas europaea* (squares), and numbers of the organotrophic bacterium *Arthrobacter globiformis* (triangles) in mixed, continuous cultures in the absence (open symbols) and presence (closed symbols) of the bacteriovorous flagellate *Adriamonas peritocrescens*. The culture was continuously supplied (D = 0.004 hr^{-1}) with mineral medium supplemented with 2 mM ammonium and variable amounts of glucose. (Modified after Verhagen and Laanbroek 1991, 1992.)

bacteria did not wash out and their numbers remained at a low but constant level in the chemostat. This phenomenon could only be explained by wall growth of the ammonia-oxidizing bacteria. The wall of the culture vessel might have offered a microhabitat where the competitive abilities of the chemolithotrophic bacteria with respect to ammonia-scavenging abilities may have been better than in the culture liquid itself. Introduction of the bacteriovorous flagellate *Adriamonas peritocrescens,* isolated from the same grassland soil as the *A. globiformis* strain (Verhagen *et al.*, 1994b), lowered the numbers of bacteria by grazing, but had no net effect on the production of nitrate from ammonia (Verhagen and Laanbroek, 1992). The flagellate apparently had a stimulating effect on the activity of the remaining ammonia-oxidizing cells, since fewer cells produced the same amount of nitrate per unit of time. Such a stimulation of nitrification in liquid cultures has also been shown by Griffiths (1989). The mechanism of this effect is not yet clear, but it is apparently not directly related to increased mineralization of organic nitrogen by the protozoa, since the total output of mineral nitrogen in the form of nitrate remained the same.

4.2. Nitrification in Ammonium-Limited Soils

In aquatic environments such as mixed continuous cultures, K_m values or specific affinities may be a useful tool to predict the outcome of competition for growth-limiting substrates between species. However, in more heterogenous environments such as soils, the utilization of these kinetic parameters might be less useful. Competition experiments between ammonia-oxidizing *N. europaea* and the organotrophic *A. globiformis,* in pot experiments and in soil columns continuously percolated with mineral medium supplemented with ammonium and glucose, confirmed the weak position of the chemolithotrophic bacteria with respect to ammonium consumption (Verhagen *et al.*, 1992, 1994a). Introduction of the bacteriovorous flagellate *A. peritocrescens* stimulated the activity of the ammonia-oxidizing cells again, whereas no effect on the total number of chemolithotrophic bacteria was observed (Verhagen *et al.*, 1993, 1994a). In addition to the stimulation of nitrification by a yet unknown factor as observed in homogenous continuous cultures, the flagellates may also have brought about a better mixing of the ammonia-oxidizing bacteria or their substrate in the soil. In the presence of growing plant roots, nitrification was completely repressed due to ammonium limitation (Verhagen *et al.*, 1994a). Here, a likely positive effect of protozoa on nitrification was overruled by the activity of the plant roots. Repression of ammonia-oxidizing bacteria as well as of potential nitrifying activities due to ammonium limitation were also observed in a pot experiment with the relatively fast-growing plant species *R. palustris* (Engelaar *et al.*, 1991). With the slower-growing *Rumex acetosa,* numbers of ammonia-oxidizing bacteria and potential nitrifying activities increased at the start of the incubation period. When

R. acetosa also reached a certain amount of biomass, numbers and activities of ammonia-oxidizing bacteria leveled off due to increasing ammonium limitation in the soils.

It had already been shown in agricultural soils by Jansson (1958), who applied labeled ammonium in the absence and presence of wheat straw, that nitrifying bacteria were regularly less effective than the organotrophic bacteria in the competition for mineralized ammonium. From pot experiments with mustard plants, Jansson (1958) concluded that the plants had only a very limited ability to compete effectively with the bacteria for nitrogen in the presence of farmyard manure. However, after nitrification had been well established in the presence of an excess of added ammonium, the nitrifying bacteria were able to use a considerable part of the ammonium chemically fixed in clay minerals, and consequently otherwise biologically unavailable ammonium became nitrified. However, it should be kept in mind that utilization of chemically fixed ammonium by nitrifying bacteria could only happen once in the same soil. In agreement with Jansson (1958), Zak *et al.* (1990) observed a decrease in nitrification in the presence of the plant species *Allium tricoccum* when compared to no-plant soils during short incubation experiments with ^{15}N-labeled ammonium. Most of the ^{15}N-labeled ammonium had been consumed by the organotrophic bacteria, since microbial bioimass contained 8.5 times as much nitrogen as *A. tricoccum* biomass 2 days after isotope addition. However, when plants and organotrophic bacteria are not nitrogen-limited, nitrification may occur (Riha *et al.*, 1986). On the basis of half-saturation values known for ammonium consumption by a soil community consisting of plant roots, organotrophic microorganisms, and ammonia-oxidizing bacteria, Rosswall (1982) predicted that ammonium-oxidizing bacteria would be the weakest competitors of them all and that nitrification would be repressed by the activity of plant roots and organotrophic bacteria under conditions of limiting ammonium supply.

4.3. Competition for Ammonium versus Allelopathy in Grassland Soils

Repression of nitrification in natural grassland soils themselves has often been observed, especially under climax vegetation. In New Zealand, for example, the nitrifying population of a low-fertility grassland soil was very small as indicated by MPN enumerations (Robinson, 1963). The population of nitrifying bacteria could be increased by field treatment with urea. In counts of chemolithotrophic nitrifying bacteria by a MPN method, Meiklejohn (1968) observed low numbers of ammonia- and nitrite-oxidizing bacteria in soils under a number of common indigenous African grasses. In contrast, numbers of nitrifying bacteria were about 100 times higher in heavily fertilized pastures. These observations all conform to the idea that nitrifying bacteria are weak competitors with respect to limiting amounts of ammonium. Studying one grassland and two different

forest succession series, Rice and Pancholy (1972, 1973) also observed a decreasing number of nitrifying bacteria with increasing successional stages of the vegetation. However, the amount of ammonium also increased with increasing succession. In addition, the amounts of tannins in the soil of intermediate and late successional stages were high. So, it was hypothesized that nitrification in plant-nutrients-limited grassland soils is inhibited by allelochemical compounds produced by plants in order to conserve nitrogen and energy. This idea of inhibition of nitrifying bacteria by plant-derived allelochemicals, which had already been raised by Theron in 1951, was partly supported by a number of experiments (Moore and Waid, 1971; Munro, 1966; Rice and Pancholy, 1973). However, as can be seen from the experiments of Moore and Waid (1971), repeated addition of root washings reduced the repressive effect of these washings on the nitrification. Purchase (1974) observed that daily washings of living or decaying grass roots did not inhibit nitrification. The effects of tannins on pure cultures of ammonium-oxidizing bacteria were dependent on the genera studied (Bohlool *et al.*, 1977).

No inhibition of nitrification by allelochemicals was observed in the root zones of *Plantago* species. A positive correlation had been found between numbers of nitrifying bacteria in the rhizosphere of these plant species and the uptake of nitrate by the plant as indicated by the nitrate reductase activities in their leaves (Smit and Woldendorp, 1981). With an excess of ammonium, accumulation of *N. europaea* and of an organotrophic *Pseudomonas* species was observed around the roots of *Plantago lanceolata* in a small axenic growth chamber that was continuously percolated with mineral medium (Both, 1990). In this experiment, ammonium was always in excess. *Nitrosomonas europaea* might have been stimulated by ammonia production by the organotrophic bacteria growing on organic nitrogen compounds released by the plant roots. As described in Section 2.1, ammonia is likely to be a better substrate for the enzyme ammonia mono-oxygenase. However, the chemolithotrophic bacteria might also have been positively affected by these compounds themselves, since the *N. europaea* strain used in this experiment is able to incorporate exogenous amino acids as has been discussed above (Frijlink *et al.*, 1992b).

Stienstra *et al.* (1994), who studied nitrification in the root zone of *Holcus lanatus* originating from grassland soils, each with a different history of fertilizer application, observed decreasing potential ammonium-oxidizing activities with increasing periods of nonfertilization (Fig. 6). The grasslands studied constituted a series of different stages of plant succession in the vegetation resulting from decreasing nutrient availabilities (Olff, 1992). The potential nitrification activity in the root zones of *Holcus lanatus* and other plant species was similar in each field but differed significantly between the fields. In addition, MPN numbers of ammonium-oxidizing bacteria in the root zone of *H. lanatus* also decreased as the period of nonfertilization increased. So, these results are not in favor of the hypothesis of repressed nitrification by allelochemicals released by the plants

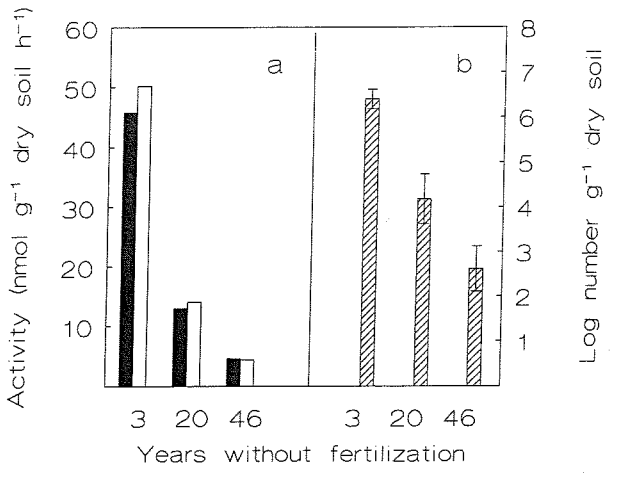

Figure 6. (a) Potential ammonia-oxidizing activities and (b) most probable numbers of ammonia-oxidizing bacteria measured in samples from extensively used grassland soils in which fertilization had been omitted since 3, 20, and 46 years, respectively. Samples for measurements of potential ammonia-oxidizing activities were collected in March 1991 from the root zones of *Holcus lanatus* (filled bars) and other dominant plant species (open bars) that are representative for a particular succession stage of the grassland vegetation: *Agrostis stolonifera* (3 years), *Anthoxanthum odoratum* (20 years), and *Agrostis capillaris* (46 years). Samples for determination of numbers were randomly taken in July 1991. Bars represent 95% confidence limits. (Modified after Stienstra *et al.*, 1994.)

under climax vegetation, but do support the idea of a diminished ammonium supply. However, it cannot be completely excluded that the production of nitrification-repressing organic compounds by *H. lanatus* may be stimulated by nutrient limitation.

4.4. Nitrification in Ammonium-Limited Grassland Soils in Relation to pH

Bramley and White (1989), who studied short-term nitrification activities in relation to pH in four pasture soils with a different history of liming, noticed that the optimum pH for an indigenous ammonium-oxidizing population was never far from the prevailing soil pH. In this study the upper 3 cm of soil, consisting of leaf litter and a dense root mat, was discarded to minimize any inhibitory effect that grass roots may have on the rate of nitrification. To study the pH optimum of the ammonium-oxidizing community in the series of grassland soils with a different history of fertilization, mentioned above (Stienstra *et al.*, 1994), the upper 10 cm, including the grass sod, was used. The pH_{KCl} values of the soils decreased from 4.9 to 4.4 as the period of nonfertilization increased. In the most recently

fertilized grassland soils, the ammonium-oxidizing community showed a pH optimum of 7.0. As the periods of nonfertilization increased, the pH optimum decreased but became less distinct. In the grassland soil that had not been fertilized the longest time, no pH optimum was observed at all in the range of pH 5.0–8.0. It was hypothesized that the value of the optimum pH indicated the physiological condition of the ammonium-oxidizing bacteria, with the most active bacteria having the highest optimum pH. The grassland soil that had been fertilized relatively recently, i.e., only 3 years ago, also contained significantly higher concentrations of nitrate. In general, nitrate consumption by plants enhances the pH of the rhizosphere, whereas the opposite occurs during consumption of ammonium (e.g., Troelstra *et al.*, 1992). Hence, nitrate consumption by the plant roots will create microsites of increased pH in an otherwise acid soil, hereby stimulating the acid-sensitive ammonium-oxidizing bacteria. So, a positive interaction might occur in these recently fertilized grassland soil between the nitrate-producing chemolithotrophic nitrifying bacteria and the nitrate-consuming plants. In addition, a shift in the ammonium-oxidizing community from acid-sensitive to acid-tolerant bacteria may also have occurred, with increased impoverishment of the soils due to the nonuse of fertilizers and annual removal of aboveground plant biomass. In contrast to acid-sensitive bacteria, acid-tolerant bacteria in heathland soils did not have a distinct pH optimum between pH 4.0 and pH 6.0 (De Boer *et al.*, 1989b).

4.5. Effect of Substrate Concentrations on MPN Enumerations of Nitrite-Oxidizing Bacteria

A most remarkable observation was made by Both and co-workers when enumerating numbers of nitrite-oxidizing bacteria in natural grassland soils. In general, numbers of these bacteria counted by a MPN procedure were highly dependent on the nitrite concentration used in the enumeration medium. Usually, the highest numbers of nitrite-oxidizing bacteria were observed at a low nitrite concentration, i.e., 0.05 mM (Fig. 3) (Both *et al.*, 1990, 1992a,b). It was only in a well-drained grassland soil on top of a former river bank that the highest numbers were observed for a relatively short period of time at the highest nitrite concentration applied, i.e., 5.0 mM (Fig. 7a) (Both *et al.*, 1990, 1992b). In pot experiments with two grassland plant species, numbers of nitrite-oxidizing bacteria determined at 0.05 mM nitrite after 14–15 weeks of plant growth were significantly higher in the root zone compared to the nonrooted soil (Stienstra *et al.*, 1993). In contrast to this, numbers determined at 5.0 mM nitrite after 11–12 weeks of incubations were significantly higher in the bulk soil. In arable soils characterized by different management practices, numbers of nitrite-oxidizing bacteria determined at 0.1 mM nitrite were always two orders of magnitude higher than those determined at 5.0 mM nitrite (Laanbroek and Gerards, 1991). Differences between numbers obtained at low and high nitrite concentrations

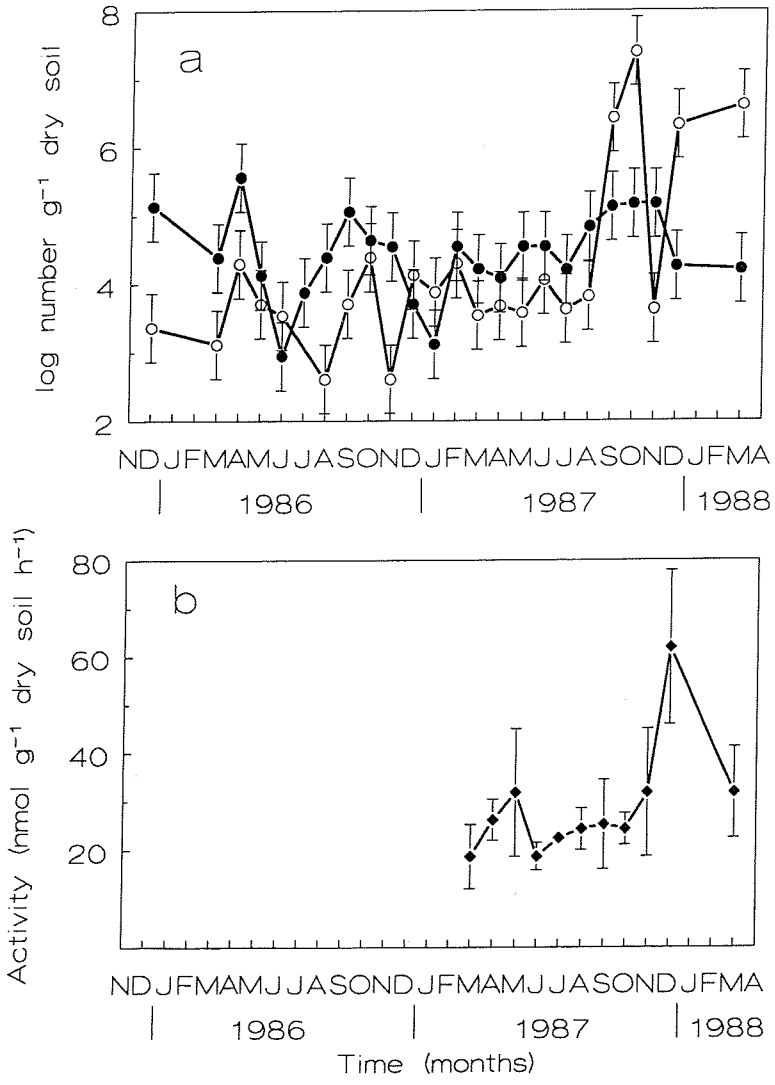

Figure 7. (a) Numbers of nitrite-oxidizing bacteria and (b) potential nitrite-oxidizing activities determined in samples collected from an extensively used grassland soil. Numbers of nitrite-oxidizing bacteria were determined in mineral medium supplemented with 0.05 mM (○) or 5.0 mM (●) nitrite. Bars represent 95% and 66% confidence limits for numbers and activities, respectively. (After Both *et al.*, 1990, 1992b.)

could be simulated by applying Monod and Haldane kinetics and different incubation times (Both and Laanbroek, 1991). According to the model they used, numbers of the nitrite-tolerant and -sensitive cells are underestimated at 0.05 mM and 5.0 mM nitrite, respectively, after applying an incubation period of 90 days. Therefore, low numbers of nitrite-oxidizing bacteria at 5.0 mM compared to 0.05 mM nitrite may indicate the predominance of nitrite-sensitive cells. It is not yet known what these cells represent. Aeration of anoxic waterlogged soils either by the presence of oxygen-releasing aerenchymatous plants or aerobic laboratory incubations in the presence of ammonium promoted the growth of nitrite-tolerant cells at 5.0 mM nitrite together with the ammonium-oxidizing cells (Both *et al.*, 1992a). No stimulation of nitrite-sensitive cells at 0.05 mM nitrite was observed in the presence of oxygen. Anoxic incubations decreased the numbers at 0.05 mM, but slightly stimulated the numbers at 5.0 mM nitrite. No effect of anoxic incubations was observed with respect to the numbers of ammonium-oxidizing bacteria. Since the behavior of the nitrite-tolerant bacteria and ammonium-oxidizing bacteria was similar toward oxic incubations in the presence of ammonium, this group of nitrite-oxidizing bacteria might represent the actively nitrifying part of the soil community, whereas the nitrite-sensitive cells enumerated at 0.05 mM nitrite may symbolize resting or organotrophically growing cells. To study the effect of starvation on the result of enumerations at low and high concentrations of nitrite, cells of different *Nitrobacter* species present in the waterlogged soils were enumerated after 18 months of starvation in the absence of nitrite (Laanbroek and Schotman, 1991). During these enumerations, no effect of the nitrite concentration on numbers was observed with nongrowing cells. Enumerations of *Nitrobacter* cells after subculturing for many generations on organic substrates were not affected by low or high nitrite concentrations (Stienstra *et al.*, 1993). So, the nature of the nitrite-sensitive cells obtained during enumerations at low nitrite concentrations is still obscure. But since these cells may have a relatively low half-saturation constant for nitrite oxidation as indicated by the model of Both and Laanbroek (1991), nitrite-sensitive cells might be responsible for scavenging traces of nitrite during low or nonnitrifying conditions in the soil.

4.6. Potential Nitrifying Activities in Grassland Soils

In addition to enumeration of chemolithotrophic nitrifying bacteria by MPN procedures described above, cell numbers have also been derived indirectly from determinations of potential nitrifying activities performed in soil slurries under optimal conditions in the laboratory according to the method of Schmidt and Belser (1982). These potential nitrifying activities do not represent *in situ* nitrate production rates, but indicate virtually the sizes of the enzyme systems able to oxidize ammonia or nitrite. In the soil of an extensively used pasture, determinations of potential nitrite-oxidizing activities were performed monthly, together with enu-

merations of nitrite-oxidizing bacteria by the MPN procedure (Both *et al.*, 1992b). Maximal values for the potential nitrite-oxidizing activities were obtained in early spring and early winter (Fig. 7b). However, these maximal values were only two to three times larger than the basic values measured in summer and midwinter. These relatively small differences between maximal and minimal values of potential nitrite-oxidizing activities were also observed by Berg and Rosswall (1986) in arable soils. Hence, the sizes of the nitrite-oxidizing enzyme systems present in the soil hardly fluctuated during seasons. Numbers of nitrite-oxidizing bacteria determined by the MPN method revealed larger seasonal fluctuations than the potential nitrite-oxidizing activities did (Fig. 7). This was also observed by Sarathchandra (1978) and Berg (1986). The latter suggested that the observed differences between MPN counts and potential nitrite-oxidizing activities may be due to the presence or absence of free-living bacteria. Under optimal conditions, a larger part of the nitrite-oxidizing community would be present as free-living cells in the soil, whereas more adverse conditions would force the larger part of the community to survive in microcolonies. Assuming a constant activity per cell, as was done by Berg, a concentration of cells in microcolonies would not effect the potential nitrite-oxidizing activity of the community, but would decrease the total number obtained in a MPN series. However, the activity per cell is dependent on the environmental conditions (Both *et al.*, 1992c; Laanbroek and Gerards, 1993; Laanbroek *et al.*, 1994). Low numbers of nitrite-oxidizing bacteria observed in a MPN series could also be due to the inability of cells from adverse soil conditions to proliferate in mineral medium, whereas these cells are still able to convert nitrite to nitrate during a measurement of potential nitrite-oxidizing activities.

Surprisingly, the potential nitrite-oxidizing activity determined monthly in the extensively used pasture soil correlated very well with the apparent saturation constant for nitrite oxidation (Both *et al.*, 1992b). Hence, the specific affinity for nitrite oxidation as indicated by the ratio between potential activity and apparent saturation constant (Button, 1985) was fairly constant throughout the year and averaged 295×10^6 liter (l) g^{-1} dry soil hr^{-1}. The average specific affinity for nitrite oxidation measured in 14 different soil samples from the same grassland location of 12×40 m, but taken in June a year later, amounted to 382×10^6 l g^{-1} dry soil hr^{-1}. This latter value was not significantly different from the annual average. Although astonishing, the meaning of this constancy in specific affinity throughout time is not obvious in a heterogenous system such as a soil where the ability of scavenging low amounts of substrates is likely to be more important than a high specific affinity.

5. Survival Mechanisms in the Absence of Mineral Nitrogen

As was previously discussed, chemolithotrophic ammonia-oxidizing bacteria are bad competitors with respect to oxygen and ammonium when compared

with organotrophic bacteria. Which means that they will lose the competition from aerobic organotrophic bacteria under conditions of limiting supply of extracellular energy sources. So, nitrifying bacteria have to survive periods of deprivation of extracellular energy sources. Since *Nitrobacter* species can grow organotrophically (Bock *et al.*, 1992; Watson *et al.*, 1989), they may use organic compounds when there is no nitrite supply. Although observations of large numbers of *Nitrobacter* cells in soils in relation to ammonia-oxidizing cells are often explained by organotrophic growth of the former (e.g., Bock and Koops, 1992), nothing is known about their competitive ability with respect to organic compounds compared to other organotrophic bacteria. Under conditions of optimal energy supply, ammonia- as well as nitrite-oxidizing bacteria accumulate intracellular storage materials in the form of poly-β-hydroxybutyrate, polyphosphate, or glycogenlike granules (Bock and Heinrich, 1969; Bock and Koops, 1992; Gay *et al.*, 1983; Smith and Hoare, 1968; Terry and Hooper, 1970; Van Gool *et al.*, 1971). Chemolithotrophically grown cells of *N. winogradskyi* and *N. vulgaris* may possess an average poly-β-hydroxybutyrate content of 10–30% of the cell dry weight (compare Bock *et al.*, 1992). Even under growth-limiting conditions in the chemostat, the production of storage materials was very likely (Laanbroek and Gerards, 1993). Apparently, chemolithotrophic cells anticipate future energy-limited situations by production of intracellular reserve polymers under conditions of sufficient extracellular energy supply.

By endogenous respiration *N. europaea* cells were able to maintain a considerable proton gradient across the membrane in the absence of an extracellular substrate (Frijlink *et al.*, 1992a). Even at low pH ranges when ammonia oxidation was impossible, *N. europaea* cells could depend on endogenous substrates for the maintenance of a significant proton gradient. This gradient could be used to drive a secondary energy-dependent process such as active transport of alanine. The ability to actively accumulate a number of amino acids (Frijlink *et al.*, 1992b), which might include recapturing "lost" endogenous compounds, will have survival value under conditions of ammonia deprivation.

From experiments with specific inhibitors it was concluded that extra- and intracellular energy sources used two distinct systems for energy generation in *N. europaea* (Frijlink *et al.*, 1992a). Two different pathways for exo- and endogenous respiration are apparently also active in *N. winogradskyi* (Eigener, 1975; Eigener and Bock, 1975). According to these authors, nitrite oxidation in *N. winogradskyi* might even be inhibited by endogenous respiration, a characteristic that seems odd with respect to maximal utilization of extracellular energy sources and concomitant preservation of intracellular storage materials. However, a delay in switching from intra- to extracellular energy sources might be profitable in the longer term when the extracellular supply of mineral nitrogen is only available for a short period. Nitrification is inhibited in environments such as fish and oxidation ponds where relatively short cycles of oxic and anoxic conditions

prevail (Diab *et al.*, 1993). Switching from anoxic to oxic conditions, nitrification started only after a 24 to 48 hr lag period. This observation may also be important with respect to the activity of nitrifying bacteria in the root zones of waterlogged, aerenchymatous plants, where oxygen concentrations will be subjected to diurnal changes due to plant activity (Buis, personal communication).

However, nothing is known about the exact survival mechanisms of chemolithotrophic nitrifying bacteria in soils, and hence this should be the subject of further studies. An adequate survival mechanism will be essential for these slowly growing bacteria with an extremely low cell yield due to growth on ammonia and nitrite as energy sources.

6. Future Perspectives

As indicated in the introduction of this chapter, the predominance of *Nitrosomonas* species in ammonia-oxidizing communities is often questioned. Nevertheless, most of the physiological studies concerning ammonia-oxidizing bacteria have been done with the species *N. europaea*. Therefore, hardly anything could be said about physiological differences between the various ammonium-oxidizing species or about possible differences in ecological niches in the soil. Differences between the ammonia-oxidizing species and genera might become apparent by studying their temporal and spatial distribution. This can be done by using specific antibodies (Bohlool and Schmidt, 1973, 1980; Schmidt, 1973; Gay *et al.*, 1983; Josserand *et al.*, 1981) or specific DNA probes (Head *et al.*, 1993; McCaig *et al.*, 1994).

The nitrite-oxidizing genus *Nitrobacter* contains several physiologically distinct species (Bock and Koops, 1992; Bock *et al.*, 1992). The distribution of physiologically different *Nitrobacter* species in an extensively used grassland soil was studied by using specific antibodies against these species (Both *et al.*, 1992b). No cross-reactions between antisera and antigenes were observed. The serotypes of *N. hamburgensis*, *N. winogradskyi* strain ATCC253g1, and *N. winogradskyi* strain *agilis* were simultaneously present throughout the year. Their numbers were always of the same magnitude. Coexistence of more than one *Nitrobacter* species in one soil sample is also reported by Josserand *et al.* (1981) and Laanbroek and Gerards (1991). On a yearly basis, numbers of *N. hamburgensis* and *N. winogradskyi* strain *agilis* in the extensively used grassland soil were significantly ($p < 0.05$) correlated with each other. During the study of spatial distribution of these nitrite-oxidizing bacteria, numbers of *N. hamburgensis* and both *N. winogradskyi* strains were correlated at a significance level of at least 5%. As revealed from principal component analyses, numbers of *N. hamburgensis* and *N. winogradskyi* strain *agilis* were always similarly affected by pairs of unknown factors, and in the case of spatial distribution numbers of the second *N. winogradskyi* strain were affected by these unknown factors in the

same way as the other *Nitrobacter* species. This could indicate that these physiologically distinct species showed the same behavior in the soil, which seems to be unlikely. The corresponding performance of the different species might also be due to unreliability of the use of antibodies raised against laboratory strains grown under optimal conditions. In contrast to the DNA probes, specific antibodies are phenotypic markers that might be affected by the prevailing environmental conditions. Hence, antibodies raised in the laboratory may be less useful for enumerating *in situ* grown bacteria. The use of species-specific DNA markers seems to be more promising to determine possible niche differences between physiologically distinct chemolithotrophic nitrifying bacteria in soils, and more so when this enumeration method could be applied on a microscale.

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