Prokaryotes (2006) 5:861–872
DOI: 10.1007/0-387-30745-1_41

CHAPTER 3.2.17

The Nitrite-Oxidizing Bacteria

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Introduction

Nitrite-oxidizing bacteria are a small group of primarily organo and/or chemoautotrophs that are difficult to grow and work with. For many years they drew very little attention, although it was realized that they provide a key link in the global nitrogen cycle between the ammonia-oxidizing bacteria, which generate nitrite, and the various denitrifying microorganisms that remove nitrate by reducing it to ammonia or molecular nitrogen, thus completing the global nitrogen cycle.

The growing public awareness relating to issues of environmental pollution in recent decades brought renewed interest in this group of bacteria, as their role in the transformation of nitric oxides became apparent. In addition, these bacteria cause economic damage to agriculture and to ground water quality by contributing to the leaching of nitrogen fertilizers (distributed in the form of ammonia) from surface soils, thus polluting aquifers with increasing concentrations of nitrites and nitrates.

Owing to their key role in the global cycling of nitrogen and their activities both as prime contributors to and scavengers of nitric oxides in the biosphere, nitrite-oxidizing bacteria became in recent years the focus of intensive ecophysiological research, in spite of the difficulties in cultivating and maintaining many of these bacteria in the laboratory.

In addition to this group of autotrophic nitrifyers, there is also a diverse group of heterotrophic bacteria capable of heterotrophic nitrite oxidation. Thus, in one such study (Sakai et al., 1996), the nitrite transforming activities of heterotrophic bacterial strains from various culture collections as well as isolates from activated sludge were studied. Of the 48 strains tested, 17 strains consumed 1–5 mM of nitrite and accumulated corresponding amounts of nitrate. Heterotrophic microorganisms are rather flexible in determining the fate of the consumed nitrite. For instance, in *Bacillus subtilis* strain I-41 (a denitrification-positive isolate), the ratio of the amount of nitrate accumulated to that of nitrite consumed varied from 0 to 100%, depending on the culture conditions. However, the mechanism of oxidation of nitrite to nitrate in these heterotrophic bacteria is very different from that in the autotrophic bacteria (Sorokin, 1991), as heterotrophic nitrite oxidation requires catalase and hydrogen peroxide (H_2O_2) generated through oxidation of organic electron donors. Also, aeration affects the nitrite- and nitrate-transforming activities of various heterotrophic bacteria. For example, *Pseudomonas pavonaceae*, a denitrification-positive strain, metabolizes both nitrite and nitrate to more reduced compounds at low oxygen pressure, and the direction of the conversion changes from reduction to oxidation at high oxygen pressure. This switching might be caused by inhibition and repression of the nitrite-reducing activity and by stimulation of nitrite-oxidizing activity by oxygen (Sakai et al., 1997).

Historic Background

Nitrite-oxidizing bacteria carry the second stage of the nitrification process, that of oxidation of nitrite to nitrate. Because nitrate is an essential ingredient of gunpowder, nitrate and nitrification were the focus of interest both to scientists and politicians for many centuries. Nitrification protocols for the manufacture of nitrates by composting organic matter in soil have existed for many centuries: from the tenth century in China and from the twelfth century in Europe (Macdonald, 1986). However, the process was considered to be of a chemical and not biological nature until the late nineteenth century. It was only in 1862 that Pasteur suggested that nitrification was of biological origin (Pasteur, 1862), and it was only when Winogradsky in 1891 succeeded in isolating a nitrite-oxidizing bacterium that the debate about whether nitrification is a one-stage process (carried by a single bacterium) or a two-stage process (carried by two distinct classes of microorganisms) was settled (Macdonald, 1986).

Nitrite Oxidation

A detailed account of the mechanism of nitrite oxidation as an energy source has been described previously (Bock et al., 2001). The key enzyme that makes the nitrite-oxidizing bacteria so important in the global nitrogen cycle is nitrite oxidoreductase (NOR), which carries out the stoichiometric reaction:

$$NO_2^- + H_2O \Leftrightarrow NO_3^- + 2H^+ + e^- \quad (1)$$

$$2H^+ + 2e^- + 0.5O_2 \rightarrow H_2O \quad (2)$$

Reaction (1) is reversible, and many nitrite oxidizers are capable of reducing nitrate to nitrite under anaerobic conditions, but the significance of the reverse reaction to their survival is not clear, as both nitrite and nitrate are rapidly reduced in anaerobic environments.

Nitrite oxidoreductase was isolated from various *Nitrobacter* strains, such as from mixotrophically grown cells of *Nitrobacter hamburgensis*; the enzyme purified from heat-treated membranes was homogeneous by the criteria of polyacrylamide gel electrophoresis and size exclusion chromatography. The monomeric form consisted of two subunits with molecular weights of 115 kDa and 65 kDa, respectively. The dimeric form of the enzyme contained 0.70 molybdenum, 23.0 iron, 1.76 zinc and 0.89 copper gram-atoms per molecule. The catalytically active enzyme was investigated by visible and electron paramagnetic resonance spectroscopy (EPR) under oxidizing (as isolated), reducing (dithionite), and turnover (nitrite) conditions. As isolated, the enzyme exhibited a complex set of EPR signals between 5–75 K, originating from several iron-sulfur and molybdenum (V) centers. Addition of the substrate nitrite or the reducing agent dithionite resulted in a set of new resonances. The molybdenum and the iron-sulfur centers of nitrite oxidoreductase from *Nitrobacter hamburgensis* were involved in the transformation of nitrite to nitrate (Meincke et al., 1992).

Systematics

As late as 1981, all bacteria capable of chemolithotrophic growth using ammonia ornitrite as energy source and capable of assimilating CO_2 via the Calvin cycle were grouped in one family, the Nitrobacteraceae (Watson et al., 1981). This classification was based on the fact that all the bacteria grouped in this family carry out oxidation reactions of inorganic nitrogen compounds, and all are capable of chemolithotrophic growth using these as energy sources. All are Gram-negative bacteria and usually found in association; as a physiological interdependence exists between the two groups, inasmuch as the ammonia oxidizers provide the substrate for the nitrite oxidizers, whereas the nitrite oxidizers are inhibited by excess ammonia.

With the advent of molecular biology and classification of bacteria through sequencing of 16S ribosomal RNA genes and other specific DNA sequences, as well as the use of monoclonal antibodies, it became evident that the two groups are not related, and that there is no phylogenetic relationship between ammonia and nitrite oxidizers. Almost all nitrite oxidizers (with the exception of *Nitrospira moscoviensis*, which seems to occupy an intermediate position; Schramm et al., 1998) are a homogenous group belonging to the α-subdivision of the Proteobacteria and are very closely related to the nonsulfur purple photosynthetic bacterium *Rhodopseudomonas palustris*. None of the ammonia-oxidizing bacteria belong to the α-subdivision, and they are all distributed between the β- and γ-subdivisions (Woese et al., 1984; Woese et al., 1985).

Comparative 16S rDNA sequencing was used to evaluate phylogenetic relationships among selected strains of ammonia- and nitrite-oxidizing bacteria (Teske et al., 1994). All characterized strains were shown to be affiliated with the proteobacteria. The study extended recent 16S rDNA-based studies of phylogenetic diversity among nitrifiers by the comparison of eight strains of the genus *Nitrobacter* and representatives of the genera *Nitrospira* and *Nitrospina*. The latter genera were shown to be affiliated with the δ-subdivision of the proteobacteria but did not share a specific relationship to each other or to other members of the δ-subdivision. All characterized *Nitrobacter* strains constituted a closely related assemblage within the α-subdivision of the Proteobacteria. As previously observed, all ammonia-oxidizing genera except *Nitrosococcus oceanus* constitute a monophyletic assemblage within the β-subdivision of the proteobacteria. Consideration of physiology and phylogenetic distribution suggests that nitrite-oxidizing bacteria of the α- and γ-subdivisions are derived from immediate photosynthetic ancestry. Each nitrifier retains the general structural features of the specific ancestor's photosynthetic membrane complex. Thus, the nitrifiers, as a group, apparently are not derived from an ancestral nitrifying phenotype.

Watson et al. (1981) identified three genera of nitrite-oxidizing bacteria (the *Nitrospira* being discovered only in 1986; Watson, 1986) and classified them on the basis of morphology and nutritional requirements. Bartosch et al. (1999) also divided the nitrite-oxidizing bacteria into four genera, *Nitrobacter*, *Nitrospina*, *Nitrococcus* and

Nitrospira, according to their reactions with monoclonal antibodies prepared against the nitrite oxidoreductase of the genus *Nitrobacter*. Among these, *Nitrobacter* and *Nitrospira* strains are capable of heterotrophic or mixotrophic growth, the rest being strict chemolithotrophs.

Because of the difficulties in culturing nitrifying bacteria on solid media, the methods for enumerating and identifying nitrifiers in environmental samples conventionally depended on incubating serial dilutions for very long periods, and then testing these for appearance of nitrate. Thus, the dominant nitrite-oxidizing species in an environmental sample was usually identified only after incubation of 3–6 months and was acertained on the basis of morphological and physiological properties such as sensitivity to salts and nutrient concentration. These observations led over the years to a general consensus that *Nitrobacter* strain were the dominant nitrite-oxidizing organisms in practically all tested environments such as soil, marine waters, wastewater treatment plants, fresh water and reservoirs; *Nitrococcus* and *Nitrospina* were rarely identified (Watson et al., 1981).

In 1995, a Gram-negative, nonmotile, nonmarine, nitrite-oxidizing bacterium was isolated by enrichment culture of a sample from a partially corroded area of an iron pipe located in a heating system in Moscow, Russia (Ehrich et al., 1995). The cells (0.9–2.2 μm × 0.2–0.4 μm in size) were helical to vibroid-shaped and often formed spirals with up to three turns 0.8–1.0 μm in width. The organism possessed an enlarged periplasmic space and lacked intracytoplasmic membranes and carboxysomes. The cells excreted extracellular polymers and formed aggregates. The bacterium grew optimally at 39°C and pH 7.6–8.0 in a mineral medium with nitrite as sole energy source and carbon dioxide as sole carbon source. The doubling time was 12 h in a mineral medium with 7.5 mM nitrite. The cell yield was low; only 0.9 mg of protein/liter was formed during oxidation of 7.5 mM nitrite. Under anoxic conditions, hydrogen could be used as electron donor with nitrate as electron acceptor. Organic matter (yeast extract, meat extract and peptone) supported neither mixotrophic nor heterotrophic growth. At concentrations as low as 0.75 g of organic matter/liter or higher, growth of the nitrite-oxidizing cells was inhibited. The cells contained cytochromes of the *b*- and *c*-type. The G+C content of DNA was 56.9 ± 0.4 mol%. This chemolithoautotrophic nitrite-oxidizer differed from the terrestrial members of the genus *Nitrobacter* with regard to morphology and substrate range and was similar to *Nitrospira marina* in both characteristics. The isolated bacterium was designated as a new species of the genus *Nitrospira*. Recent studies suggest that this genus dominates most of the nitrite oxidation activities in natural as well as artificial habitats (see below).

With the introduction of molecular technologies to environmental microbiology, the true dominant nitrite oxidizers could be identified in situ in environmental samples, either by polymerase chain reaction (PCR, using specific rDNA primers) or fluorescence in situ hybridization (FISH, using specific probes). These studies led to the surprising conclusion that *Nitrospira* strains are the dominant nitrite oxidizers in most environmental samples tested so far, whereas *Nitrobacter* sp. are seldom identified without prior enrichment (Burrell et al., 1998; Burrell et al., 1999; Schramm et al., 1998; Juretschko et al.,1998; Okabe et al., 1999).

There is, however, no doubt that within the next years, with the advent of oligonucleotide microchip technology, oligonucleotide microchips will dominate as genosensors for determinative and environmental studies in microbiology (Guschin et al., 1997). Precise identification of bacterial communities and of the genes switched on by each member of these communities under various environmental conditions will become routine in the environmental microbiology lab, and these procedures will enable the fine resolution of the community of nitrifying bacteria and its activities in different biotopes.

DNA sequencing methodologies also were used to identify bacteria in situ, in biofilms and activated sludge particles (Schramm et al., 1998). Bacterial aggregates from a chemolithoautotrophic, nitrifying fluidized bed reactor were investigated with microsensors and rDNA-based molecular techniques. The microprofiles of O_2, NH_4^+, NO_2^- and NO_3^- demonstrated the occurrence of complete nitrification in the outer 125 μm of the aggregates. FISH analysis showed that the dominant populations were of *Nitrospira* spp. and *Nitrosospira* sp. and that they formed separate, dense clusters which were in contact with each other and occurred throughout the aggregate. Significantly, no ammonia- or nitrite-oxidizing bacteria of the genera *Nitrosomonas* or *Nitrobacter*, respectively, could be detected by FISH. To identify the nitrite oxidizers, a 16S ribosomal DNA clone library was constructed and screened by denaturing gradient gel electrophoresis (DGGE), and selected clones were sequenced. The organisms represented by these sequences formed two phylogenetically distinct clusters affiliated with the nitrite oxidizer *Nitrospira moscoviensis*. The dominant *Nitrospira* sp. formed clusters with the nitrosospira sp. and occurred throughout the aggregate while the second, smaller, morphologically and genetically different population of *Nitrospira* sp. was

restricted to the outer nitrifying zones. The phylogeny of bacteria belonging to the genus *Nitrobacter* was investigated by sequencing the whole 16S rRNA gene (Orso et al., 1994). The average level of similarity for three *Nitrobacter* strains examined was high (99.2%), and the similarity level between *Nitrobacter winogradsky* and *Nitrobacter* sp. strain LL, which represent two different genomic species, was even higher (99.6%). When all of the *Nitrobacter* strains and their phylogenetic neighbors *Bradyrhizobium* and *Rhodopseudomonas* species were considered, the average similarity level was 98.1%. When complete sequences were used, *Nitrobacter hamburgensis* clustered with the two other *Nitrobacter* strains, though this was not the case when partial sequences were used. The two *Rhodopseudomonas palustris* strains examined exhibited a low similarity level (97.6%) and were not clustered.

In another study (Okabe et al., 1999), the in situ spatial organization of ammonia-oxidizing and nitrite-oxidizing bacteria in domestic wastewater biofilms and autotrophic nitrifying biofilms was investigated by using microsensors and FISH, performed with 16S rDNA-targeted oligonucleotide probes. The combination of these techniques made it possible to relate in situ microbial activity directly to the occurrence of specific nitrifying bacterial populations. In situ hybridization revealed that bacteria belonging to the genus *Nitrobacter* were not detected; instead, *Nitrospira*-like bacteria were the main nitrite-oxidizing bacteria in both types of biofilms. *Nitrospira*-like cells formed irregularly shaped aggregates consisting of small microcolonies, which bound the clusters of ammonia oxidizers. Whereas most of the ammonia-oxidizing bacteria were present throughout the biofilms, the nitrite-oxidizing bacteria were restricted to the active nitrite-oxidizing zones, which were inside the biofilms. Microelectrode measurements showed that the active ammonia-oxidizing zone was located in the outer part of a biofilm, whereas the active nitrite-oxidizing zone was located just below the ammonia-oxidizing zone and overlapped the location of nitrite-oxidizing bacteria, as determined by FISH.

The use of fluorescent monoclonal antibodies was also very useful for the rapid quantification and in situ detection of specific nitrifiers in a mixed bacterial habitat such as a biofilm (Noda et al., 2000). In that study, 12 monoclonal antibodies against *Nitrosomonas europaea* (IFO14298) and 16 against *Nitrobacter winogradsky* (IFO 14297) enabled a direct cell count of *N. europaea* and *N. winogradsky*. Moreover, the distribution of N. europaea and N. winogradsky in a biofilm could be examined. Most of *N. winogradsky* existed near the surface and most of *N. europaea* existed within the polyethylene glycol (PEG) gel pellet.

Using DNA sequences from the intergenic spacer (IGS) region of the ribosomal operon, and two primers derived from 16S and 23S rDNA conserved sequences, Navarro et al. (1992a) amplified these sequences by PCR. The PCR products, cleaved by four base cutting restriction enzymes, were used to differentiate *Nitrobacter* strains. This method is convenient for the genotypic characterization of *Nitrobacter* isolates and was successfully used to characterize natural populations of *Nitrobacter* from various soils and a lake. Considerable diversity was demonstrated in various soils, and in both water and sediments of the lake.

Navarro et al. (1992b) also studied the genomic diversity of *Nitrobacter* strains by determining rDNA gene restriction patterns as well as hybridization characteristics and DNA base compositions. The DNA hybridization (S1 nuclease method) revealed five DNA genomic groups, and these groups formed three genomic *Nitrobacter* species.

Mobarry et al. (1996) prepared a hierarchical set of five 16S rRNA-targeted DNA probes for phylogenetically defined groups of autotrophic ammonia- and nitrite-oxidizing bacteria. Their environmental application was demonstrated by quantitative slot blot hybridization and whole-cell hybridization of nitrifying activated sludge and biofilm samples. In situ hybridization experiments revealed that *Nitrobacter* and *Nitrosomonas* species occurred in clusters and frequently were in contact with each other within sludge flocs.

The nitrite oxidoreductase (NOR) from the facultative nitrite-oxidizing bacterium *Nitrobacter hamburgensis* X14 was used to develop a probe for the gene *norB* (Kirstein and Bock, 1993). Sequence analysis of DNA fragments revealed three adjacent open reading frames in the order *norA*, *norX* and *norB*. The deduced amino acid sequence of protein NorB contained four cysteine clusters with striking homology to those of iron-sulfur centers of bacterial ferredoxins. Protein NorB shares significant sequence similarity to the β-subunits (NarH and NarY) of the two dissimilatory nitrate reductases (NRA and NRZ) of *Escherichia coli*. Additionally, the derived amino acid sequence of the truncated open reading frame of norA showed striking resemblance to the α-subunits (NarG and NarZ) of the *E. coli* nitrate reductases. Additionally, the derived amino acid sequence of the truncated open reading frame of norA showed resemblance to the α-subunits of the *E. coli* nitrate reductases.

Monoclonal antibodies prepared against nitrite oxidoreductases of nitrite oxidizers also

were used successfully for the identification of nitrite oxidizers (Bartosch et al., 1999). Immunoblot analyses performed with three monoclonal antibodies (MAbs) that recognized the nitrite oxidoreductase (NOR) of the genus *Nitrobacter* were used for taxonomic investigations of nitrite oxidizers. These MAbs were able to detect the nitrite-oxidizing systems (NOS) of the genera *Nitrospira*, *Nitrococcus* and *Nitrospina*. When the genus-specific reactions of the MAbs were correlated with 16S rDNA sequences, they reflected the phylogenetic relationships among the nitrite oxidizers. In ecological studies, the immunoblot analyses demonstrated that *Nitrobacter* or *Nitrospira* cells could be enriched from activated sludge by using various substrate concentrations.

The microbiology of the biomass from a nitrite-oxidizing sequencing batch reactor (NOSBR) as well as the seed sludge used were investigated by microscopy, by culture-dependent methods, and by molecular biological methods (Burrell et al., 1998). The NOSBR was fed with an inorganic salts solution and nitrite as the sole energy source. It was operated for 6 months, and 16S ribosomal DNA clone libraries were prepared both from the seed sludge and from the reactor. Analysis of the seed sludge revealed that it contained three clones (4% of biomass) that were closely related to the autotrophic nitrite-oxidizer *Nitrospira moscoviensis*, whereas the NOSBR sludge itself was overwhelmingly dominated by bacteria closely related to the *N. moscoviensis* (89%). Only two clone sequences were similar to those of the genus *Nitrobacter*. Near-complete insert sequences of eight clones of *N. moscoviensis* isolated from the NOSBR and one clone from the seed sludge were determined and phylogenetically analyzed. This report was the first to show the presence of bacteria from the *Nitrospira* genus in wastewater treatment systems.

Nitrite-oxidizing bacteria belonging exclusively to the genus *Nitrospira* also dominated the nitrite-oxidizing community of a phosphate-removing biofilm from a sequencing batch biofilm reactor (Gieseke et al., 2001).

In another study (Burrell et al., 1999), a sequencing batch reactor (SBR) was operated to selectively grow a nitrite-oxidizing microbial community, and it was found that the nitrite oxidation was due the presence of bacteria from the *Nitrospira* genus and not the *Nitrobacter* genus, which were in very low numbers. It was hypothesized that the unknown nitrite-oxidizing bacteria in wastewater treatment plants are a range of species related to *Nitrospira moscoviensis*.

Oxidation of nitrite to nitrate in aquaria is typically attributed to bacteria belonging to the genus *Nitrobacter* that are members of the α-subdivision of the class Proteobacteria. To identify bacteria responsible for nitrite oxidation in aquaria, clone libraries of rRNA genes were developed by Hovanec et al. (1998) from biofilms from several freshwater aquaria, and analysis of the rDNA libraries, along with results from denaturing gradient gel electrophoresis (DGGE) on frequently sampled biofilms, indicated the presence of putative nitrite-oxidizing bacteria closely related to other members of the genus *Nitrospira*. Hybridization experiments with rRNA from biofilms of freshwater aquaria demonstrated that *Nitrospira*-like rRNA comprised nearly 5% of the rRNA extracted from the biofilms during the establishment of nitrification. Nitrite-oxidizing bacteria belonging to the α-subdivision of the class Proteobacteria (e.g., *Nitrobacter* spp.) were not detected in these samples. Aquaria that received a commercial preparation containing *Nitrobacter* species did not show evidence of *Nitrobacter* growth and development but did develop substantial populations of *Nitrospira*-like species. Time series analysis of rDNA phylotypes on aquaria biofilms by DGGE, combined with nitrite and nitrate analysis, showed a correspondence between the appearance of *Nitrospira*-like bacterial ribosomal DNA and the initiation of nitrite oxidation. In total, the data suggest that *Nitrobacter winogradsky* and close relatives were not the dominant nitrite-oxidizing bacteria in freshwater aquaria. Instead, nitrite oxidation in freshwater aquaria appeared to be mediated by bacteria closely related to *Nitrospira* sp.

Isolation and Nutritional Requirements

The information in this section is compiled from the chapter by Bock and Koops (1992) on Nitrobacter in the second edition published in the previous edition of {The Prokaryotes}.

Obligate and facultative lithoautotrophic nitrite oxidizers can be successfully isolated only if mineral nitrite media free of any organic contaminants are used, otherwise heterotrophs will overgrow nitrite oxidizers. A sample of 1–2 g (soil, stone, mud etc.) is placed in 100 ml of enrichment medium selective for the growth of either terrestrial or marine strains (Table 1). For the enrichment of dominant nitrifiers, a combination of the most probable number (MPN) technique followed by the serial dilutions method is suitable. In the highest dilutions, nitrite oxidation can be detected only after several months because of the slow growth of the nitrite oxidizers. Plating samples from enrichment cultures on rich and poor solid agar media

Table 1. Four culture media[a] for lithoautotrophic and mixotrophic growth of nitrite oxidizers.

Ingredient	1a	2b	3b	4c
Distilled water (ml)	1,000	1,000	1,000	300
Seawater (ml)				700
$NaNO_2$ (mg)		2,000	2,000	69
KNO_2 (mg)	300			
$MgSO_4 \cdot 7H_2O$ (mg)	187.5	50	50	100
$CaCl_2 \cdot 2H_2O$ (mg)	12.5			6.0
$CaCO_3$ (mg)		3.0	3.0	
KH_2PO_4 (mg)	500	150	150	
K_2HPO_4 (mg)	500			1.74
$FeSO_4$ (mg)	10	0.15	0.15	
Chelated iron[b] (mg)				1.0
Na_2Mo_4 (μg)				30
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (μg)		50	50	
$MnCl_2 \cdot 4H_2O$ (μg)				66
$CoCl_2 \cdot 6H_2O$ (μg)				0.6
$CuSO_4 \cdot 5H_2O$ (μg)				6.0
$ZnSO_4 \cdot 7H_2O$ (μg)				30
$KHCO_3$ (mg)	1,500			
NaCl (mg)		500	500	
Sodium pyruvate (mg)			550	
Yeast extract[c] (mg)			1,500	
Peptone[c] (mg)			1,500	

[a]pH is adjusted to 7.4–7.8.
[b]13%, Geigy.
[c]Difco.

can check contamination by heterotrophs. It may take a year to obtain pure cultures with the technique described above.

Medium 1a for terrestrial strains is from Aleem and Alexander (1958), media 2b and 3b also for terrestrial strains are from Bock and Stackebrandt (1983), and medium 4c for marine strains is from Watson and Waterbury (1971).

Habitats

As described earlier, the nitrite-oxidizing bacteria are ubiquitous, and are usually found in association with the ammonia-oxidizing bacteria, which provide them with their energy source. In spite of many reports describing their sensitivity to light (Guerrero and Jones, 1996; Guerrero and Jones, 1997) and various toxicants (Deni and Penninckx, 1999; Jenicek et al., 1996), nitrite-oxidizing bacteria are abundant in practically all terrestrial, marine and fresh water habitats. Nitrifying bacteria play a significant role in erosion of rocks (Lebedeva et al., 1978) primarily because they release nitrates, which dissolve carbonates, in exchange for CO_2 they assimilate. This process was found to be a major cause of deterioration of historic monuments in Europe (Mansch and Bock, 1998). Proliferation of nitrifiers in sewage is also the basis for nitrogen removal from domestic and industrial waste in water treatment plants, including chemical industry wastewater. Nitrite oxidizers grow within a wide range of pH values up to 10 (Sorokin, 1998a; Sorokin et al., 1998b) and salinities (limits are difficult to determine as nitrifying bacteria resist very low water activities, such as occur when living within desiccated rocks).

Although nitrite oxidizers are considered obligate aerobes (even though many can utilize nitrate in absence of oxygen as an electron acceptor), growth of nitrite oxidizers can be observed in totally anaerobic environments such as deep in the mud at the bottom of deep anaerobic wastewater reservoirs, in the absence of nitrate or oxygen, in presence of significant concentrations of sulfides (Abeliovich, 1987). Also, nitrifying bacteria were isolated from a marine anaerobic sediment at depths of up to 8 cm (Blackburn, 1983). Nothing is at present known about the metabolism and survival mechanisms of these nitrite-oxidizing bacteria living under strict anaerobic, reducing conditions.

Light (particularly blue light) is frequently mentioned as an inhibiting factor for the nitrification process. However, the reports on the effects of light are sometimes contradictory. Nitrifiers are found in all habitats exposed to sunlight, either at the surface of fresh water or marine habitats, so obviously they must have efficient protection mechanisms to overcome photoinhibition and damage caused by direct sunlight.

Sorokin et al. (1998b) and Sorokin (1998a) have isolated five strains of lithotrophic, nitrite-oxidizing bacteria from sediments of three soda lakes after enrichment at pH 10 with nitrite as sole energy source; these strains were described as a new species of the genus *Nitrobacter*, *N. alkalicus*. Nitrite oxidation to nitrate occurred at pH 10. The nitrifiers had pear-shaped budding cells morphologically similar to those of *Nitrobacter* and formed tiny colonies on mineral nitrite agar at pH 10.

In a study aimed at evaluating the potential of wastewater treatment plants to contaminate receiving waterways, survival of *Nitrobacter* cells associated with particles in water treatment plant discharges was studied using immunofluoresence methods (Bonnet et al., 1997). It was found that *Nitrobacter* colonies can settle in freshwater sediments in a week (10^6 cells per gram of dry sediment) and can therefore colonize river sediments.

Chemolithotrophic nitrifying bacteria are dependent on the presence of oxygen for the oxidation of ammonium via nitrite to nitrate. The success of nitrification in oxygen-limited environments largely depends on the oxygen sequestering abilities of both ammonium- and

nitrite-oxidizing bacteria. Oxygen consumption kinetics were determined with cells grown in mixed culture in chemostats at different growth rates and oxygen tensions (Laanbroek and Gerards, 1993). Reduction of oxygen tension in the culture was found to repress the oxidation of nitrite before the oxidation of ammonium was affected, and hence nitrite accumulated.

In addition to the competition between ammonium- and nitrite-oxidizing bacteria, there is competition with organotrophic bacteria for the available oxygen as well. The outcome of the competition is determined by their specific affinities for oxygen as well as by their population sizes. The effect of mixotrophic growth of nitrite-oxidizing *Nitrobacter hamburgensis* on the competition for limiting amounts of oxygen was studied in mixed continuous culture experiments with the ammonium-oxidizing *Nitrosomonas europaea* at different oxygen concentrations (Laanbroek et al., 1994). The specific affinity for oxygen of *N. europaea* was in general higher than that of *N. hamburgensis*, and in transient state experiments, when oxic conditions were switched to anoxic, *N. hamburgensis* was washed out and nitrite accumulated. However, at low oxygen concentration, the specific affinity for oxygen of *N. hamburgensis* increased and became as great as that of *N. europaea*, and owing to its larger population size, the nitrite-oxidizing bacterium became the better competitor for oxygen and ammonium accumulated. Therefore Laanbroek et al. suggest that continuously oxygen-limited environments present a suitable ecological niche for the nitrite-oxidizing *N. hamburgensis*.

Biofilms

Biofilms present a unique biotope for using molecular DNA techniques as well as FISH and various sensors. The combination of microsensor and molecular techniques is highly useful for studies on the microbial ecology of biofilms in general, and in particular for the identification of activity sites of nitrifying bacteria.

Thus, the distribution of nitrifying bacteria of the genera *Nitrosomonas*, *Nitrosospira*, *Nitrobacter* and *Nitrospina* was studied by Schramm et al. (2000) in a membrane-bound biofilm system in which gradients of oxygen, pH, nitrite and nitrate were determined by means of microsensors, while the nitrifying populations along these gradients were identified and quantified using FISH in combination with confocal laser scanning microscopy. It was found that the oxic part of the biofilm was dominated by ammonia oxidizers and by members of the genus *Nitrobacter*, and *Nitrospira* sp. were virtually absent in this part of the biofilm, whereas they were most abundant at the oxic-anoxic interface. In the totally anoxic part of the biofilm, cell numbers of all nitrifiers were relatively low. These observations suggest the microaerophilic behavior of an as yet uncultured *Nitrospira* sp. as a factor affecting its environmental competitiveness.

De Beer and Schramm (1999), using microsensors and molecular techniques (such as in situ hybridization with 16S rDNA-targeted oligonucleotide probes), showed that there exists in biofilms grown in bioreactors a complex nitrifying community, consisting of members of the genera *Nitrosomonas*, *Nitrosospira*, *Nitrobacter* and *Nitrospira*.

In nitrifying biofilms from a trickling filter of an aquaculture water recirculation system, it was found that nitrification was restricted to a narrow zone of 50 μm of the very top of the film. Ammonia oxidizers formed a dense layer of cell clusters in the upper part of the biofilm, whereas the nitrite oxidizers showed less-dense aggregates in the proximity of the ammonia oxidizers. Both ammonia and nitrite oxidizers were not restricted to the oxic zone of the biofilm but were also detected in substantially lower numbers in the anoxic layers and even occasionally at the bottom of the biofilm (Schramm et al., 1996).

Enumeration

Conventional methodologies for counting nitrifiers are gradually being replaced by new technologies, although the MPN method still dominates, this because all methods based on specific probes or antibodies still leave open the possibility that some species do not crossreact with the probe used. On the other hand, the issue of optimal conditions for maximal yields using MPN counts has not been yet resolved.

One example is a new amperometric enzyme-linked immunoassay for specific enumeration of *Nitrobacter*, which uses an electrode made of glassy carbon on which an immunological reaction is carried out. The detection limit was approximately 3×10^6 *Nitrobacter* cells/ml (Sanden and Dalhammar, 2000). It was shown that this method could be applied for the enumeration of *Nitrobacter* in activated sludge and other environmental samples. The reason for the attempts to develop new methodologies is the lengthy incubation periods required for stable MPN counts: maximum most probable numbers of the ammonia-oxidizing group were attained in 20–55 days (median, 25) and MPN estimates of nitrite oxidizers required a much longer incubation (103–113 days; Matulewich et al., 1975). In addition, Both et al. (1990a) and (1990b) studied the effect of two concentrations of nitrite in the

incubation medium, 0.05 and 5.0 mM, and found that numbers of nitrite-oxidizing bacteria were highly dependent on the nitrite concentration as well as on the soil sampled.

Neither the influence of pH or nitrite concentration in the incubation medium could account exclusively for the MPN-enumeration result, which raises the issue of whether to use more than one incubation medium for the enumeration of nitrite-oxidizing bacteria. Enumeration of nitrite-oxidizing bacteria in soil samples by the MPN technique often showed relatively high cell number at a low nitrite concentration, when compared to the number of ammonium-oxidizing bacteria. When different *Nitrobacter* species as well as nongrowing cells differing in age were incubated 5 months at 20°C in presence of various nitrite concentrations and pH values, it appeared that the growth of cells taken from an early stationary phase culture of all these species was not affected by high nitrite concentrations or low pH. Growth of 8- and 18-month-old nongrowing cells of *Nitrobacter hamburgensis* was also insensitive to high nitrite concentration (5 mM). The growth of 8- and 18-month old resting cells of *N. vulgaris* was repressed only by a combination of 5 mM nitrite and a low pH. Growth of 8-month-old nongrowing cells of *N. winogradsky* was sensitive to 5 mM irrespective of pH, but growth of 18-month-old cells were inhibited by 5 mM nitrite only at a low pH. The growth of 8- and 18-month-old resting cells of *N. winogradsky* serotype *agilis* was repressed by low pH rather than by high nitrite concentration (Laanbroek and Schotman, 1991). These results emphasize the problems associated with using MPN counts for estimating size of nitrifier populations in natural samples.

A molecular approach based on PCR that was expected to detect and quantify nitrifying bacteria was also tested using specific primers of the genus *Nitrobacter*. In this study, coupled in parallel was a ^{14}C-radiotracing method used to measure potential nitrification; it was shown that *Nitrobacter* represented less than 0.1% of the total bacterial community (Berthe et al., 1999).

In another study, also aimed at counting *Nitrobacter* populations in situ by PCR, two primers from the 16S rDNA gene were used to generate a 397-bp fragment by amplification of *Nitrobacter* species DNA, and it was found that the PCR had a lower detection threshold (10^2 *Nitrobacter* cells per gram of soil) than did the MPN or fluorescent antibody method (Degrange and Bardin, 1995). In contrast, another study (Feray et al., 1999) found the best recovery yield was obtained with the immunofluorescence technique (21.3%), and the poorest detection level was reached with the MPN method (3.1%).

Wastewater

Tertiary wastewater treatment, aimed at removing all nitrogen wastes from the effluents, is costly in terms of the oxygen required for oxidizing ammonia to nitrate. However, as far as the nitrification—denitrification process is concerned, oxidation of nitrite to nitrate is an unnecessary step, as denitrification can just as well proceed from nitrite. Therefore, attempts are being made to devise operational parameters that will inhibit the activity of nitrite-oxidizing bacteria in wastewater treatment plants.

Thus, it was found (Rhee et al., 1997) that accumulation of nitrite occurred during the aerobic phase of a sequencing batch reactor (SBR) operating to remove nitrogen from synthetic wastewater: the activity of autotrophic nitrite oxidizers was reduced in the SBR and the free ammonia was the main inhibitor of nitrite oxidation.

It has been shown experimentally (Garrido et al., 1997) that it is possible to convert all ammonium to approximately 50% nitrate and 50% nitrite in the effluent of a biofilm air-lift suspension reactor, this with oxygen concentrations between 1 and 2 mg/liter. The ammonia- and nitrite-oxidizing bacterial populations occurring in the nitrifying activated sludge of an industrial plant treating sewage with high ammonia concentrations were also studied using in situ hybridization with a set of hierarchical 16S rDNA-targeted probes (Juretschko et al., 1998). Although a *Nitrobacter* strain was isolated, members of the genus *Nitrobacter* were not detectable in the activated sludge by in situ hybridization. A specific 16S rDNA-targeted probe for *Nitrospira* demonstrated that a *Nitrospira*-like bacterium was present in significant numbers (9% of the total bacterial counts).

According to Blackall (2000), even though it is common knowledge that *Nitrosomonas* and *Nitrobacter* are the major ammonia and nitrite oxidizers, today we know that these organisms may not play any role in the transformations for which they have achieved such acclaim. Using the above-mentioned methodologies, *Nitrospira*-like bacteria were found to be the dominant nitrite oxidizers in both enriched and full scale nitrifying systems.

Resistance, Inhibition and Biodegradation

Because inhibition of nitrite oxidation is economically advantageous in wastewater treatment plants, procedures were developed for identification of nitrification inhibitors in wastewater.

These include fractionation of wastewater samples and a nitrification inhibition assay with pure cultures of *Nitrobacter* to identify the inhibitory effect. In such a study, a series of unsaturated fatty acids and two monoterpenes were found to be inhibitory in an industrial wastewater sample (Svenson et al., 2000).

A shorter nitrification-denitrification cycle occurs in the presence of free ammonium. Although the occurrence of this cycle depends on both a high ammonia concentration and high pH, it was found that the pH of the wastewater is the decisive parameter (Surmacz-Gorska et al., 1997). However, a study aimed at identifying nitrification at high pH values in soda lakes and soda soils with pH 9.5–11 led to the isolation of a nitrifier morphologically similar to *Nitrobacter* that formed tiny colonies on mineral nitrite agar at pH 10 (Sorokin, 1998a).

Hwang et al. (2000), in a detailed study of the inverse relation between alkalinity and ammonia with respect to inhibition of nitrite oxidation, showed that when the molar ratio of carbonate alkalinity to ammonia increased from 4.1 to 9.4 (thus increasing the concentration of free ammonia), the ammonium removal rate doubled. At the same time the higher concentration of free ammonia in the medium was a selective inhibitor for *Nitrobacter*, causing an enhanced nitrite build-up in a biofilm reactor.

As for the effect of heavy metals, laboratory evaluations were conducted to study the toxic effects of copper and nickel on a culture of strictly obligatory nitrifiers (*Nitrosomonas* sp. and *Nitrobacter* sp.) in continuous flow stirred tank reactors. *Nitrosomonas* sp. was found to be equally or more sensitive than *Nitrobacter* sp. (Lee et al., 1997).

Attempts were made to develop a process for the simultaneous removal of organic halogens and nitrogen from kraft pulp mill effluents. A nitrifying biofilm reactor removed organic halogens from bleached kraft pulp mill effluents including chlorophenols from synthetic wastewater (Kostyal et al., 1997; Kostyal et al., 1998). In another study, none of several chemicals tested (nonylphenol, naphthalene, 2-methylnaphthalene, di-2-ethylhexylphthalate and toluene) inhibited nitrification when added to soil at various concentrations (Kirchmann et al., 1991). However, in many cases municipal wastewater did contain substances that inhibited nitrification to varying degrees (Jonsson et al., 2000).

Reports conflict on the effect of toluene on indigenous microbial populations. Nitrite oxidation potential (NOP) was reduced after incubation with high toluene concentrations for 45 days (Fuller and Scow, 1996). Trichloroethylene is also inhibitory to the soil indigenous nitrifying population (Fuller and Scow, 1997).

Volatile fatty acids such as formic, acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric and *n*-caproic acid as well as trimethylamine are inhibitory to nitrification, but only at high concentrations, suggesting that volatile fatty acids and trimethylamine alone cannot account for the inhibition of the nitrification activity in domestic wastewater (Eilersen et al., 1994).

Nitrifying bacteria were also found to be resistant to monochloramines, and the combination of increased concentrations of oxidized nitrogen with decreased total chlorine in treated water was used as an indicator of bacterial nitrification (Cunliffe, 1991).

The need to set limits on nitrite oxidation in wastewater treatment plants created a demand for assays to determine the degree of inhibition of nitrification. One such assay, developed by Grunditz and Dalhammar, was based on inhibition of pure cultures of *Nitrosomonas* and *Nitrobacter* isolated from sewage sludge (Grunditz and Dalhammar, 2001).

As for the effects of light, nitrifying bacteria (both ammonia and nitrite oxidizers) are capable of recovery from photoinhibition in the dark (see above). Recovery of oxidizing activities is both dose and wavelength dependent (Guerrero and Jones, 1996). The light-absorbing pigment was identified as a porphyrin-like pigment with an absorption maximum at 408 nm, accumulating at the late exponential phase of growth. This photoreceptor was found at higher concentrations in ammonia oxidizers than in nitrite oxidizers (Guerrero and Jones, 1997).

Lithotrophy

An evaluation of field data from historical buildings in Germany, carried on by Mansch and Bock (1998), showed that ammonia and nitrite oxidizers were found in 55 and 62% of the samples, respectively, and that nitrifying bacteria will colonize natural stone within several years of exposure. The highest cell numbers were in some cases found underneath the surface. Nitrifying bacteria showed a preference for calcareous material with a pore radius of 1–10 μm. Cell numbers of nitrifying bacteria did not correlate with the nitrate content of the stone material. Their data strongly suggest that microbial colonization of historical buildings is enhanced by anthropogenic air pollution. A comparison of samples taken between 1990 and 1995 from buildings throughout Germany showed that in eastern Germany significantly more colonization with facultatively methylotrophic bacteria and nitrifying bacteria existed. The same was true for natural stone from an urban exposure site when compared to material from a rural exposure site.

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