Re/Views in Environmental Science & Bio/Technology **1:** 115–141, 2002.

Review

Origin, causes and effects of increased nitrite concentrations in aquatic environments

Sarah Philips[1], Hendrikus J. Laanbroek[2] & Willy Verstraete[1]*
[1]*Laboratory of Microbial Ecology and Technology, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium;*
[2]*Centre for Limnology, Netherlands Institute of Ecology, Rijksstraatweg 6, 3631 AC Nieuwersluis, Netherlands*
*(*author for correspondence, e-mail: Willy.Verstraete@rug.ac.be)*

Key words: denitrification, nitrification, nitrite accumulation, nitrite toxicity

Abstract

Literature frequently mentions increased nitrite concentrations along with its inhibitory effect towards bacteria and aquatic life. Nitrite accumulation has been studied for decades, and although numerous causal factors have already been commented on in literature, the mechanism of nitrite accumulation is not always clear. From the broad range of parameters and environmental factors reviewed in this paper, it is obvious that the causes and consequences of nitrite accumulation are not yet completely understood. Among others, pH, dissolved oxygen, volatile fatty acids, phosphate and reactor operation have been found to play a role in nitrite accumulation, which results from differential inhibition or disruption of the linkage of the different steps in both nitrification and denitrification. In the case of nitrification, this differential inhibition could lead to the displacement or unlinking of the ammonia oxidisers and nitrite oxidisers. In this paper, the idea is formulated that the nitrifier population forms a role model for the total microbial community. Increased nitrite concentrations would in this aspect not only signal a disruption of nitrifiers, but possibly also of the total configuration of the microbial community.

Abbreviations: AMO – Ammonia mono-oxygenase; Anammox – Anaerobic ammonium oxidation; AOB – Ammonia-oxidising bacteria; ATP – Adenosine triphosphate; COD – Chemical oxygen demand; DNRA – Dissimilatory nitrate reduction to ammonium; DO – Dissolved oxygen; EU – European union; FA – Free ammonia; F/M – Food to micro-organism ratio; FNA – Free nitrous acid; HAO – Hydroxylamine oxidoreductase; HRT – Hydraulic residence time; NDBEPR – Enhanced biological phosphate removal with nitrification and denitrification; NO – Nitric oxide; NOB – Nitrite-oxidising bacteria; NOR – Nitrite oxidoreductase; OLAND – Oxygen limited autotrophic nitrification denitrification; RBC – Rotating biological contactor; Sharon – Single reactor high activity ammonia removal over nitrite; SRT – Sludge residence time; TAN – Total ammoniacal nitrogen; TOC – Total organic carbon; VAS – Volatile attached solids

1. Introduction

Nitrite is a common intermediate in at least three different oxidative or reductive biochemical pathways that occur in water or soil: nitrification, denitrification and (dissimilatory or assimilatory) nitrate reduction to ammonium. If these processes do not proceed under steady state conditions, a risk of accumulation of toxic nitrite exists. Apart from these conventional nitrification / denitrification pathways, recent technological processes have been developed that make use of nitrite as an intermediate.

1.1 *Denitrification*

Denitrification involves the reduction of oxidised forms of nitrogen – nitrate (NO_3^-) and nitrite (NO_2^-) – into gaseous end products namely dinitrogen (N_2) or nitrous oxide (N_2O). In anoxic environments the respiratory reduction of NO_3^- to N_2 is an alternative to

O_2 respiration for the denitrifying bacteria (Sørensen 1978). The sequential production of intermediates via a linear pathway of four reductive steps mediated by four enzymes in the manner originally proposed by Payne (1973) has been widely accepted (eq. 1). The values between parenthese in equation 1 are the oxidation states of the different nitrogen forms.

$$NO_3^{-(5+)} \Rightarrow NO_2^{-(3+)} \Rightarrow NO^{(2+)} \Rightarrow N_2O^{(1+)} \Rightarrow N_2{}^{(0)} \quad (1)$$

Reduction of each specific nitrogen oxide is catalysed by a specific enzyme, generally referred to as the nitrogen oxide (NO_3^-, NO_2^-, NO or N_2O) reductase. In each reaction of the pathway, the oxidised nitrogen substrates serve as e-acceptor in respiration usually coupled to the oxidation of organic compounds as e-donors for energy generation.

As an example, the denitrification of nitrate as e-acceptor with glucose as carbon source can be expressed by equation 2.

$$5C_6H_{12}O_6 + 24NO_3^- + 24H^+ \rightarrow 12N_2 + 30CO_2 + 42H_2O \quad (\Delta G = -560\ \text{kJ mol}^{-1}\text{N}) \quad (2)$$

From this equation it follows that 2.68 g e-donor or Chemical Oxygen Demand (COD) are needed per g of NO_3^--N. In practice of wastewater treatment this ratio should be taken >4, to ensure a sufficiently fast and complete conversion to N_2. It should be noted that denitrification results in an increase of pH. The ability to denitrify is widespread among bacteria of unrelated systematic affiliations. Some bacteria can use non-carbon e-donors e.g., sulphur or H_2 (Zumft, 1992) according to equations 3 and 4.

$$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O \quad (3)$$

$$8NO_3^- + 5HS^- + 3H^+ \rightarrow 4N_2 + 5SO_4^{2-} + 4H_2O \quad (4)$$

1.2 *Nitrate reduction to ammonium*

1.2.1 *Dissimilatory nitrate reduction to ammonium*

In contrast to denitrification discussed above, the process of dissimilatory nitrate reduction to ammonium (DNRA) does not have N_2 but NH_4^+ as final compound, although both processes have nitrite as first intermediate. DNRA is performed by fermentative bacteria (e.g., Enterobacteriaceae, *Aeromonas*, *Vibrio* spp., *Clostridium sp.*), whereas denitrification is mediated by respiratory organisms (e.g., *Pseudomonas* and *Bacillus* species) (Caskey & Tiedje 1980; Cole 1996; Focht & Verstraete 1977). Nitrate and nitrite fermentation to ammonium is in direct competition with denitrification. The theoretical energy yield of these competing reactions (Table 1) indicates that if evaluated on energy yield per e-donor (H_2), denitrification provides more potential energy. However, if evaluated on energy available per nitrate (e-acceptor) consumed, then the DNRA is slightly more favourable. Moreover, DNRA has a capacity of accepting eight electrons versus only five for denitrification. Thus, DNRA is favoured when e-acceptor is limiting relative to the e-donor i.e., low levels of nitrate or nitrite, while denitrification is favoured when the e-donor (carbon) is in limited supply (Smith 1982; Tiedje et al. 1982). DNRA is performed by two types of enzymes: membrane bound nitrate reductase and nitrite reductase, whereby the various bacteria capable of DNRA contain not just one but two to four nitrate reductases (Cole 1996).

Table 1. Theoretical energy yield and electron-accepting capacity of denitrification and dissimilatory nitrate reduction to ammonium (after Tiedje et al. 1982)

Reaction	Energy yield $\Delta G^{0'}$ (kJ mol^{-1})		Electrons accepted per NO_3^-
	H_2	NO_3^-	
Denitrification			
$2\,NO_3^- + 5\,H_2 + 2\,H^+ \rightarrow$			
$N_2 + 6\,H_2O$	–224	–559	5
Dissimilatory nitrate reduction to ammonium			
$NO_3^- + 4\,H_2 + 2\,H^+ \rightarrow$			
$NH_4^+ + 3\,H_2O$	–150	–599	8

1.2.2 *Assimilatory nitrate reduction to ammonium*

Next to the dissimilatory nitrate reduction to ammonium, also assimilatory nitrate reduction to ammonium exists. Although the pathways of both processes look superficially similar, their enzymes and functions are not. In assimilatory nitrate reduction, the ammonium produced is incorporated into cell material as organic nitrogen and is a nutritional supplement for many bacteria if reduced nitrogen for assimilation is in short supply (Sørensen 1978).

1.3 *Nitrification*

Nitrification is the process whereby the reduced ammoniacal nitrogen is biologically oxidised to nitrite (nitritation) and then to nitrate (nitratation) with O_2

as terminal e-acceptor. The process is catalysed by two phylogenetically unrelated groups of autotrophic bacteria, the ammonia-oxidising bacteria (AOB) and the nitrite-oxidising bacteria (NOB). Almost all nitrifying bacteria are obligate chemolitho-autotrophic, i.e., they fulfil their carbon requirements via fixation of CO_2, via the Calvin cycle, for biosynthesis to organic carbon (i.e., auto-) and therefore use the energy for growth they obtain from the oxidation of ammonium or nitrite as sole energy source (i.e., chemolitho-). Some nitrite-oxidising bacteria have however been shown to be able of heterotrophic lifestyle under certain conditions (Focht & Verstraete 1977). The first step of nitrification is the oxidation of ammonia to nitrite over hydroxylamine (NH_2OH), involving the membrane bound ammonia mono-oxygenase (AMO) and the hydroxylamine oxidoreductase (HAO), and is carried out by ammonia-oxidising or nitroso-bacteria (AOB). The ammonia is initially oxidised to hydroxylamine in an endothermic reaction (eq. 5), after which the hydroxylamine is further converted to nitrite in an energy generating reaction (eq. 6) using oxygen from water and an additional molecular oxygen as terminal e-acceptor. The resulting pathway is given in eq. 7.

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (5)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (6)$$

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

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O$$

$$(\Delta G = -275 \text{ kJ mol}^{-1}\text{N}) \quad (7)$$

Subsequently the formed nitrite is further oxidised to nitrate by the nitrite-oxidising or nitro- bacteria (NOB), making use of the membrane-bound nitrite oxidoreductase (NOR) (eq. 8). The overall energy generating reaction of nitrification (eq. 7 + eq. 8) is given in equation 9.

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

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

$$NO_2^- + 0.5O_2 \rightarrow NO_3^-$$

$$(\Delta G = 75 \text{ kJ mol}^{-1}\text{N}) \quad (8)$$

$$NH_3 + 2O_2 \rightarrow NO_3^- + H^+ + H_2O$$

$$(\Delta G = 350 \text{ kJ mol}^{-1}\text{N}) \quad (9)$$

Although nitrification has been studied for more than a century, modern microbiologists still do not fully understand just how the nitrification sequence first begins (Alleman & Preston 1991). Literature provides both the un-ionised ammonia and the ionised ammonium as substrate for nitrifiers. Suzuki's work (Suzuki et al. 1974) strongly suggested that NH_3 rather than NH_4^+ is the true substrate of AMO, while for example Smith et al. (1997a) assumed that correlations found in their results reflect that NH_4^+ is the substrate for nitrification. Suzuki et al. (1974) based their assumption on the observation that the value of the half-saturation constant (K_m) for (NH_3+NH_4^+)-N (total ammoniacal nitrogen = TAN) decreased for increasing pH, while the K_m for NH_3 remained constant, independent of the pH. However, experiments by Groeneweg et al. (1994) did not result in a constant K_m for NH_3 over a broad range of pH values, although these authors suggest that the peak activity reflects a pH optimum for the AMO/HAO complex. In support to the findings of Suzuki et al. (1974), bacterial membranes are permeable to ammonia but not to ammonium (Kleiner 1985). However, transport of ammonia across the cytoplasmic membrane is doubted to be a prerequisite for ammonia oxidation (Groeneweg et al. 1994), as the AMO is located at the outer side of the membrane (Bock et al. 1991). Stein et al. (1997) concur with Suzuki et al. (1974), but find NH_3 a curious substrate as at physiologically relevant pH values (pH 6–8), the NH_3-NH_4^+ equilibrium is shifted primarily toward NH_4^+. Although the use of ammonia or ammonium is not unequivocal in literature, in this text the terms ammonia oxidation and ammonia oxidisers will be applied.

Along with the use of ammonia for energy generation, some of the ammonia is assimilated into cell tissue. As the fixation of CO_2 costs autotrophic bacteria about 80% of the energy generated by substrate oxidation (Kelly 1978) and as for each carbon-atom fixed, nitrifiers have to oxidise about 35 molecules of NH_3 or 100 molecules of NO_2^- (Wood 1986), growth yield of the nitrifiers is low. Moreover, growth rate is very slow compared to heterotrophic organisms. Assuming that $C_5H_7NO_2$ is the empirical cell formula for nitrifying bacteria (Brown & Caldwell 1975), the synthesis equations for ammonia oxidisers and nitrite oxidisers are given in eq. 10 and 11 respectively (Sharma & Ahlert 1977).

$$13NH_4^+ + 15CO_2 \rightarrow 10NO_2^- + 3C_5H_7NO_2 + 23H^+ + 4H_2O \quad (10)$$

$$10NO_2^- + 5CO_2 + NH_4^+ + 2H_2O \rightarrow 10NO_3^- + C_5H_7NO_2 + H^+ \quad (11)$$

It was long-time commonly accepted that in wastewater treatment plants, *Nitrosomonas* and *Nitrobacter* are the most common bacteria for nitritation and nitratation, respectively. However, the predominance of *Nitrosomonas* species in ammonia-oxidising communities in soils is often questioned (Laanbroek & Woldendorp 1995). In addition, researchers recently submitted that *Nitrosospira*-like bacteria are nearly ubiquitous and are the dominant AOB in many environmental samples and wastewater treatment systems (Hiorns et al. 1995; Schramm et al. 1998). Yet, the role and importance of *Nitrospira* might strongly depend on the specific treatment plant conditions, as upon surveys performed in 11 nitrifying wastewater treatment plants *Nitrosospira*-related sequences could only be detected in two samples, whereas all 11 plants investigated harboured nitrosomonads (Purkhold et al. 2000). Also the role of *Nitrobacter* is questioned. Burrell et al. (1998, 1999) showed that the conversion of nitrite to nitrate is more likely carried out by *Nitrospira.* They also reported the presence of the *Nitrospira* strain in activated sludge. Moreover, from investigations by *in situ* identification methods (e.g., Fluorescent *In Situ* Hybridisation or FISH), Blackall (2000) and Daims et al. (2000) concluded that *Nitrospira*-like bacteria are the dominant NOB in both enriched and full scale nitrifying systems. These observations were also made for aquaria, as data obtained from developing clone libraries of rRNA genes from several freshwater aquaria (Hovanec et al. 1998) suggest that *Nitrobacter winogradsky* and close relatives were not the dominant NOB. Instead, nitrite oxidation in freshwater aquaria appeared to be mediated by bacteria closely related to *Nitrospira moscoviensis* and *Nitrospira marina*.

2. Accumulation of nitrite

There have been reports of high nitrite concentrations occurring in both terrestrial and aquatic ecosystems. An important remark regarding the phenomenon of nitrite accumulation was given by Nijhof and Klapwijk (1995). They pointed out that considerable confusion arises from the use of terms such as "accumulation" or "build-up", without indicating whether a continuing concentration rise due to a net nitrite production is meant, e.g., due to a relatively insufficient nitrite oxidation rate, or just a high equilibrium concentration.

Usually the presence of nitrite is undesired in wastewater treatment or other domains, but some recent processes prefer nitrite as an intermediate. The so-called nitrite-shunt is a shortened nitrification process until nitrite and subsequent denitrification of the nitrite. Examples are the Sharon (Single reactor High Activity Ammonia Removal Over Nitrite), OLAND (Oxygen Limited Autotrophic Nitrification Denitrification) and Anammox (ANaerobic AMMonium Oxidation) processes (Hellinga et al. 1997; Kuai & Verstraete 1998; Strous 2000). Shortened nitrification and denitrification are more economical compared to traditional nitrogen removal, as it reduces the need of O_2 and of organic carbon source (Verstraete & Philips 1998).

As nitrite is an intermediate of both nitrification and denitrification, it can accumulate in aerobic as well as anoxic environments. Nitrite can be produced from ammonium, through nitrification, and from nitrate, through a number of nitrate reductive pathways. For instance, in fast flowing aerobic small streams, ammonia oxidation via nitrification is mainly responsible for elevated nitrite levels, whereas in slow-flowing conditions nitrite concentrations are more attributed to anaerobic nitrate-reducing processes (Kelso et al. 1997).

As indicated before, complete denitrification proceeds via a linear pathway of four reductive steps and is mediated by four enzymes. It has been found however that not all organisms classified as denitrifiers can execute the entire pathway as they do not express all these enzymes or have all the genes. According to Focht and Verstraete (1977), a distinction can be made between nitrate-respiring and denitrifying bacteria. The formation of nitrite can thus result from the selection of micro-organisms reducing nitrate only to nitrite (Wilderer et al. 1987; Casey et al. 1999). Drysdale et al. (2001) go further, dividing the heterotrophs present in NDBEPR (Enhanced Biological Phosphate Removal with Nitrification and Denitrification) sludge into 5 functional groups delineating their capacity for nitrate and/or nitrite reduction under anoxic conditions (Table 2). Or, an additional mechanism for nitrite accumulation during denitrification could be the repression of the synthesis of nitrite reductase.

Nitrite accumulation is a common trait of dissimilatory nitrate reduction to ammonia due to either inhibitory effect of nitrate on the nitrite reductase or repression of this enzyme. Nitrite accumulates especially when high concentrations of nitrate are present (Kelso et al. 1997).

Table 2. Functional groups in the heterotrophic microbial community of anoxic NDBEPR (Enhanced Biological Phosphate Removal with Nitrification and Denitrification) sludge according to the capacity for nitrate and/or nitrite reduction (after Drysdale et al. 2001)

Functional group	Denitrification capacity	(%)*
True denitrifiers	both nitrate and nitrite reduction	8.6
Incomplete denitrifiers	nitrate reduction to nitrite with no further reduction of the nitrite	31.0
Incomplete nitrite reducers	nitrate and nitrite reduction, but nitrates inhibit nitrite reduction	20.5
Exclusive nitrite reducers	only nitrite reduction	2.6
Non-denitrifiers	no nitrate nor nitrite reduction	37.3

*Numbers were assessed through spread plate technique.

Under normal conditions for nitrification, ammonia and nitrite oxidation rates are reported to be highly coupled, both in rates as in space, preventing nitrite accumulation. As nitrifiers are known sensitive organisms with respect to environmental conditions, the coupling in rates between AOB and NOB can easily be disturbed. Wastewater treatment plants frequently fail to establish stable nitrification, which is often attributed to the slow growth of nitrifying bacteria. Moreover, different species of AOB and NOB differ in their *in situ* growth kinetics, their substrate and oxygen affinities and their sensitivities to several environmental factors, which is discussed below.

An accumulation of nitrite often results from a modification of the growth kinetics of AOB and NOB. An imbalance could occur if the activity of the NOB is inhibited to a greater extent than the activity of the AOB. NO_2^- is accumulated if the oxidation of ammonia occurs at a greater rate than the nitrite oxidation. According to Smith et al. (1997b), the rate of ammonia oxidation has to exceed the nitrite oxidation by only a small margin in order to generate nitrite accumulation. In their experiments for example, the difference between the nitritation and the nitratation rate was barely 3%, giving nevertheless rise to significant nitrite accumulations.

As for the spatial coupling, several researchers already mentioned that clusters of AOB and NOB are in contact with each other in sludge flocs. This is nicely illustrated in Figure 1. Based on *in situ* hybridisation experiments, Mobarry et al. (1996) demonstrated a close physical association between *Nitrosomonas* sp. and *Nitrobacter* sp. in nitrifying samples from a continuously stirred tank reactor. *Nitrobacter* cells formed small clusters that were usually found in contact with *Nitrosomonas* cell clusters. Upon examination of both wastewater sludges and autotrophic nitrifying biofilms, it was found that the majority of the AOB formed dense spherical microcolonies. *Nitrospira*-like cells formed irregularly shaped aggregates which clustered around the clusters of AOB (Okabe et al. 1999). Juretschko et al. (1998) and Schramm et al. (1998) illustrated that both in activated sludge flocs and in the biofilm of a nitrifying fluidised bed reactor, *Nitrospira*-like cells were in the vicinity of *Nitrosococcus mobilis*-microcolonies. Similar observations were made regarding *Nitrospira* respectively *Nitrobacter* located in close vicinity to AOB (Daims et al. 2000). The spatial structure of AOB and NOB is not only important in water environments. Geostatistical analysis of a soil transect indicated that ammonia and nitrite-oxidising bacteria are not independently spatially located and a spatial association between both groups of bacteria is suggested (Grundmann & Debouzie 2000). In an enrichment of acid-tolerant nitrifying bacteria (pH 4) De Boer et al. (1992) observed aggregated *Nitrosospira*-like bacteria surrounded by *Nitrobacter*-like bacteria.

This juxta-positioning of the AOB and NOB may reflect the syntrophic association between these two groups of bacteria. Both AOB as NOB benefit from the close physical association. On the one hand, the close physical association is useful for energetic reasons: NOB are able to efficiently intercept the nitrite (i.e., their substrate) produced by the AOB, helping to cope with the poor energy yield of nitrite oxidation. On the other hand, AOB like the presence of the NOB as the latter relieve them from the toxic nitrite. It therefore aids in the defence against the toxicity of nitrite by preventing its accumulation or formation of toxic by-products, such as NO which can interact with bacterial enzymes (Stein & Arp 1998). Juxta-positioning could thus provide a competitive advantage over solitary occurrence. Disturbing the spatial organisation could lead to nitrite accumulation. Poughon et al. (1999)

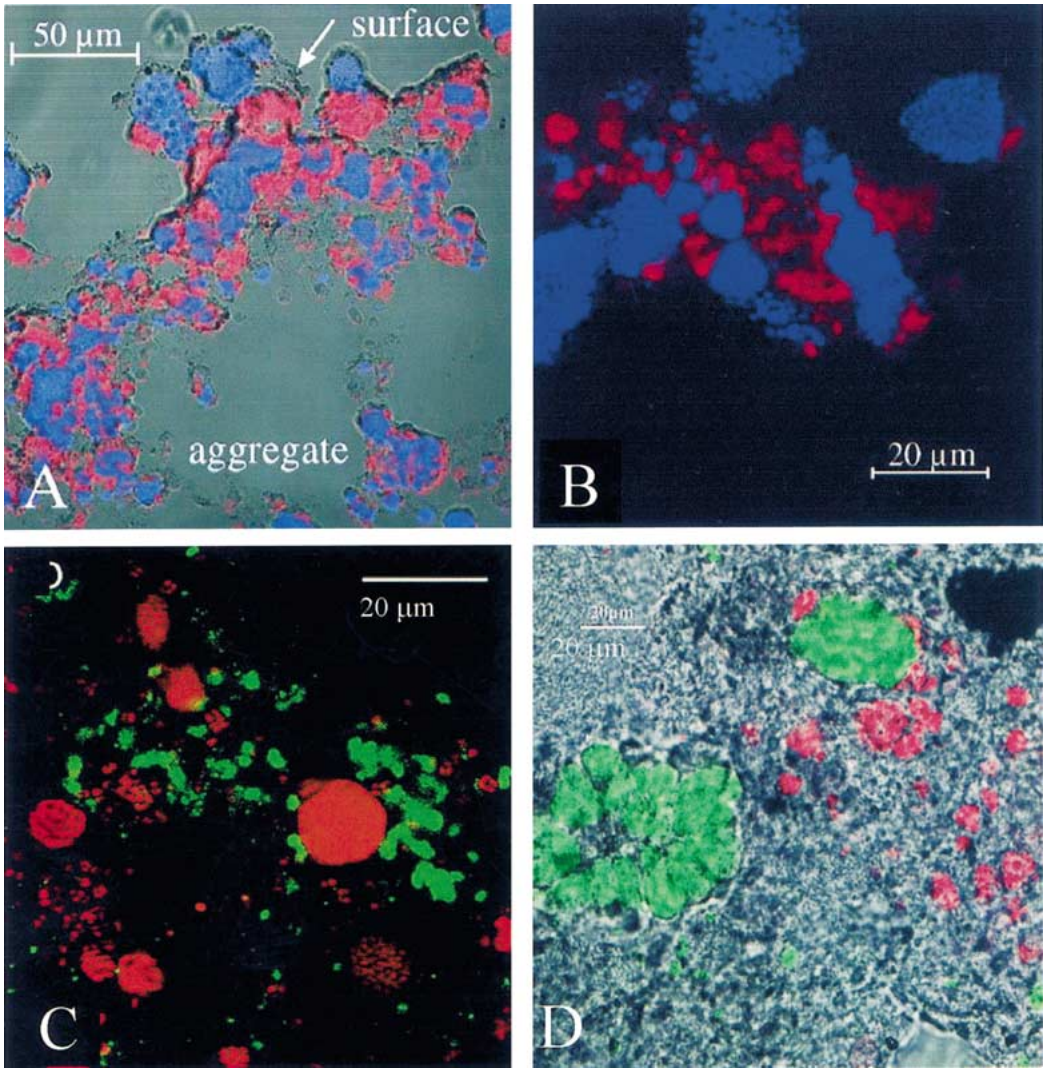

Figure 1. In situ identification of nitrifying bacteria after simultaneous hybridisation of AOB and NOB, demonstrating their close spatial distribution in (image A and B) bacterial aggregates from a chemolithotrophic nitrifying fluidised bed reactor, (image C) a nitrifying biofilm, or (image D) activated sludge. A and B: Blue AOB *Nitrosospira* (CY5-labelled probe NSV443) and red NOB *Nitrospira* (CY3-labelled probe NSR1156) (Schramm et al. 1998); C: Cells of AOB are stained red (TRITC-labelled probe Nso190) and cells of *Nitrospira*-like bacteria are green (FITC-labelled Ntsp1026 probe) (Okabe et al. 1999); D: Green *Nitrosococcus mobilis* (FLUOS-labelled probe NmV) and red stained *Nitrospira*-like bacteria (CY3-labelled probe Ntspa1026aA18) (Juretschko et al. 1998).

found confirmation in their experiments with a nitrifying fixed bed column that output transient nitrite peaks are higher when changes in the process conditions produce a rearrangement of biomass distribution.

Because of the tandem of ammonia and nitrite oxidation, the pool size of nitrite typically remains low under steady state conditions (Bock et al. 1989; Ward 2000). It is widely accepted (Randall & Buth 1984b) that under normal conditions the rate of nitritation is lower than the rate of nitratation so that nitrite does not accumulate. In some cases however, this tandem has not been installed yet. For example during the start-up phase of nitrifying biofilms nitrite peaks can be observed. Due to the apparent slower growth of NOB – e.g., minimum doubling times of 7–8 hours for AOB and 10–13 hours for NOB have been mentioned (Bock 1978; Bock et al. 1986) – good co-operation and juxta-positioning is not possible from the early start. Most likely, AOB grow first as micro-colonies that produce nitrite and then are colonised by NOB aggregates. This in time separated growth and installation of AOB and NOB results in an initial

Table 3. Some values for the yield (g biomass g^{-1} N) of AOB and NOB encountered in literature

AOB	NOB	Reference
0.04–0.13	0.02–0.07	Randall and Buth (1984)
0.03–0.13	0.02–0.08	Sharma and Ahlert (1977)
0.05–0.08	0.02–0.05	Alleman and Preston (1991)
0.076	—-	Neufeld et al. (1986)
0.30	0.08	Focht and Verstraete (1977)
0.45	0.45	Rittman et al. (1999)

nitrite peak. As AOB catalyse the first reaction of the two-step nitrification, making the NOB wait for their substrate, and because the AOB have a higher yield (Table 3), dominance of AOB and accumulation of nitrite is to be expected for the start-up phase of a nitrifying bioreactor (Schramm et al. 2000). It was demonstrated (Bovendeur 1989) that in developing biofilms nitratation capacity develops with some delay after the development of nitritation, leading to initial higher nitrite concentrations. Eventually however, the nitratation capacity will exceed the nitritation capacity. Therefore, Bovendeur (1989) deems that a net nitrite production will not occur during nitrification in full-grown biofilms. Also Venterea and Rolston (2000a) accord transient nitrite accumulations following ammonium addition to lag effects as high ammonium levels stimulate AOB while NOB growth rates are initially limited by low substrate levels. As such, these authors suggest that some degree of nitrite accumulation following ammonium addition is a consequence of the nature of nitrification itself, being a two-step sequential process carried out by distinct organisms. Moreover, after periods of starvation, NOB demonstrated a relatively retarded activity as compared to AOB (Gerards et al. 1998; Tappe et al. 1999).

Also spatial coupling of nitrification and denitrification can prevent nitrite accumulation in the bulk water phase. Okabe et al. (1999) found that apart from NOB, a wide variety of heterotrophs were found to surround and coexist with AOB. According to Okabe et al. (1999) this may suggest that various heterotrophs could utilise soluble organic compounds secreted from the AOB. Denitrifying bacteria among these heterotrophs are thus in an ideal position as the AOB also produce nitrite from their nitrifying activities, in this way supplying denitrifiers present with both carbon and nitrite allowing denitrification. The AOB in turn also benefit from the presence of the heterotrophs as the latter liberate them from the toxic nitrite.

The former discussion indicates that uncoupling or displacement of spatial organisation could alter the metabolism and growth rates of nitrifiers possibly resulting in nitrite accumulation. If an extrapolation would be made to the whole microbial population in activated sludge, it may be hypothesised that elevated nitrite levels not only signal an upset within the nitrification-denitrification processes, but could also be a signal of disturbance in the configuration of the entire microbial population.

Origins of nitrite accumulation can be summarised into spatial uncoupling of AOB and NOB or of AOB and denitrifiers, imbalance of nitritation and nitratation rates, inhibition of nitratation, incomplete denitrification pathway or even uncoupling of the activities of the different reducing enzymes in denitrification.

3. Toxicity of nitrite

3.1 *Higher organisms*

The occurrence of high concentrations of nitrite is an important water quality concern as it is highly toxic to human, fauna and flora. In the stomach nitrite forms carcinogenic N-nitroso-compounds which can have implications in the pathology of gastric cancer (Bruning-Fann & Kaneene 1993b). Although the relationship between nitrite consumption and cancer is still controversial, nitrosamines formed by the reaction of nitrite with various proteins during digestion are known to be mutagenic and carcinogenic (Weng et al. 1992). Nitrite has also been named as a possible cause of migraine headache (Gerber 1997). Moreover, passage of nitrite into the bloodstream results in the irreversible conversion of haemoglobin to methaemoglobin, thus compromising the oxygen-binding capacity (Van Leeuwen 2000), causing respiratory deficiencies in aquatic animals and human beings (Bradberry et al. 1994). Especially infants are sensitive for methaemoglobinemia (Bruning-Fann & Kaneene, 1993b), leading to the "blue-baby syndrome". Next to humans, nitrite is capable of inducing methaemoglobinemia in a wide range of species, i.e., cattle, sheep, swine, dogs, guinea pigs, rats, chickens and turkeys. In rats, chronic nitrite exposure causes pathological changes in a variety of tissues, alterations in motor activity and brain electrical activity and alters gastric mucosal absorption (Brunning-Fann & Kaneene 1993a).

Table 4. Some examples of the toxicity of nitrite to aquatic fauna

Species	Test	Nitrite mg N L^{-1}	Reference
Gilthead seabream (12-day old larvae)	LC50 24 h	607	Parra and Yufera (1999)
Australian crayfish	LC50 24 h	42.9	Meade and Watts (1995)
Australian crayfish	LC50 48 h	37.1	Meade and Watts (1995)
Australian crayfish	LC50 96 h	25.9	Meade and Watts (1995)
European eel	LC50 96 h	144	Kamstra et al. (1996)
Tiger prawn	LC50 96 h	14	Chen and Chin (1988)
Juvenile grass carp	LC50 96 h	10.6	Alcarez and Espina (1997)
Shortnose sturgeon	LC50 96 h	10	Fontenot and Isely (1998)
Nile tilapia	LC50 96 h	8–81	Atwood et al. (2001)
Amphibians	LC50 15 d	<2	Marco et al. (1999)
Range of fish	LC50	0.1–1.0	Eddy and Williams (1994)
Silver perch	EC5	2.78	Frances et al. (1998)
Zebrafish	mortality	0.3–1.5	Pullium et al. (1999)

LC50: Concentration at which 50% mortality is observed.
EC 5: Concentration at which growth is reduced by 5%.

Because of its high toxicity, regulatory measures are established on the concentrations allowed in water. For example, the quality criteria for drinking water put forward by the EU set the maximum nitrite content at 0.5 mg L^{-1} (= 0.15 mg N L^{-1}) (European Council Directive 1998). The WHO (1996) established a guideline value for nitrite in drinking water of 3 mg NO_2^- L^{-1} or 0.9 mg N L^{-1}. The European Union guideline for rivers supporting salmonid fish amounts 0.003 mg NO_2^--N L^{-1} (European Economic Community 1978).

Some values of nitrite toxicity to aquatic fauna are given in Table 4. Nitrite has an influence on several biological functions. When present in the water, nitrite is immediately incorporated in the hemolymph and midgut of tiger shrimps via branchial chloride uptake of NO_2^-, and accumulated in the tissues (Cheng & Chen 2000). The same researchers suggest that nitrite uptake by the Kuruma shrimp *Penaeus japonicus* results in reductions of hemolymph Cl^-, Na^+ and protein levels leading to a decrease in hemolymph osmolality (Cheng & Chen 1998). Nitrite exposure significantly decreased the temperature tolerance of *Penaeus setiferus* (white shrimp) postlarvae (Alcaraz et al. 1997) and induced behavioural and morphological changes in amphibians (Marco & Blaustein 1999), such as reduced feeding activity, disequilibrium and paralysis, abnormalities and eventually death (Marco et al. 1999). In case of fish, nitrite has resulted in branchial Cl^- uptake inhibition, impairment of the acid-base balance and the electrolyte, reduction of the oxygen carrying capacity of blood by oxidation of hemoglobin to methemoglobin (Alcarez & Espina 1997), changes in gill histopathology (Frances et al. 1998), gross growth efficiency and survival (Alcaraz & Espina 1997) or extracellular hyperkalemia (Grosell & Jensen 2000). Exposure to 7.8 mg NO_2^--N L^{-1} resulted in gill lamellar thickening and epithelial lifting along with a proliferation of mucous cells for the greenlip abalone (Harris et al. 1998). Data obtained by Gonzales et al. (2000) suggest that environmentally realistic concentrations of nitrite may affect the dynamics of conjugative metabolism in fish. A review on the toxicity of nitrite to fish giving other effects of nitrite has been written by Lewis and Morris (1986).

Addition of chloride ions (Cl^-) to culture water seems reduce the nitrite toxicity in most freshwater fish (Lewis & Morris 1986; Atwood et al. 2001), increasing the nitrite tolerance more than 30-fold (Wisse & Tomasso 1989). According to Doblander and Lackner (1997) part of the nitrite taken up will be detoxified by erythrocytes in fish.

3.2 *Micro-organisms*

In addition to higher organisms, nitrite has also a known influence upon micro-organisms and their processes. It is a known bacteriostatic molecule due to its affinity for the metal ions in the centre of enzymes (Wild et al. 1995). Literature informs on inhibition by nitrite of ammonia oxidation activity (Figure 2)

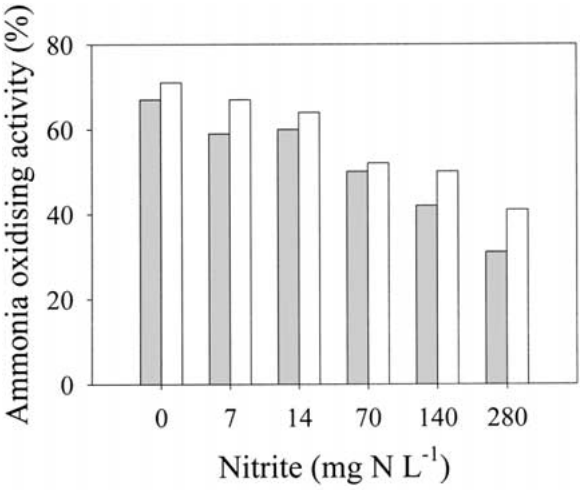

Figure 2. Ammonia-oxidising activity relative to the initial activity in *Nitrosomonas europaea* cells exposed to nitrite after a 24-h incubation (pH 8) in the absence of ammonium under aerobic (grey bars) or anaerobic (white bars) conditions, while exposed to increasing concentrations of nitrite (after Stein & Arp 1998).

(Anthonisen et al. 1976; Groeneweg et al. 1994; Stein & Arp 1998), nitrite oxidation (Anthonisen et al. 1976), denitrification (Abeling & Seyfried 1993; Holub et al. 2000), both anoxic (Meinhold et al. 1999) and aerobic phosphate removal (Meinhold et al. 1999; Pak & Chang 2000), methanogenesis (Klüber & Conrad 1998a,b; Percheron et al. 1999; Quevedo et al. 1996) and cell growth (Cui et al. 1992; Almeida et al. 1995a). It has been suggested that especially ammonium-starved cells are sensitive for nitrite (Gerards et al. 1998). Addition of 70 mg nitrite-N L^{-1} to anoxic rice field soil slurries resulted in complete inhibition of methanogenesis for at least 24 days (Klüber & Conrad 1998a). Addition of 28 or 14 mg N L^{-1} showed similar effects, however with only 15–19 and 7–11 days of inhibition of CH_4 production. Other denitrification products (NO, N_2O) also caused complete but largely reversible inhibitions of methanogenesis. Tests with *Methanosarcina barkeri* and *Methanobacterium bryantii* revealed that the strongest inhibition was caused by NO followed by NO_2^- or N_2O, respectively, for the two bacteria (Klüber & Conrad 1998b). The authors concluded additionally that this inhibitory effect was not primarily due to an increase of the redox potential, but to toxic effects on methanogenic bacteria and perhaps other members of the microbial community. Even processes that make use of nitrite accumulation such as the Anammox process exhibit toxicity towards nitrite (Jetten et al. 1999). Table 5 summarises a number of processes influenced by nitrite. Results from tests where nitrite was added to activated sludge pointed to an amplification of the toxicity of nitrite if the latter was dosed in combination with proteins or amino acids (Philips & Verstraete 2000). Batch incubation tests of *Nitrosomonas europaea* indicated that the hydroxylamine oxidation activity was not strongly affected by nitrite, but that specifically the ammonia oxidation activity of the cells decreased (Stein & Arp 1998). Almeida et al. (1995a) observed an inhibition of 50% of the initial oxygen uptake rate by *Pseudomonas fluorescens* at approximately 390 mg N L^{-1}. However, at levels of nitrite below 14 mg N L^{-1}, a stimulatory effect of nitrite on the oxygen uptake rates of *Pseudomonas fluorescens* grown under aerobic conditions was observed. Although growth of *Pseudomonas* was inhibited (60%) at ca. 100 mg N L^{-1}, specific rates of nitrite and nitrate reduction were stimulated by 30%.

3.3 *Mechanisms of nitrite toxicity*

According to Sijbesma et al. (1996) nitrite acts as a protonophore, i.e., an uncoupler that increases the proton permeability of membranes by a shuttling mechanism. An uncoupler is an agent that stimulates basal electron transport, inhibits ATP synthesis, stimulates ATP hydrolysis and inhibits various exchange reactions catalysed by the ATP-ase (Rottenberg 1990). Almeida et al. (1995a) generated results supporting this hypothesis: a decreased growth of *Pseudomonas fluorescens* in the presence of high nitrite concentrations indicates a lower efficiency of respiratory-chain-linked energy conservation, due to an increase of the proton permeability by the nitrite, counteracting the proton pumping effect of ATP-ase. Furthermore, it has been suggested that toxic effects on the cell may be exerted by the nitrite un-dissociated fraction (HNO_2) (Almeida et al. 1995a), which is capable of increasing proton permeability of the cell membrane by shuttling protons between the two sides. Almeida et al. (1995a) found indications that growth inhibition depended only on the nitrous acid concentration.

Besides its direct toxicity, some researchers claim that derivatives of nitrite are responsible for the observed effects. Nitroso and nitro products can be generated through photoreaction of nitrite with phenols or other aromatic compounds (Bilski et al. 1988). Nitrite may react in the environment or in foods with secondary amines to form N-nitrosamines which

Table 5. Illustrations of the inhibition of bacterial processes by nitrite

Process	Inhibition %	Nitrite mg N L^{-1}	Reference
Ammonia oxidation	60	280	Stein and Arp (1998)
Ammonia oxidation	0–80	80–3000	Groeneweg et al. (1994)
Ammonia oxidation	50	42–70	Muller et al. (1995)
Anammox	100	>280	Jetten et al. (1999)
Anoxic phosphate removal	n.s.	5–10	Kuba et al. (1996)
Anoxic phosphate removal	100	>8	Meinhold et al. (1999)
ATP synthesis	n.s	380	Almeida et al. (1995a)
Denitrification	100	308	Almeida et al. (1995a)
Denitrification at pH 6	100	250	Glass et al. (1997)
Growth of *Clostridium sporogenes*	50	140	Cui et al. (1992)
Growth of *Pseudomonas denitrificans*	27	100	Kornaros and Lyberatos (1997)
Growth of *Pseudomonas fluorescens*	60	98	Almeida et al. (1995a)
Growth of *Pseudomonas fluorescens*	100	308	Almeida et al. (1995a)
Methanogenesis	100	70	Klüber and Conrad (1998a)
Nitrification activity	40	100	Dahl et al. (1997)
Nitrifying capacity of activated sludge	20	100	Philips and Verstraete (2000)
Oxygen respiration rate	80	n.s	Spector (1998)
Oxygen uptake rate of activated sludge	30	100	Philips and Verstraete (2000)
Phenol degradation	75	>185	Holub et al. (2000)

n.s. not specified by the author.

are potent carcinogens and even low and occasional exposure may be hazardous (Atlas & Bartha 1993).

The observed repression of ammonia oxidation upon introduction of substrate to ammonium-starved *Nitrosomonas europaea* cells, has been attributed to the destruction of the enzyme system of the AOB by nitrite or a metabolite (Gerards et al. 1998).

In addition to nitrite itself, metabolites of nitrite chemistry have been suggested to cause toxicity. In order to determine the specificity of nitrite as inhibitor of ammonia oxidation activity, Stein and Arp (1998) added NO or N_2O, being chemicals related to nitrite chemistry, to incubations of *Nitrosomonas europaea*. As externally added NO or N_2O had little effect on ammonia oxidation activity, it was initially suggested by these researchers that nitrite itself rather than a known product of nitrite chemistry caused the activity loss. However, according to the same researchers it would be more logical that nitrite would be converted to a reactive species. A possible mechanism herewith could be that nitrite is reduced by AMO to a reactive species such as NO or a nitrosyl radical. That reactive species in turn forms a metal-nitrosyl complex by targeting a copper or iron molecule close to a bacterial enzyme active centre (Stein & Arp 1998; Zumft 1993). The observed discrepancy by Stein and Arp (1998) between the hypothesis of NO toxicity and absence of effect when adding NO could be explained by the observation of von Schulthess et al. (1995) that it makes a difference whether NO is brought form outside to the cells or whether it is produced by the organism itself. Not the bulk concentration but the concentration in the close environment of the enzymes determines the activity. von Schulthess et al. (1995) pulsed actively denitrifying micro-organisms with nitrite, which resulted in an immediate accumulation of NO due to inactivation of the nitric oxide reductase. The accumulated NO subsequently induced severe inhibition of the other reductase enzymes in the denitrification pathway. Moreover, in research concerning the anaerobic biocorrosion of iron and stainless steel powder, Kielemoes et al. (2000) postulated that the inhibitory effect of higher nitrite concentrations could be partly due to the chemical formation of NO from the reaction of nitrite and Fe(0) and its toxic effect on the micro-organisms acting at the steel surface. In yet another research, the appearance of a yellow colour in the effluent of activated sludge reactors fed with milkpowder and dosed with nitrite is suggested to be due to interaction of NO derived

form the nitrite with proteins in the milkpowder, thus forming yellow nitroso compounds (Philips & Verstraete 2000). The former examples illustrate that possibly NO and not nitrite causes inhibition.

NO is a very reactive agent with potentially toxic oxidation products such as nitrogen dioxide (NO_2) (Kuhlen et al. 1999). As a free radical NO is inherently reactive and mediates cellular toxicity by damaging critical metabolic enzymes and by reacting with superoxide to form an even more potent oxidant, peroxynitrite (Bredt 1999). Mallick et al. (2000) found results to support the hypothesis that accumulation of nitrite is the prerequisite for NO production in the case of the chlorophycean microalga *Scenedesmus*.

3.4 *Influence of nitrite on bulking in wastewater treatment*

Accumulation of nitrite has economical implications if it occurs in wastewater treatment plants. The presence of nitrite, as well as nitrate, has a significant stimulatory effect on the proliferation of low F/M (food to micro-organism ratio) filamentous organisms in nitrogen (N) and nitrogen and phosphorus (N, P) removal activated sludge systems (Musvoto et al. 1999; Tonkovic 1998). The mechanism of this effect could not be revealed. However, Casey et al. (1994) formulated a hypothesis for the cause of low F/M bulking saying that floc-formers, which denitrify nitrate fully to N_2 are inhibited in their oxygen uptake under subsequent aerobic conditions by formed NO, therefore advantaging growth of filaments. This hypothesis was further elucidated in Casey et al. (1999b). Under conditions in which the sludge (containing facultative aerobic organisms) is subject to alternating anoxic-aerobic conditions with nitrite present throughout the anoxic zone or period, NO is considered to accumulate intracellularly by floc-forming organisms, and not by filaments, as the latter only denitrify to nitrite and thus do not produce NO. The intracellular NO inhibits the aerobic respiration and utilisation of substrate by the floc-formers in the subsequent aerobic period. Hereby it should be stressed that specifically intracellular nitric oxide causes the inhibition. Extracellular nitric oxide is unstable, particularly under aerobic conditions where it reacts rapidly with oxygen.

In stead of aerobic respiration, the floc-formers shift to aerobic denitrification of the nitrite, maintaining intracellular NO and thereby its inhibitory effect. Because of the lower yield of this respiration with nitrite compared to oxygen (Casey et al. 1999a), filaments gain advantage over the floc-formers and proliferate. In the absence of nitrite during the change from anoxic to aerobic conditions, however, floc-formers are not inhibited and filaments gain no advantage under aerobic conditions (Casey et al. 1999b). This inhibition of the utilisation of O_2 under aerobic conditions results from the interaction of NO, accumulated intracellular during prior anoxic conditions, with the Fe-containing complexes of one of the enzymes specific to aerobic respiration, the cytochrome oxidase. Fe-NO complexes are created which are incapable of transferring electrons to oxygen at the oxidase (Casey et al. 1999a).

Dosing nitrite (0–100 mg N L^{-1}) to activated sludge reactors also caused bulking (Philips & Verstraete 2000), but the occurrence of bulking seemed to be strongly dependent on the history of the sludge.

4. Abiotic factors influencing nitrite accumulation

Literature makes notice of several parameters influencing nitrite accumulation, either individually or in combination with other factors. Abiotic factors like temperature, pH and nutrients have a strong influence on the growth and activity of micro-organisms.

4.1 *pH*

Surmacz-Gorska et al. (1997) found in their experiments that the pH of the wastewater was the decisive parameter in NOB activity inhibition. Tonkovic (1998) on the other hand came to the conclusion from sewage plant monitoring data with lab scale reactor tests that nitrite accumulation is independent of the pH, but is rather due to low dissolved oxygen, suppressing *Nitrobacter*.

In case of denitrification nitrite production occurs significantly at high pH ranges (Merkel et al. 1993). Also Schuch et al. (2000) observed that a more alkaline pH (>7.8) resulted in a lower denitrification rate and in an increasing nitrite concentration in the effluent (26 mg N L^{-1} at pH 9). Similar results were obtained in case of nitrification by Glass and Silverstein (1998), who observed a significantly increased nitrite accumulation (250, 500 or 900 mg NO_2^--N L^{-1}) in sequencing batch reactors when mixed liquid pH was increased during nitrification (pH 7.5, 8.5 or 9.0 respectively). On the other hand, Furumai et al.

(1996) found an accumulation of nitrite at pH-values lower than 7.4 or at low alkalinity in a denitrifying granular filter.

Also in soils, pH has been reported to have an influence on nitrite accumulation. According to Venterea and Rolston (2000b) distinct periods of high nitrite accumulation occurred below critical pH values in California agricultural soils. Then again, due to the acid-tolerance of nitrite-oxidising cells accumulation of nitrite is hardly expected in acid soils, as the oxidation of ammonia in acid soils seems to be limited by the characteristics of the AOB (Laanbroek & Woldendorp 1995). Concerning the activity of NOB, pH was found to strongly interact with initial ammonium concentration (Mauret et al. 1996).

4.2 *Ammonium (NH_4^+) or free ammonia (NH_3)*

Notwithstanding the major importance of the pH at such, it more often influences other parameters playing even a greater role in nitrite accumulation. Among others the equilibrium of ammonium and ammonia in water is dependent on the pH according to the equation and formula below (eq. 12 and 13). The term free ammonia (FA) is understood to mean non-dissociated or un-ionised ammonia. This term is commonly used in literature and will therefore be applied in this text.

$$NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O \quad (12)$$

$$[NH_3 - N]_{free} = \frac{[TAN] \times 10^{pH}}{K_a/K_w + 10^{pH}} \quad \text{with}$$

$$K_a K_w = \exp[6334/(273 + t)] \quad (13)$$

TAN = Total ammoniacal nitrogen = ammonium + free ammonia

K_a = Ionisation constant for ammonium (e.g., K_a at 20 °C = $10^{-9.24}$)

K_w = Ionisation constant for water (e.g., K_w at 20 °C = 0.69 10^{-14})

t = temperature in °C

Although NH_4^+ can cause nitrite accumulation (e.g., Horan and Azimi 1992; Smith et al. 1997a), the effect of free ammonia seems to be more pronounced. FA is a competitive inhibitor of nitrite oxidoreductase activity, which is located on the cell membrane of NOB (Yang & Alleman 1992). Boundary conditions for FA inhibition of both AOB and NOB were first depicted by Anthonisen et al. (1976) (Figure 3). Because several environments and operating conditions were tested,

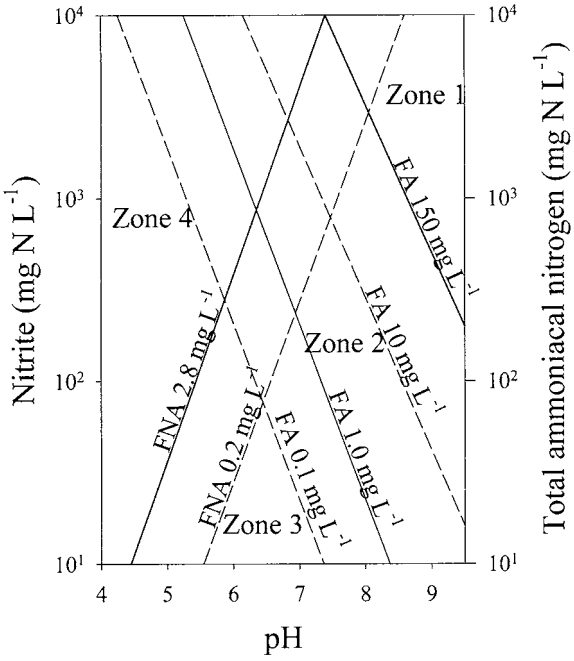

Figure 3. Relationship between concentrations of free ammonia (FA) and free nitrous acid (FNA) and inhibition to nitrifiers. The dashed lines mark the lower limit and the solid lines mark the upper limit of the range of boundary conditions of zones of nitrification inhibition. Zone 1 = Inhibition of nitritation and nitratation by FA; Zone 2 = Inhibition of nitratation by FA; Zone 3 = Complete nitrification; Zone 4 = Inhibition of nitratation by FNA (after Anthonisen et al. 1976).

the different zones in Figure 3. are not well-delineated, but are delimited by a range of boundary conditions. (see further). According to Alleman (1984) the slightly less basic pH optimum of *Nitrobacter* (7.2–7.6) compared to *Nitrosomonas* (7.9–8.2) appears to be reflected in the higher sensitivity of *Nitrobacter* to FA which is aggravated at a higher pH. For loadings of 1 g NH_4^+-N m^{-2} d^{-1} and higher, nitrite concentrations up to 300 mg N L^{-1} were obtained in the effluent of an upflow submerged filter at dissolved oxygen concentrations of 4 to 5 mg O_2 L^{-1} (Ceçen & Gönenç 1995). A study of nitrification in mixed culture (Mauret et al. 1996) produced threshold concentrations for the start of NOB inhibition between 6.6 and 8.9 mg NH_3-N L^{-1}. Whatever the temperature, Mauret et al. (1996) observed a transient nitrite build-up of roughly 50% of the initial ammonia concentration for threshold concentrations of FA of 8.91 mg N L^{-1}. Villaverde et al. (2000) and Rols et al. (1994) remark that the inhibition phenomenon is specific and depends both upon the concentration of nitrifying organisms

Table 6. Comparison of literature values of free ammonia inhibition of nitrite oxidation in wastewater treatment. Th.c. = Threshold concentration, i.e. lowest concentration exhibiting some degree of inhibition

NH_4^+ (mg N L^{-1})	NH_3 (mg N L^{-1})	pH	T (°C)	Observed effect	Remarks	Reference
	0.02	<7.2	20	Th.c. inhibition of nitrite-oxidation	Continuous flow	Prakasam and Loehr (1972)
	0.08–0.82			Th.c. inhibition of nitrite-oxidation		Anthonsisen et al. (1976)
	0.12	<7.2	20	> 90% inhibition of nitrite-oxidation	Continuous flow	Prakasam and Loehr (1972)
	0.5	7	18	Th.c. inhibition of nitrite-oxidation		Muller et al. (1995)
13	0.6	7.8–8.1		> 95% nitrite accumulation	Sequential batch reactor.	Alleman and Irvine (1980)
up to 1060	0.7–40	6.5–8.0		No effect	Enriched culture	Wong-Chong and Loehr (1978)
	1–3		20	Temporary 50% nitrite accumulation	Non-acclimated biomass	Turk and Mavinic (1989)
16	1.06	8.1	25	55% inhibition of nitrite-oxidation	2.5 mg O_2 L^{-1}	Balmelle et al. (1992)
40	2.95	8.1	25	90% inhibition of nitrite-oxidation	2.5 mg O_2 L^{-1}	Balmelle et al. (1992)
	5			Sustained nitrite accumulation	Intermittant contact with NH_3	Turk and Mavinic (1986)
	5–20		20	Sustained nitrite accumulation	Non-acclimated biomass	Turk and Mavinic (1989)
100	6.64	8.1	25	100% inhibition of nitrite-oxidation	2.5 mg O_2 L^{-1}	Balmelle et al. (1992)
35	8.9	7	15	50% nitrite accumulation		Mauret et al. (1996)
490	13	7.8		100% inhibition of nitrite-oxidation	Fill-and-draw	Verstraete et al. (1977)
up to 840	14–32	8.0		Nitrite accumulation	Cultures	Wong-Chong and Loehr (1978)
40	15.5	9.2		Transient nitrite accumulation	Sequential batch reactor.	Sauter and Alleman (1980)
500	20	8.2		> 90% inhibition of nitrite-oxidation	Oxidation ditch	Murray et al. (1975)
500	0.07–0.4	8.0		Nitrite accumulation up to 300 mg N L^{-1}	Conventional activated sludge	Surmacz-Gorska et al. (1997)
	>3			50% reversible inhibition	Mixed AOB/NOB-population	Abeling and Seyfried (1992)
	9	8.1	25	100% inhibition of NOB	Batch *Nitrosomonas* enriched	Rols et al. (1994)
650	24	8.0		Transient nitrite accumulation	Batch activated sludge system	Wong-Chong and Loehr (1975)
	0.2			Th. c. inhibition nitrite-oxid.	2 months after start-up	Villaverde et al. (2000)
80	0.5	7.8		52% nitrite accumulation	Submerged biofilter	Fdz-Polanco et al. (1996)
	0.5–0.7			Th. c. inhibition nitrite-oxid.	6 months after start-up	Villaverde et al. (2000)
100	1.5	7.5–8.5	20–25	80–90% nitrite accumulation	Submerged biofilter	Villaverde et al. (1997)

as upon the threshold concentration (on volume basis) of FA causing inhibition. In doing so, Rols et al. (1994) established the threshold of inhibition of NOB between 0.5 and 3 mg NH_3-N mg^{-1} viable NOB biomass. For the same reason, Suthersan and Ganczarczyk (1986) introduce the FA over biomass ratio as a specific inhibition effect measure. The inhibiting effect of FA on NOB may be the result of a combination of several factors such as initial NH_4^+ concentration, pH and temperature (Balmelle et al. 1992). It is further said that the inhibition by FA is such that the effect of temperature, alkalinity and ammonium load is masked when the concentration of free ammonia is above certain values (Fdz-Polanco et al. 1994, 1996; Villaverde et al. 1997). A comparison between FA threshold concentrations, i.e. concentrations at which inhibition begins, resulting from different studies is made in Table 6.

Notwithstanding the severe effects of high free ammonia on nitrite concentrations, Sutherson and Ganczarczyk (1986) and Turk and Mavinic (1989) reported that the biomass gets acclimated to FA, and nitrite build-up cannot be maintained for a long time. Both AOB and NOB were capable of acclimating to

FA levels as high as 40 mg NH_3-N L^{-1} (Turk & Mavinic 1989). Also Rols et al. (1994) believe that the history of the sludge (in their case enrichment in the presence of high FA concentrations) or the operation of the reactor are factors altering the growth dynamics of NOB towards acclimatisation to NH_3. Results of experiments with submerged biofilters (Villaverde et al. 2000) also suggest that NOB develop the capability to resist higher concentrations of FA after exposure for long periods of time to constant FA concentrations: the threshold value of 0.2 mg NH_3-N g^{-1} VAS (Volatile Attached Solids) after 2 months of operation increased to 0.5–0.7 mg NH_3-N g^{-1} VAS after 6 months, for constant VAS concentration and NH_3 loads (66.4 mg NH_3-N d^{-1} = 2 g NH_4^+-N d^{-1}). Moreover, the inhibitory effect of FA on NOB is attenuated when the temperature is within the optimum range for NOB growth, i.e., 10–20 °C (Balmelle et al. 1992). Furthermore, the inhibition of NOB seems to be reversible as nitrite ceased to accumulate in river sediments when FA concentrations declined below 50 μg N L^{-1} (Smith et al. 1997a).

4.3 *Free nitrous acid (HNO_2)*

Apart from the NH_4^+/NH_3equilibrium, pH also influences the equilibrium between nitrite and the unionised, free nitrous acid (FNA). HNO_2 has been found to be toxic to nitrifiers. Also here, the adjective 'free' points to the non-dissociated state of the nitrous acid. According to the nitrous acid equilibrium, the concentration of FNA will increase as the pH decreases:

$$H^+ + NO_2^- \leftrightarrow HNO_2 \quad (14)$$

$$[HNO_2 - N]_{free} = \frac{[NO_2^- - N]}{K_n \times 10^{pH}} \quad \text{with}$$

$$K_n = \exp[-2300/(273 + t)] \quad (15)$$

K_n = Ionisation constant for nitrous acid (e.g., K_n at 20 °C = $10^{-3.4}$)

An inhibition mechanism that has been proposed for HNO_2 toxicity is that it acts as an uncoupler by donating a proton inside the cell. That intracellular proton directly interferes with the transmembrane pH gradient required for ATP synthesis (Glass et al. 1997).

The concentrations of NH_3 and HNO_2 in function of total ammoniacal nitrogen (TAN=NH_4^++NH_3) and total nitrite concentrations (NO_2^-+HNO_2), pH and temperature were given by Anthonisen et al. (1976). From these, boundary conditions of zones of nitrification inhibition were determined (Figure 3). A range of boundary conditions, depending on various operating conditions, delimits each zone. Zone 1 (FA > 10–150 mg L^{-1}) marks the inhibition of AOB and NOB by free ammonia, while in zone 2 (0.1–1.0 mg L^{-1} < FA < 10–150 mg L^{-1}) FA inhibits only NOB. Complete nitrification is possible in zone 3 (FA < 0.1–1.0 mg L^{-1} and FNA < 0.2–2.8 mg L^{-1}). In zone 4 NOB are inhibited by free nitrous acid (FNA > 0.2–2.8 mg L^{-1}). Because the concentrations of these two forms depend on the solution pH, FA is the main inhibitor of nitrification at high pH (>8), for lower concentrations of free ammonia than of free nitrous acid inhibit the nitrifiers, whereas FNA is the main inhibitor at low pH (<7.5).

Prakasam and Loehr (1972) obtained 0.02 mg HNO_2-N L^{-1} as a threshold concentration of nitrite oxidation inhibition, which is lower than the threshold boundary range concentrations of 0.2–2.8 mg FNA L^{-1} or 0.06–0.83 mg HNO_2-N L^{-1} found by Anthonisen et al. (1976).

Also denitrification is inhibited by nitrous acid, from concentrations of 0.13 mg HNO_2 L^{-1} (0.04 mg N L^{-1}) at a pH of 6.8 (Abeling & Seyfried 1993). Glass et al. (1997) estimated the threshold inhibitory concentration for HNO_2 less than 0.02 mg HNO_2-N L^{-1} at pH 6. In this case, the biomass concentration appeared to have no effect on nitrite inhibition of denitrification.

4.4 *Hydroxylamine (NH_2OH)*

According to Hu (1990) hydroxylamine exhibited acute toxicity to *Nitrobacter* and this may also cause nitrite build-up in a nitrifying system. Hydroxylamine has been found to severely inhibit *Nitrobacter* (Castignetti & Gunner 1982; Stüven et al. 1992). No nitrite oxidation occurred when 0.42 mg NH_2OH-N L^{-1} was present. Addition of 2.5–5 mg NH_2OH-N L^{-1} to a submerged filter system significantly enhanced nitrite accumulation during nitrification (Hao & Chen 1994). Moreover, this inhibitory effect of hydroxylamine on NOB was found to be irreversible. Yang and Alleman (1992) noted that the nitrite build-up in activated sludge batch cultures, correlated with the accumulation of free hydroxylamine (un-ionised or NH_2OH), and not necessarily with FA nor with low dissolved oxygen concentration. The pKa value of ionised hydroxylamine at 25 °C equals 7.99 (Yang & Alleman 1992).

4.5 *Nitrate (NO_3^-)*

In case of denitrification, nitrate in high concentrations has been found to inhibit the nitrite reduction due to competition for common electron donors (Almeida et al. 1995b; Van Rijn et al. 1996). Kornaros et al. (1996) found in addition that nitrate has an inhibitory effect on the synthesis and activity of nitrite reductase.

Observations of river sediments (Kelso et al. 1997) suggested that nitrite accumulation resulted predominantly from inhibition of the DNRA nitrite reductase system by nitrate. During DNRA, nitrite accumulates especially when high concentrations of nitrate are present. Such a nitrite accumulation was found by Bonin (1996) in the presence of 140 mg NO_3^--N L^{-1} in marine sediments.

4.6 *Temperature and seasonality*

Increased nitrite concentrations in wastewater treatment plants or even nitrogen-receiving surface waters have been found to vary with the seasons in the year. Tonkovic (1998) noted that nitrite accumulates in an activated sludge plant especially over the summer period. Also Gelda et al. (1999) found higher nitrite concentrations in a wastewater treatment facility without denitrification over the summer period (June to September). In the river Lahn (Germany) maximum nitrite concentrations were measured from May until July (mid spring–mid summer) (Von Der Wiesche & Wetzel 1998). A measuring campaign in the Lough Neagh river system (Northern-Ireland) demonstrated that minimum nitrite concentrations (0–19 μg N L^{-1}) were normally observed during winter months, while maximum concentrations (90–235 μg N L^{-1}) occurred during summer months (Smith et al. 1995). An increase in temperature from 20 to 30 °C in a compartmented (anoxic-oxic) labscale activated sludge installation resulted in a simultaneous decrease in suspended solids concentration and a temporary nitrite accumulation (from 0 to 6 mg N L^{-1}) which stabilised after a few days at 0.5 mg N L^{-1} (Philips, unpublished observations). In the "activated sludge model No. 1" (Henze et al. 1987) simulating the activated sludge processes in a wastewater treatment plant, the nitrification process is described as a single-step reaction with "autotrophs" converting ammonia to nitrate. At temperatures above 25 °C however, nitrite formation may become a severe problem and therefore nitrification should be expressed as a two-step reaction with nitrite as intermediate (Nowak et al. 1994). Knowles et al. (1965) determined that the ammonia and nitrite oxidation rates increase 2.6 and 1.8 times per 10 °C in a physiologically relevant temperature range. This is in contrast with the Arrhenius equations, from which it can be calculated that if temperature increases from 20 to 30 °C, ammonia and nitrite oxidation rates increase by a factor 2.04 and 2.15 respectively (Muller et al. 1995). The relation between the growth rates of the nitrifiers executing the two constituting steps of nitrification, the AOB and NOB, changes with temperature. It is thought that the optimum temperature for nitritation is higher than that for nitratation (Wortman & Wheaton 1991). According to Knowles et al. (1965), the maximum specific growth rate of *Nitrobacter* is significantly higher than that of *Nitrosomonas* at temperatures between 10 to 20 °C. At temperatures higher than 25 °C however, the maximum specific growth rate of *Nitrobacter* is approximately in the same range as that of *Nitrosomonas*, possibly leading to nitrite accumulation. Mulder and van Kempen (1997) go further and claim that at higher temperatures, the growth rate of the NOB is lower than that of the AOB (Figure 4). The Sharon process makes use of this property to wash out the NOB. In this way the nitrification is stopped at nitrite so that less oxygen is needed and subsequent denitrification demands less external carbon source (Hellinga et al. 1997; Jetten et al. 1997).

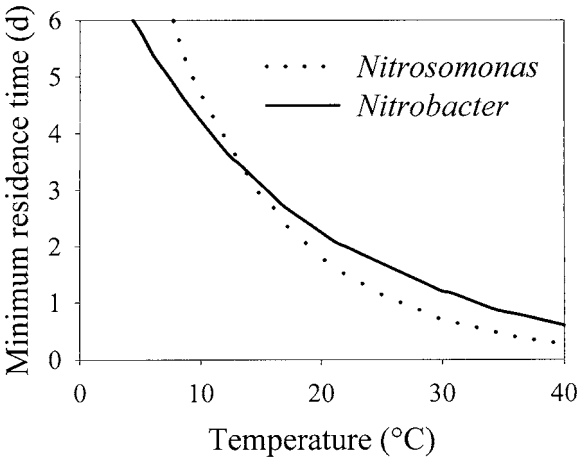

Figure 4. Effect of temperature on the minimal required cell residence time for ammonia and nitrite oxidation. Above 14 °C it is possible to wash out the NOB while maintaining the AOB (after Jetten et al. 1997, with temperature coefficients of Hunik et al. 1994).

Studies on nitrifying biofilms as a model for riverbeds indicate a critical temperature domain (16–22 °C) in which the activity of NOB seems to be more inhib-

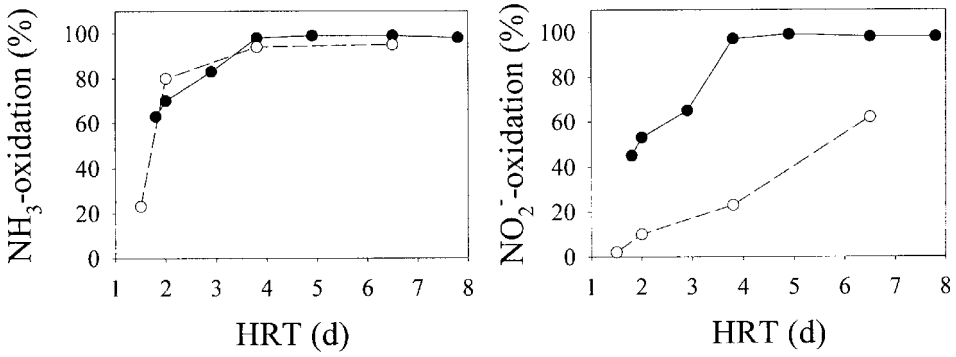

Figure 5. Effect of O_2 concentration, sufficient O_2 (—●—) and 0.5 mg O_2 L^{-1} (—○—), on ammonia and nitrite oxidation in pure cultures of nitrifying bacteria after reaching steady state conditions for various hydraulic retention times (HRT) (after Hanaki et al. 1990b).

ited than the nitritation by AOB (Von Der Wiesche & Wetzel 1998). A modelling analysis on data collected from a nitrogen polluted lake (Gelda et al. 1999) demonstrated that the distinct seasonal differences in NO_2^-, i.e., peak nitrite concentrations over the July to October interval (summer) compared to the rest of the year, are largely the result of differences in rate and temporal patterns of the two stages of nitrification. The abrupt nitrite peaks in September/October reflect intervals of higher nitritation than nitratation rates followed by periods of higher nitratation than nitritation rates.

Next to higher temperatures, lower temperatures (<14 °C) have often been found to result in nitrite accumulation in activated sludge (Randall & Buth 1984b). Though, Mauret et al. (1996) did not find a significant specific effect of temperature on the accumulation of nitrite, because although at 15 °C NOB activity is reduced, AOB activity goes down proportionally. According to Koch and Siegrist (1997) more nitrate reducers are present when temperatures during growth are low.

As follows from the formula describing the equilibrium of NH_3/NH_4^+ and of HNO_2/NO_2^-, temperature plays a role in the respective concentrations in the waterphase. An increase in temperature causes an increase in FA concentration, according to eq. 13. Earlier it was mentioned that increased temperatures result in higher activity of nitrifying bacteria. However, Fdz-Polanco et al. (1994) showed that rising temperatures differentially affect AOB and NOB via the formation of NH_3 that is more inhibitory to NOB. For NOB the inhibition of the higher NH_3 (>1 mg NH_3-N mg^{-1} VAS) concentrations at higher temperatures outweighs the activity stimulant allowing nitrite accumulations of 80% (Fdz-Polanco et al. 1994).

4.7 *Dissolved oxygen concentration (DO)*

When it comes to nitrification, the dissolved oxygen concentration is an utmost important parameter for both AOB as for NOB. Low oxygen concentrations induced for instance a marked decrease in the rate of NO_2^- production by pure cultures of *Nitrosomonas* sp. (Goreau et al. 1980). However, AOB seem to be more robust towards low DO than NOB. Accumulation of nitrite at low DO is usually explained by the difference in saturation constant in terms of DO (K_0) between AOB and NOB (Hanaki et al. 1990b). In other words, oxygen deficiency due to low DO more significantly influences the activity of NOB than that of AOB (Leu et al. 1998). This was nicely illustrated by Hanaki et al. (1990b) (Figure 5). According to Hunik et al. (1994) the half-saturation constant for O_2 is 0.16 mg O_2 L^{-1} and 0.54 for *Nitrosomonas europaea* and *Nitrobacter agilis* respectively. However, values for the half-saturation constant given in literature for activated sludge vary in the range of 0.25–0.5 mg O_2 L^{-1} and 0.34–2.5 mg O_2 L^{-1} respectively (Barnes & Bliss 1983). This is due to the experience that the oxygen concentration in a sludge floc or biofilm not necessarily equals that of the waterphase. The saturation constant is therefore dependent on the biomass density, the floc size, the mixing intensity and the rate of diffusion of O_2 in the floc (Münch et al. 1996).

According to tests performed by Leu et al. (1998), the oxygen deficiency resulting from ammonium degradation in the inner layer of mixed films caused the accumulation of nitrite. This indicates that the activity of NOB is more influenced by oxygen deficiency in deep mixed biofilms under low organic matter condition than that of AOB. Strangely, a thicker biofilm does not necessarily lead to higher nitrite accu-

mulations during nitrification. According to Harada et al. (1987) the accumulation of nitrite in the bulk phase increased with a decrease in biofilm thickness. Possibly this can be explained by the hypothesis that in thick biofilms, oxygen is not only transported by means of diffusion, but also by advection through channels.

When the O_2 supply was limited, nitrite accumulated in a mixed culture of *Nitrosomonas europaea* and *Nitrobacter winogradsky* (Laanbroek & Gerards 1993). The ammonia-oxidising population had a greater specific affinity for O_2 than the nitrite-oxidising bacteria. Cessation of the oxygen supply resulted in an immediate accumulation of nitrite in the mixed cultures of *Nitrosomonas europaea* and *Nitrobacter hamburgensis* that had been growing at 80% O_2 saturation (Laanbroek et al. 1994). Apparently the AOB win the competition for the limiting amounts of oxygen in the transient state between 80 and 0% O_2 when switched from oxic to anoxic. Moreover, AOB have been found to develop an ability to endure the fluctuation of DO, but NOB did not (Peng et al. 2000).

In a study with a suspended growth reactor, nitrite oxidation was strongly inhibited by low DO (<0.5 mg L^{-1}). Together with unaffected ammonia oxidation this resulted in accumulation of nitrite to 60 mg N L^{-1} (Hanaki et al. 1990b). Moreover, according to Hanaki et al. (1990b) these low DO levels even result in higher (doubled) growth yields of AOB while the growth yield of NOB was unchanged, which can possibly give rise to increased nitrite concentrations. Experiments by Laanbroek et al. (1994) resulted in yields of *Nitrosomonas europaea* at oxygen concentrations of 0 and 80% air saturation equal to 13.8 and 6.6 10^{12} cells mol^{-1} NH_4^+ respectively, which also implies more than a doubling of the growth yield for low DO levels.

Ceçen and Gönenç (1995) found that the bulk oxygen to bulk ammonia ratio rather than the ammonia concentration itself is the most crucial parameter in the accumulation of nitrite. In nitrification, these researchers found a considerable degree of nitrite accumulation at bulk O_2/bulk NH_3 ratios lower than 5. Both bulk oxygen and bulk ammonia concentrations should be monitored and their ratio should be at least 5 to prevent nitrite accumulation. Because this requirement is not often achieved in zero-order range full-scale systems, high nitrite concentrations can be expected in the effluent (Ceçen & Gönenç 1995). Also Joo et al. (2000) recorded an important

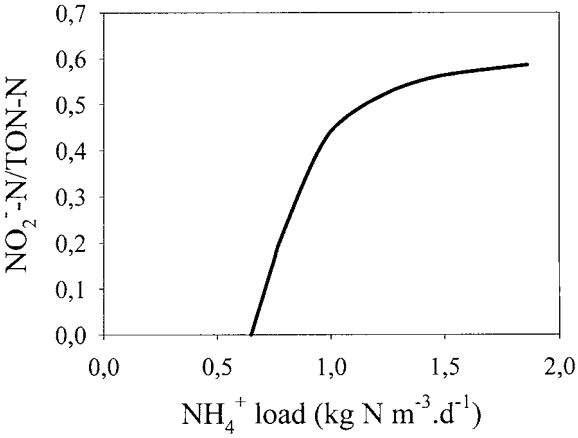

Figure 6. Effect of NH_4^+ load on nitrite accumulation expressed as part of nitrite in the amount of total oxidised nitrogen (TON = NO_2^- + NO_3^-) (after Joo et al. 2000).

connection between O_2 and NH_4^+ load. Experiments with an upflow biological aerated filter (low superficial airflow = 0.21 cm s^{-1}) showed that NO_2^- accumulation increased for increasing NH_4^+ loads (Figure 6). Figure 6 demonstrates that when ammonium load increased from 0.6 kg NH_4^+-N m^{-3} to 1 kg NH_4^+-N m^{-3}, the ratio $NO_2^-/(NO_2^- + NO_3^-)$ rapidly increased to 0.5. According to Joo et al. (2000) this indicates that indirectly oxygen was the limiting component, all the more since pH was so that inhibition by NH_3 was negligible.

It is essential for good nitrification to keep DO at a sufficiently high level, but this imposes costs. Knowledge on the effect of low DO and the acceptable DO for complete nitrification is essential for process economy, as the amount of energy consumption for air supply accounts for a high percentage of the total energy consumption in a wastewater treatment plant.

In the process of denitrification, oxygen inhibits the synthesis of nitrate reductase only partially, while the synthesis of nitrite reductase is completely suppressed. Therefore, at the start of anoxic conditions after an aerobic period, initially only the nitrate reduction starts, leading to a temporary nitrite accumulation (Krul & Veeningen 1977). These findings were confirmed by recent investigations. Ferguson (1994) also attributed inhibition of nitrite reductase to DO. At a bulk DO of 5.6 mg O_2 L^{-1}, the rate of NO_3^- reduction in activated sludge was reduced to 33% of the anoxic rate, while the rate of (NO_3^-+NO_2^-) disappearance was only 4% of the anoxic rate (Oh & Silverstein 1999b). Linked to the observed nitrite accumula-

Table 7. Critical concentrations (mM) of some volatile fatty acids with respect to inhibition of nitrification and denitrification (after Eilersen et al. 1994, 1995)

Volatile fatty acid	Ammonia oxidation	Nitrite oxidation	Nitrite reduction	Nitrate reduction
Formic acid	—	2	—	—
Acetic acid	—	115	—	—
Propionic acid	—	68	196	74
n-Butyric acid	—	33	—	—
Isobutyric acid	6	8	32	30
n-Valeric acid	37	75	57	36
Isovaleric acid	6	7	18	18
n-Caproic acid	36	81	110	105

The critical concentration is the concentration at which activity falls to 50% of the activity in the absence of the inhibiting compound.

tion, this also proofs the higher sensitivity of nitrite reductase towards oxygen. It was established by Casey et al. (1999a) that for denitrifying organisms grown under anoxic conditions, and subsequently subjected to aerobic conditions, O_2 affects the synthesis of the reductases in the order N_2O, NO, NO_2^- and least of all NO_3^- reductase. Thus under the anoxic period of alternating anoxic-aerobic systems, NO_3^- reductase is synthesised at higher levels than NO_2^- reductase, resulting in an increase in nitrite concentrations (Casey et al. 1999b). Moreover as discussed earlier, nitrite oxidation is more sensitive to low DO, which can be found under the un-aerated period in alternating anoxic-aerobic conditions, than ammonia oxidation. Consequently, under the aerobic conditions of alternating anoxic-aerobic systems nitrite is not oxidised at the same rate as ammonia and therefore accumulates. Conceivably, both production of nitrite under aerobic (nitrification) as under anoxic conditions (denitrification) can contribute to extracellular nitrite accumulation under alternating anoxic-aerobic conditions (Casey et al. 1999b).

4.8 *Volatile fatty acids*

Formic, acetic, propionic and n-butyric acid all inhibited nitrite oxidation, but exhibited no significant effect on ammonia oxidation (Eilersen et al. 1994). Examples of critical concentrations of volatile fatty acids are summarised in Table 7. The inhibitor concentration I_k is the concentration at which activity falls to 50% of the activity in the absence of the inhibiting compound. Curiously, the iso-forms have considerable lower I_k values. Also Takai et al. (1997) indicated non-dissociated fatty acids to be responsible for the inhibition of nitrite oxidation, while ammonia oxidation was not inhibited.

When used as e-donor (5 mM) in denitrification by *Pseudomonas stutzeri*, acetic and propionic acid also lead to nitrite accumulation, while at that concentration no accumulation was noticed for butyric, valeric or caproic acid (Van Rijn et al. 1996). Eilersen et al. (1995) provided concentrations of volatile fatty acids provoking 50% inhibition of nitrite reduction (Table 7). Values for this critical concentration for acetic and propionic acid are high compared to the 5 mM leading to nitrite accumulation in the work of Van Rijn et al. (1996). For isobutyric, isovaleric and n-valeric acid the un-dissociated form appeared to act as the inhibitor (Eilersen et al. 1995).

In a study on the effect of carbon compounds commonly found in agricultural pollutants on the biochemical pathways of nitrite accumulation in freshwater sediments, Kelso et al. (1999) found that formate especially and acetate to a much lesser extent can give rise to nitrite accumulations up to 1.3 mg N L^{-1} caused by partial inhibition of the denitrification pathway.

4.9 *Organic matter*

Literature has reported on the influence of organic matter on nitrification, and possible nitrite accumulation. An enormous build-up of nitrite occurred upon increasing the organic matter concentration (Leu et al. 1998), due to oxygen deficiency. When organic matter is present, heterotrophs compete with nitrifiers for oxygen (Zhang et al. 1995), and generally the heterotrophs win thanks to their higher affinity for oxygen. According to Okabe et al. (1996), increased C/N ratios retarded particularly the NOB. Zhang et al. (2000) found that nitrite accumulation in a nitrifying biofilm was related to fulvic acids loading. Fulvic acids loadings higher than 0.02 kg TOC $h^{-1}.m^{-3}$ caused nitrite build-up as high as 11.4 mg N L^{-1}, AOB were only inhibited by fulvic acids loadings higher than 0.05 kg TOC $h^{-1}.m^{-3}$. Dependency of nitrite concentration on influent COD (Chemical Oxygen Demand) or HRT (Hydraulic Residence Time) in combined nitrifier and heterotroph populations is complicated (Hanaki et al. 1990a) as different combinations of these two factors had different effects on nitrite concentrations in the effluent.

In the case of denitrification, Wilderer et al. (1987) found that glucose as carbon source promoted nitrite accumulation. This was explained in terms of the fermentative conditions allowing an enrichment of the biocommunity for bacteria that reduce nitrate only to nitrite. Gomez et al. (2000) investigated sucrose, ethanol and methanol as carbon sources in a denitrifying submerged biofilter. Nitrite accumulation occurred with sucrose as carbon source; with ethanol and methanol nitrite accumulation was practically zero.

Also the lack or deficiency of organic carbon has been reported to influence the accumulation of nitrite during denitrification (Martienssen et al. 1995; Oh & Silverstein 1999a). This accumulation nearly immediately disappeared upon addition of methanol as carbon source in the anoxic phase (Martienssen & Schöps 1997). Oh and Silverstein (1999a) on the other hand found that nitrite accumulation during denitrification due to carbon (acetate) limitation (C/N = 1/1) required several weeks to be eliminated after C/N ratio was increased to 2/1.

4.10 *Phosphate (PO_4^{3-})*

Nitrite oxidation might be affected by phosphorus deficiency (Nowak et al. 1996). In a biological pretreatment plant for rendering plant effluent, treating highly nitrogenous wastewaters ($t > 25\,^{\circ}C$), nitrite oxidation was substantially reduced at phosphate levels below 0.2 mg PO_4^{3-}-P L^{-1}. The phosphate half-saturation coefficient for NOB is about one order of magnitude higher than for AOB (0.2 $\leftrightarrow$ 0.03 mg P L^{-1}) (Nowak et al. 1996). *Nitrobacter* especially is unable to oxidise nitrite to nitrate in the absence of phosphates, the so-called phosphate block. Phosphate, but in this case rather its presence than its absence, has also a profound effect on denitrification, causing nitrite accumulation: intermediate nitrite accumulation during nitrate reduction is higher in a medium with PO_4^{3-} than in a medium devoid of phosphate. Moreover, nitrite reduction is markedly depressed by the presence of phosphate. Nitrite reduction rates by a *Pseudomonas* sp. strain decreased from 4.9 to 2.7 mg NO_2^--N g^{-1} protein min^{-1} as phosphate concentration was increased from 0 to 15.5 mg P L^{-1} (Barak & Van Rijn 2000). According to Barak and Van Rijn (2000) the latter could be caused by a competitive use of a c-type cytochrome, needed for electron transport to both nitrite reductase and the site responsible for active phosphate transport.

4.11 *Inhibitory compounds*

Of a dozen compounds tested by Tomlinson et al. (1966), only chlorate, cyanate, azide and hydrazine were more inhibitory to the oxidation of nitrite than of ammonia. Azide (50% at 0.3 μM *in vivo*) has been shown to be a strong selective inhibitor of nitrite oxidation (Ginestet et al. 1998). Chlorate (20 mM) has been used to stop nitrite oxidation in activated sludge systems (Surmacz-Gorska et al. 1996). In tests with gold-mine service water, it was shown that chlorine (3–13 mg L^{-1}), chlorine dioxide (2–8 mg L^{-1}), bromine (>8 mg L^{-1}) and cyanide (>2 mg L^{-1}) caused selective inhibition of the nitritation (Jooste & Vanleeuwen 1993). The nitrifiers appeared however to be able to adapt to bromine.

In a nitrification reactor followed by a denitrification column, NOB was more adversely affected by the salt content (NaCl) than AOB resulting in accumulation of nitrite at salt contents above 2% (Dinçer & Kargi 1999). Changing an aquarium from freshwater to seawater resulted in an immediate increase in ammonium and subsequently nitrite. Reestablishment of nitrite oxidation took 40 days, in contrast to the faster recovery of ammonia oxidation (20 days) (Hovanec et al. 1998).

Tang et al. (1992) examined the toxicity of 43 organic chemicals to *Nitrobacter*. From these chemicals 1,2-Dichlorobenzene, 1,2,4-Trichlorobenzene, Cyclohexane, Octanol, 1-Clorohexane, 2,4-Dichlorophenol, 2,4,6-Trichlorophenol and 4-Aminophenol had a IC50 (concentration causing 50% inhibition) below 1 mM. Also p-Nitrobenzaldehyde, p-nitraniline and N-methylaniline have been found to be inhibitory to nitrite oxidation (Hockenbury & Grady 1977). Acid Orange 7, an azo dye commonly used in textile, pharmaceutical, food and cosmetic industries inhibits all stages of the nitrification process, although the NOB were more sensitive than the AOB (He & Bishop 1994).

In low concentrations (e.g., 0.7 mg L^{-1}), nickel is more inhibitory towards NOB, leading to nitrite build-up. The sensitivity of the nitrifiers for nickel increases with lower temperatures ($14\,^{\circ}C \leftrightarrow 30\,^{\circ}C$), which could indicate a synergistic toxic effect between nickel and temperature (Randall & Buth 1984a). Contradictory to the latter, from a study on the toxic responses of heavy metals on nitrifiers, *Nitrosomonas* sp. was found to be equally or even more sensitive than *Nitrobacter* sp. towards nickel and copper (Lee et al. 1997). Jooste and Vanleeuwen (1993) on the

contrary found that nickel (II) did not significantly affect the nitrification process. In a biofilm from a RBC (Rotating Biological Contactor), nickel at 50 mg L^{-1} exhibited 10% inhibition of nitrite oxidation, while cadmium (50 mg L^{-1}) gave 50% inhibition and copper nil (Wang 1984). In still another research, Cu (26 mg L^{-1}), Fe (22 mg L^{-1}), Pb (1.98 mg L^{-1}), Cr (0.23 mg L^{-1}) and Cd (0.024 mg L^{-1}) were found not to affect nitrite reduction and were not toxic to the bacteria (Arquiaga et al. 1993). Moreover, the introduction of metals (Cd, Cr, Pb, Cu and Fe) in concentrations (from 0.02 mg L^{-1} for Cd to 22 mg L^{-1} for Fe) typical for navy shipyard wastewater did not inhibit nitrite removal efficiencies (Kamath et al. 1991).

4.12 *Light*

Light is inhibiting to both AOB and NOB, through the oxidation of cytochrome c caused by light in the presence of O_2. Olson (1981) and Vanzella et al. (1989) found evidence that NOB were more sensitive to sunlight than AOB. In coastal marine samples, Olson (1981) found a 50% inhibition of nitrite oxidation at a light intensity of 6.64 μmol m^{-2} s^{-1}, as opposed to a 50% inhibition of ammonia oxidation at light intensity of 18.26 μmol m^{-2} s^{-1} (full sunlight = 2490 μmol m^{-2} s^{-1}). This results in the spatial separation of the two stages of nitrification in marine environments, from which the position of a nitrite maximum in near surface seawater can be explained (Olson 1981). Treatment with a low light dose for extended periods was more damaging to NOB (Guerrero & Jones 1996). Bock (1965) attributes this greater sensitivity of NOB to the relatively low cytochrome c content of *Nitrobacter* compared to *Nitrosomonas*. Guerrero and Jones (1996) concluded that the effect of light depends on the type of nitrifier as well as on the conditions of the environment. They also found that phototolerance of NOB was altered by increased cell concentrations which made these organisms light susceptible. A measuring campaign in a wastewater reservoir in Israel (Kaplan et al. 2000) identified light as a major factor hindering nitrification and especially inhibiting nitratation causing the accumulation of nitrite during late spring and summer.

Nitrite reduction in a *Pseudomonas* strain is susceptible to photo-inhibition while the rate of nitrate reduction and the aerobic respiration remained unchanged (Barak et al. 1998). The inhibition of the nitrite reduction was dose dependent with respect to light: compared to reduction rates in darkness a 15% inhibition was found at a light intensity of 5% of full sunlight against a 67% inhibition at full (100%) sunlight intensity (2000 μmol m^{-2} s^{-1}). Concerning the effect of different light spectra, Barak et al. (1998) further found that exposure to green light led to the highest inhibition of nitrite reduction. As a possible mechanism, it was suggested that light may impair the electron transfer from cytochrome c to nitrite reductase.

A totally different source of nitrite accumulation influenced by light is the production of significant concentrations of nitrite (40–118% relative to initial levels) from the photodegradation of humic substances (Kieber et al. 1999).

5. Operation of reactor or treatment plant

In literature quite a number of experimental examples are given on the role of operational parameters, such as hydraulic (HRT) or sludge residence time (SRT), start-up, mode of operation, in nitrite accumulation.

The effluent nitrite increased with a decrease of HRT in pure nitrification systems and in combined systems (nitrifiers and heterotrophs receiving glucose) with an influent COD of 160 and 500 mg L^{-1}. However, longer HRT also gave higher effluent nitrite when higher substrate concentrations (1000 mg COD L^{-1}) were added (Hanaki et al. 1990a). After study of dynamics of denitrifying bacteria in a model biocommunity, Martienssen and Schops (1999) concluded that at C/N ratios <2.5 and at an optimal HRT of 3 days, bacterial communities containing balanced ratios of nitrite-producing and of nitrite-reducing bacteria are established. In these communities a complete denitrification without accumulation of nitrite proceeded. Longer HRT resulted in increasing portions of the nitrite-accumulating *Staphylococcus sp*. Moreover, in the case of nitrification, according to Okabe et al. (1996), a higher C/N ratio retarded an accumulation of nitrifying bacteria, particularly NOB. The latter is due to a competition for oxygen between heterotrophs and nitrifiers (Zhang et al. 1995).

Besides the residence time in the reactor, also the length of the sequential anoxic and aerobic periods within the residence time is of significance. As the induction of the different denitrification enzymes proceeds sequentially, the different intermediates of denitrification accumulate temporarily after the switch

from aerobic to anoxic conditions. The length of the anoxic period is therefore important to achieve complete denitrification. Longer SRT enable the presence of higher numbers of complete denitrifiers (Arquiaga et al. 1993). According to Baumann et al. (1997) an anoxic period of 4 hours is sufficient to synthesise all enzymes and have complete denitrification.

Experiments by Rols et al. (1994) showed that the threshold of the FA inhibition and the resultant degree of nitrite accumulation depend both on the history of the sludge and on the hydraulic regime (mixed or plug flow) as well as the mode of operation (batch or continuous) of the reactor. Also Kone and Behrens (1981) state that nitrite accumulation is triggered by discontinuous operation such as batch reactors. For example, start-up in batch resulted in 65% nitrite accumulation, while only an accumulation of 30% was noticed for start-up in continuous flow mode (Villaverde et al. 2000). According to Fdz-Polanco et al. (1996) plug flow hydrodynamics in a nitrifying submerged biofilter will favour nitrite accumulation in situations of FA inhibition. Another aspect that should be considered is the more readily recovery of AOB than NOB from starvation, explaining the occasionally observed nitrite accumulation after ammonium becomes again available (Gerards et al. 1998; Tappe et al. 1999). Temporary sludge storage without addition of any substrate by means of applying idle periods ranging from 6 to 20 days in sequencing batch reactors showed that nitrite oxidation was more sensitive to long idle phases, resulting in temporary nitrite accumulation (Morgenroth et al. 2000). As for biofilm characteristics, Nijhof and Klapwijk (1995) concluded that diffusional transport mechanisms and differences in biofilm characteristics could explain the level of nitrite concentration observed in their fish culture systems recirculating over trickling filters.

6. Conclusions and remarks

The position of nitrite in the nitrogen cycle is unique, as it is involved in both aerobic and anaerobic transformations. Its accumulation depends on the balance of the rates of its formation and transformation. Parameters influencing these balances are numerous and very divers.

The fact that, despite these fragile balances, nitrite accumulation is not the rule in aquatic systems, is in view of the different characteristics of the nitrite-generating and -removing organisms (both oxidatively and reductively) indicative of close regulation between these microbial populations and their activities and physiological characteristics. It could be reasoned that such close regulation is related to close cell-to-cell contact. This aspect needs to be examined in much more detail. Moreover, the occurrence of nitrite in water systems could be considered as a signal par excellence of an uncoupling of nitrifiers in particular, or even of an overall uncoupling of the microbial community. Hence, in this respect, monitoring for significant levels of nitrite in ecosystems could correspond to overall monitoring of an important part of ecosystem equilibrium.

Acknowledgements

The Fund for Scientific Research – Flanders is acknowledged for providing a PhD Scholarship to S. Philips. We wish to thank Elke Vincke, Kris Pynaert, Jan Kielemoes, Roeland Grommen and Tom Van de Wiele for critical reading this manuscript.

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