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REVIEW PAPER

Partial nitrification—operational parameters and microorganisms involved

Banashri Sinha · Ajit P. Annachhatre

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Abstract Nitrite is a common intermediate in at least three different oxidative or reductive biochemical pathways that occur in nature (nitrification, denitrification and dissimilatory or assimilatory nitrate reduction). Nitrite accumulation or partial nitrification has been reported in literature for decades. In engineered systems, partial nitrification is of interest as it offers cost savings in aeration as well as in the form of lesser need for addition of organic carbon as compared to the conventional denitrification. A broad range of operating parameters and factors has been reviewed in this paper which are essential for achieving partial nitrification. Of these, pH, dissolved oxygen (DO), temperature, free ammonia (FA) and nitrous acid concentrations, inhibitory compounds are important factors in achieving partial nitrification.

Two groups of bacteria, namely ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) are involved in nitrification. Chemolitho-autotrophic AOB are responsible for the rate-limiting step of nitrification in a wide variety of environments, making them important in the global cycling of nitrogen. Characterization and identification of the bacterial populations in an engineered system which have been considered to be a ''black box'', has been made possible by using non-cultivation based techniques such as fluorescent in situ hybridization technique (FISH), polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), Sequencing and other techniques involving quantitative chemical analyses of specific biomarkers including quinones. Accordingly, this paper also attempts to give examples of how various molecular techniques can be used for characterizing various microorganisms involved in biological nitrogen removal.

Keywords Ammonia-oxidizing bacteria · DO · Fluorescence in situ hybridization · Operating parameters · Partial nitrification · PCR-DGGE · pH · Temperature

Abbreviations

Anammox	Anaerobic ammonium oxidation
AMO	Ammonia Monooxygenase
AOB	Ammonia-oxidizing bacteria
cDNA	Complementary deoxyribonucleic acid
Cd	Cadmium
ClO_2^-	Chlorite ion
ClO_3^-	Chlorate ion
COD	Chemical oxygen demand
Cr	Chromium
Cu	Copper

B. Sinha · A. P. Annachhatre (✉)
Environmental Engineering and Management,
Asian Institute of Technology, P.O. Box 4,
Klong Luang, Pathumthani 12120, Thailand
e-mail: ajit@ait.ac.th

DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EPA	Environmental Protection Agency
FA	Free ammonia
Fe	Iron
FISH	Fluorescence in situ hybridization
FNA	Free nitrous acid
HAO	Hydroxylamine oxidoreductase
HNO_2	Nitrous acid
HRT	Hydraulic residence time
MCRT	Mean cell residence time
MK	Menaquinone
N_2	Nitrogen gas
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_3	Ammonia
NH_4^+	Ammonium ion
NH_2OH	Hydroxyl amine
NO	Nitric oxide
N_2O	Nitrous oxide
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
NOB	Nitrite-oxidizing bacteria
NOD	Nitrogenous oxygen demand
NOR	Nitrite oxidoreductase
Pb	Lead
PCR	Polymerase chain reaction
Q	Ubiquinone
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
Sharon	Single reactor high activity ammonia removal over nitrite
SMBR	Submerged membrane bioreactor
SRT	Sludge residence time
TAN	Total ammoniacal nitrogen
TOC	Total organic carbon
VAS	Volatile attached solids
WWTP	Wastewater treatment plant

1 General background and objectives

Nitrifying bacteria and nitrification processes are integral part of most aerobic biological treatment plants. In treatment plants, the microbial population represents a delicate balance of organisms, each interacting with and influencing other members of the population. The basic aim in the operation of a biological treatment plant is to create conditions that favor the desired reactions. In order to achieve this condition a wise and thorough manipulation of the various operating parameters need to be done, where the desired population outcompetes the various other bacterial populations present within the reactor.

Nitrification is the process whereby the reduced ammoniacal nitrogen is biologically oxidized to nitrite (nitritation) and then to nitrate (nitratation) with O_2 as terminal e-acceptor. This is followed by denitrification of nitrate and nitrite to nitrogen gas which is released into the atmosphere. The process is catalyzed by two phylogenetically unrelated groups of autotrophic bacteria, the ammonia-oxidizing bacteria (AOB) and the nitrite-oxidizing bacteria (NOB). The first step of nitrification, the oxidation of ammonia to nitrite, is performed by AOB. The second step of nitrification, the oxidation of nitrite to nitrate, is performed by NOB. Almost all nitrifying bacteria are obligate chemolitho-autotrophic, i.e., they fulfill 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-). The heterotrophic denitrifiers need an organic carbon source for their growth and hence it necessitates the addition of a source of organic carbon like methanol, ethanol, molasses, etc. during conventional denitrification. The reactions involved during nitrification are dealt in details in the next section.

The urgency of reconsidering the current practices (conventional technologies of domestic wastewater treatment) in the light of sustainability becomes evident. The present-day wastewater treatment practices can significantly be improved through the introduction of new microbial treatment techniques. Partial nitrification and denitrification has been one of the most desired reactions in a nitrification treatment plant. The scientific understanding of the nitrite nitrification process (Sect. 3 of this paper) is increasingly important in the areas of biological nutrient

removal (BNR) systems for sewage treatment as well as various strong nitrogenous waste treatments including leachate and animal wastes. Partial nitrification techniques have been denoted for quite a while, as very promising for improved sustainability of wastewater treatment as it offers cost savings in aeration as well as savings in the form of lesser need for addition of organic carbon as compared to the conventional denitrification. Details of some novel processes for nitrogen removal have been reviewed and discussed extensively by Khin and Annachhatre (2004).

After the successful isolation of some species of AOB and NOB, researches continued to investigate their diversity by applying molecular techniques. Till date, *Nitrosomonas* and *Nitrosospira* are the most extensively studied AOB and *Nitrobacter* and *Nitrospira* are the most numerously found NOB. Both AOB as well as NOB are sensitive to process operating conditions such as DO, pH, temperature, alkalinity and presence of toxic compounds. Recent developments in microbiology like fluorescence-labeled, ribosomal RNA (rRNA)-targeted oligonucleotide probes or quinone profile are being widely used as a tool for the direct, cultivation-independent identification and investigation of individual microbial cells in complex environmental samples or in their natural environments. These tools can be used to assess the efficiency of the reactor operation and the growth of the desired population. This paper attempts to substantiate the use of these tools for the microbial shift study for better understanding of the reactor performance by some examples.

Reviews related to partial nitrification and on the recent developments are available as researches have proceeded along the years. All researches along the partial nitrification or nitritation line explore the inhibitory factors (for example free NH_3 or toxic compounds) that have a selective inhibition on the nitrite oxidizers and a minimal affect on the ammonia oxidizers, or the competitive factors (temperature, dissolved oxygen (DO) which do not inhibit nitrite oxidizers but can give a competitive advantage to ammonia oxidizers over the nitrite oxidizers. Some of the works investigating nitrite build-up during nitrification were performed by Wallace and Nicholas (1969), Aleem (1970), Painter (1970), Focht and Chang (1975), Anthonisen (1976), Sharma and Ahlert (1977), Suthersan and Ganczarczyk (1986), Jayamohan and Ohgaki (1988), Hanaki and Wantawin (1990) and Philips et al. (2002) which focused on a number of factors, such as the free ammonia (FA) concentration, pH, temperature, dissolved oxygen (DO) concentration and inhibitory compounds. The ways to accomplish the aim have been described by many authors, but the general conclusion is not clear and easy. This is an attempt to summarize the information of recent years and to present an overview of the general operating parameters and conditions involved during partial nitrification as well as give an insight of the types of bacteria involved in nitrification by use of non-cultivation based molecular techniques.

2 Biochemistry of nitrification

The overall stoichiometric reactions in the oxidation of ammonia to nitrate can be written as follows (EPA 1975):

$$NH_4^+ + 1.5O_2 \rightarrow 2H^+ + H_2O + NO_2^- + 58 - 84\,\text{kcal} \quad (1)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- + 15.4 - 20.9\,\text{kcal} \quad (2)$$

Reaction 1 and 2 are believed to serve as energy-yielding reactions for two autotrophic bacteria, represented by the genera *Nitrosomonas* and *Nitrobacter*, respectively. If the empirical formulation $C_5H_7NO_2$ for the gross composition of biomass is considered acceptable for ammonia and nitrite oxidizers, the following reactions can be written to represent growth (EPA 1975):

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

$$5CO_2 + NH_4^+ + 10NO_2^- + 2H_2O \rightarrow 10NO_3^- + C_5H_7NO_2 + H^+ \quad (4)$$

Although about 99% of carbon dioxide in solution exists in the form of dissolved carbon dioxide (Umbreit et al. 1957), the carbonic

acid-bicarbonate equilibrium is operative in accordance with the following equation:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \quad (5)$$

The free acid (H^+) produced in reactions 1, 3 and 4 reacts to produce carbonic acid (H_2CO_3), according to Eq. 5. Rewriting Eqs. 1, 3 and 4 by combining with Eq. 5 yields

$$NH_4^+ + 1.5O_2 + 2HCO_3^- \rightarrow 2H_2CO_3 + H_2O + NO_2^- + 58 - 84\,\text{kcal} \quad (6)$$

$$13NH_4^+ + 23HCO_3^- \rightarrow 8H_2CO_3 + 19H_2O + 10NO_2^- + 3C_5H_7NO_2 \quad (7)$$

$$NH_4^+ + 10NO_2^- + 4H_2CO_3 + HCO_3^- \rightarrow 10NO_3^- + C_5H_7NO_2 + 3H_2O \quad (8)$$

The energy released as a result of Eq. 6 is utilized in reaction 7. The two can be combined to form an overall synthesis-oxidation reaction, if the yield coefficient for the ammonia oxidizers is known. The same can be said of Eqs. 2 and 8 for the nitrite oxidizers. On the basis of representative measurements of yields and oxygen consumption, the following overall equation for nitrifier's synthesis and nitrification has been suggested (EPA 1975):

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \rightarrow 0.021C_5H_7NO_2 + 0.98NO_3^- + 1.041H_2O + 1.88H_2CO_3 \quad (9)$$

The implications of the equation are significant. The stoichiometric coefficients imply that per mole of ammonium removed, the nitrification process requires a significant amount of oxygen, produces a small amount of biomass, and results in substantial destruction of alkalinity through the production of hydrogen ions. The alkalinity–pH relationship, expressed in Eqs. 6–8, is of particular importance from the viewpoint of waste treatment plant operation. As a result of a number of experimental studies, on suspended as well as attached growth systems, it has been found that 1.4–1.7 mol (6.0–7.4 mg) alkalinity is destroyed per mole (milligram) NH_4^+ -N oxidized to nitrate.

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-oxidizing or nitrosobacteria (AOB). The ammonia is initially oxidized to hydroxylamine in an endothermic reaction (Eq. 10), after which the hydroxylamine is further converted to nitrite in an energy generating reaction (Eq. 11) using oxygen from water and an additional molecular oxygen as terminal electron acceptor. The resulting pathway is given in Eq. 12 (Hooper 1989):

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (10)$$

$$NH_2OH + H_2O \rightarrow NO_2 + 5H^+ + 4e^- \quad (11)$$

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

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O \ (\Delta G = -275\,\text{kJ mol}^{-1}\text{N}) \quad (12)$$

Two of the electrons produced in the second reaction are used to compensate for the electron input of the first reaction, whereas the other two are passed via an electron transport chain to the terminal oxidase, thereby generating a proton motive force. Subsequently the formed nitrite is further oxidized to nitrate by the nitrite-oxidising or nitro-bacteria (NOB), making use of the membrane-bound nitrite oxidoreductase (NOR) (Eq. 13). The overall energy generating reaction of nitrification (Eq. 12 + Eq. 13) is given in Eq. 14.

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (13)$$

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

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \ (\Delta G = 75\,\text{kJ mol}^{-1}\text{N}) \quad (14)$$

$$NH_3 + 2O_2 \rightarrow NO_3^- + H^+ + H_2O \ (\Delta G = 350\,\text{kJ mol}^{-1}\text{N}) \quad (15)$$

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 (Eccleston and 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.

It is generally accepted that ammonia (NH_3) and not ammonium (NH_4^+) is used as substrate, and the ammonia/ammonium ratio may therefore affect growth. Usually nitrite oxidation proceeds more rapidly than ammonia oxidation, so that nitrite rarely builds-up in the environment. This is probably due to a low S_{min} (minimum substrate concentration capable of supporting steady-state biomass) value and a relatively high substrate-utilization rate of the nitrite oxidizers (Rittmann 2001). It is known that the two equations related to synthesis and energy can be combined to form an overall, if the yield coefficient for the ammonia oxidizers is known. Nothing conclusive synthesis-oxidation reaction equation has yet been stated for partial nitrification with nitrite as the end product.

3 Shortcut biological nitrogen removal (SBNR)

It has already been mentioned in the earlier sections that nitrification involves two sub-processes; oxidation of ammonia to nitrite and oxidation of nitrite to nitrate. 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 then subsequent denitrification of the nitrite. In this way a significant advantage can be taken in nitrogen removal, by using a shortcut biological nitrogen removal (SBNR) process. The most critical condition that is needed for the success of the SBNR process is to suppress nitrite oxidation without excessively retarding the ammonia oxidation rate. Generation and maintenance of a nitritation reactor requires that either the NOB are washed out from the biomass or their spatial distribution is such that they can no longer find suitable conditions under which to reestablish them (for example the inner part of the biofilm; Kim et al. 2003). Unfortunately, ammonia- and nitrite-oxidizing bacteria can be found almost everywhere and therefore it might be difficult to find conditions favoring one over the other (Egli et al. 2003). One powerful tool to achieve this condition is biochemical selection by inhibition of nitrite oxidation. Nitrite accumulation studies have been performed focused on several factors, such as FA concentration, pH, temperature, DO concentration (Turk and Mavinic 1989; Kuai and Verstraete 1998) and heterotrophic nitrification (Rhee et al. 1997).

The method through which the nitrite pathway (ammonia–nitrite–nitrogen gas) is achieved is usually by outcompeting the NOB by the AOB. In this step, 50–90% of the ammonia is biologically converted to nitrite, using nitrifying bacteria and the next step involves the denitrification of nitrite which can be achieved either by the anaerobic autotrophic ammonia oxidizer or by the anaerobic denitrifiers respectively. Examples of some processes are the Single reactor High Activity Ammonia Removal Over Nitrite (SHARON) process, Anaerobic AMMonium Oxidation (ANAMMOX) process, the combined SHARON–ANAMMOX process, the Completely Autotrophic Nitrogen removal Over Nitrite (CANON) and Oxygen Limited Autotrophic Nitrification Denitrification (OLAND) process. In SHARON (Hellinga et al. 1998; van Loosdrecht and Jetten 1998) process, a completely mixed reactor is operated at short residence time (1–1.5 days) and high temperature (30–40°C) leading to the selective wash out of nitrite oxidizers. This results in only partial oxidation of ammonia to nitrite and subsequently reduction of the latter to nitrogen gas in the denitrification process. ANAMMOX process (Mulder et al. 1995; Schmidt et al. 2003) allows nitrite reduction with ammonium as electron donor to nitrogen gas. It combines almost equimolar amounts of ammonium and nitrite to form nitrogen gas (Jetten et al. 1999). This process can be combined with partial nitrification (SHARON) leading to a direct net conversion of ammonium to N_2 gas, which makes complete autotrophic ammonia removal possible as a sustainable pathway of nitrogen removal from wastewater. It targets wastewater streams high in ammonium (>0.2 g l^{-1}) and low in organic carbon

(C:N ratios lower than 0.15). CANON (Third et al. 2001) is the combination of partial nitrification and ANAMMOX in a single, aerated reactor. This process has been tested extensively on laboratory scale (Sliekers et al. 1998, 2003). Although ANAMMOX requires strict anoxic conditions, nitrifiers and ANAMMOX organisms are able to coexist under oxygen-limited conditions. Therefore, CANON would need process control to prevent nitrite build-up by oxygen excess under ammonia limitation (fluctuation of ammonia load). The OLAND process is described as a new process for one-step ammonium removal without addition of COD (Kuai and Verstraete 1998). Recently, it was confirmed that OLAND is based on the CANON concept (Pynaert et al. 2004; Philips et al. 2002). The formation of thick biofilm could create a favorable condition for nitrifiers and ANAMMOX organisms to coexist even under normal oxygen conditions.

Shorter nitrification and denitrification are more economical for many reasons in comparison with the traditional method of nitrogen removal (Turk and Mavinic 1986, 1989; Verstraete and Philips, 1998). This method saves organic energy up to 40% of COD in denitrification process, which should reduce the need for an extra external source of organic carbon (Abeling and Seyfried 1992). Shorter hydraulic retention time should allow the volume of the reactors to diminish, and thus diminish investment costs. Lower oxygen demand of about 25% gives lower exploitation costs (Surmacz-gorska et al. 1997). A number of researchers (Prakasam and Loehr 1972; Murray et al. 1975; Votes et al. 1975; Laudelout et al. 1976; Sauter and Alleman 1980; Blaszczyk et al. 1981) have identified the potential advantages associated with the implementation of a shortcut in nitrogen removal for highly nitrogenous wastes, via the production and reduction of nitrite (Fig. 1).

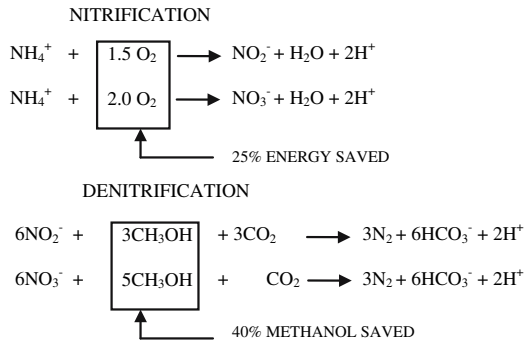


Fig. 1 Importance of partial nitrification

4 Two groups of bacteria involved in nitrification

4.1 Ammonia-oxidizing bacteria

The first step of nitrification, the oxidation of ammonia to nitrite, is performed by AOB. After the first reports on successful isolation of chemolitho-autotrophic AOB at the end of the 19th century, researches have continued to investigate their diversity in natural and engineered systems by applying enrichment and isolation techniques. These efforts resulted in the description of numerous species of ammonia oxidizers; now with the modern molecular biological techniques more species have been discovered. There are five recognized genera, with several species in each (Koops and Pommerening-Roser 2001; Purkhold et al. 2000; see Table 1). *Nitrosomonas* is the most extensively studied and usually the most numerous in soil whereas *Nitrosospira* is an aquatic ammonia-oxidizer.

Up to now, there are 25 cultured species of ammonia-oxidizing bacteria (Koops and Pommerening-Roser 2001), all with different salt requirements and substrate affinities for ammonia and/or urea. It was found that when acetate was added up to concentrations of 0.2 g TOC l^{-1} a stimulatory effect on the ammonia oxidizing capacity was observed whereas, values higher than 0.3 g TOC l^{-1} resulted in competition between heterotrophs and autotrophs with a detrimental effect over the latter. This decrease in ammonia oxidizing activity was due to a competition for substrates between both groups of bacteria (Corral et al. 2005). Molar concentrations of 100 mM of NaCl, KCl and Na_2SO_4 caused 40% inhibition on the maximum specific ammonia oxidizing activity. The ammonia oxidation activity increased in the presence of 85 mM, while higher salt concentrations up to 425 mM provoked a slight decrease of activity and

Table 1 Differential characteristics of the genera of the ammonia-oxidizing bacteria

Characteristics	*Nitrosococcus*	*Nitrosolobus*	*Nitrosomonas*	*Nitrosospira*	*Nitrosovibrio*
Cell shape	Spherical to ellipsoidal	Pleomorphic lobate	Straight rods	Tightly coiled spirals	Slender curved rods
Cell size (micro-m)	1.5–1.8 × 1.7–2.5	1.0–1.5 × 1.0–2.5	0.7–1.5 × 1.0–2.4	0.3–0.8 × 1.0–8.0	0.3–0.4 × 1.1–3.0
Flagellation of motile cells	Tuft of flagella	Peritrichous	Polar to subpolar	Peritrichous	Polar to subpolar
Arrangement of intracytoplasmic membranes	Peripheral or central stacks of vesicles	Compartmentalizing	Peripheral flattened vesicles	Invaginations	Invaginations

http://filebox.vt.edu/users/chagedor/biol_4684/Cycles/Nitrification.html (last visit on 18/09/06)

when 513 mM were added the activity was reduced to 10% (Corral et al. 2005).

The physiology of conventional 'aerobic' ammonia oxidizers is not completely understood. Only recently, it was discovered that these organisms also have an anaerobic metabolism. The proteobacterial ammonia oxidizers can obtain their energy for growth from either aerobic or anaerobic ammonia oxidation. Recently published data gave first evidence for anaerobic ammonia oxidation by *Nitrosomonas* (Schmidt et al. 1997, 1998, 2002; Jetten et al. 1999). These results indicate a complex role of nitrogen oxides (NO and NO_2) in the metabolism of 'aerobic' ammonia oxidizers. *Nitrosomonas eutropha* can oxidize ammonia in the absence of DO [Schmidt et al. 1997, 1998] replacing molecular oxygen by nitrogen dioxide or nitrogen tetroxide. Most likely ammonia (NH_3) and not ammonium (NH_4^+) is the substrate for the oxidation process (Suzuki et al. 1974; Bock et al. 1991). The main products are nitrite under oxic conditions (DO > 0.8 mg O_2/l), while under anoxic conditions (DO < 0.8 mg O_2/l) nitrogen gas, nitrite and nitric oxides are the main products (Schmidt and Bock 1997). The distribution, diversity and ecology of the ammonia-oxidizers are given in details in a recent review publication (Kowalchuk and Stephen 2001).

4.2 Nitrite-oxidizing bacteria

The second step of nitrification, the oxidation of nitrite to nitrate, is performed by NOB of which there exist eight pure cultures (Koops and Pommerening-Roser 2001), differ in ecophysiological requirements. For example, members of *Nitrospira* generally are regarded as obligately chemolithotrophic, whereas members of *Nitrobacter* can also thrive on organic compounds for energy generation (Bock 1976). Members of *Nitrospira* prefer relatively low nitrite concentrations (Ehrich et al. 1995) and are found as the most abundant nitrite oxidizer in wastewater treatment systems (Daims et al. 2001a, b). Four phylogenetically distinct groups of NOB have been described (Koops and Pommerening-Roser 2001; Table 2).

For NOB the oxidation of nitrite to nitrate is the energy generation process. There is some evidence that *Nitrospira* is the more specialized nitrite oxidizer. The other genera are more versatile, are all able to use organic energy sources beside the major source nitrite, being facultative autotrophs and anaerobes, able to grow on heterotrophic substrates such as pyruvate and also capable of the first step of denitrification (the reduction of nitrate to nitrite) (Schmidt et al. 2003; Koops and Pommerening-Roser 2001).

The application of molecular methods revealed that yet uncultured *Nitrospira*-like microorganisms and not *Nitrobacter* spp., are the dominating nitrite oxidizers in most WWTPs. *Nitrospira*-like nitrite oxidizers are also of major importance in other ecosystems like drinking water distribution systems or soil (Wagner and Loy 2002). *Nitrospira*-like nitrite oxidizers are probably *K*-strategists (with high substrate affinities and low maximum activity or growth rate) for oxygen and nitrite and thus outcompete *Nitrobacter* under substrate limiting conditions in WWTPs. This hypothesis would also explain why *Nitrobacter* and *Nitrospira*

Table 2 Differential characteristics of the genera of the nitrite-oxidizing bacteria

Characteristics	*Nitrobacter*	*Nitrococcus*	*Nitrospina*	*Nitrospira*
Cell shape	Pear-shaped/ pleomorphic rods	Spherical	Slender straight rods	Loosely coiled spirals
Cell size (micro-m)	0.5–0.8 × 1.0–2.0	1.5	0.3–0.4 × 1.7–6.6	0.3–0.4 × 0.8–1.0
Flagellation of motile cells	Polar to lateral	Polar	Not observed	Not observed
Arrangement of intracytoplasmic membranes	Polar flattened vesicles	Randomly arranged tubules	None	Invaginations
Capability of using organic substances	Heterotrophc growth	None	None	Mixotrophic growth

http://filebox.vt.edu/users/chagedor/biol_4684/Cycles/Nitrification.html (last visit on 18/09/06)

co-exist in reactors with temporarily higher nitrite concentrations (Wagner and Loy 2002).

The nitrifying bacteria are unusually stringent in their requirements for inorganic nutrients. Variation in the concentration of any of the elements may lead to disruption of the organism's performance. This is particularly true of those elements that might be called micronutrients, i.e., copper, manganese, molybdenum, and zinc. Copper is necessary as it is believed to activate the enzyme involved in the first step of ammonia oxidation. The activity of AMO in cell extracts of N. europaea is dramatically increased by the addition of copper. However, higher concentrations than necessary lead to immediate reduction in cell activity (Ensign et al. 1993). Molybdenum has been discovered to stimulate the activity of *Nitrobacter*. Concentrations as low as 1×10^{-9} M create order of magnitude increases in cell mass produced (Finstein and Delwiche 1965). Careful control of all nutrients is essential in maintaining a large population of nitrifying organisms.

5 Identification of ammonia-oxidizers using molecular methods

A major goal in microbiology is the rapid and accurate identification of bacteria in their natural environments. Culture-based methods are time consuming and are often too selective, particularly for fastidious or uncultured bacteria, and therefore this approach does not reflect the exact composition of mixed bacterial communities or microbial diversity. During recent years, molecular techniques like PCR and subsequent hybridization or sequencing and fluorescence in situ hybridization (FISH) techniques have revolutionized all fields of microbiology, and sensitive detection and exact identification of bacteria are possible.

5.1 Fluorescence in situ hybridization technique

The recent development of FISH with 16S rRNA-targeted oligonucleotide probes has made it possible to analyze complex in situ microbial community structures in environmental and engineered systems. In situ hybridization is the process of annealing a small fragment of DNA or RNA to a specific target strand of RNA in a morphologically preserved cell. Under the right conditions, this fragment or probe, will bind to a specific genetic sequence within that cell (Fig. 2). Probe specificity is freely adjustable and the target may be as specific as a single organism or as broad as all prokaryotes. Symbiotic associations as well as identification of the major population can be revealed by FISH performed with a set of fluorescently labeled 16S rRNA-targeted oligonucleotide probes (Fig. 3) without the bias of cultivation (Biesterfeld et al. 2001).

To date, several works have been published for the identification of the AOB. The species and characteristics of AOB were found to be strongly affected by the reactor operational conditions such as substrate composition and concentration in a WWTP. The results indicated that effective control of the microbial ecology by controlling the conditions of the reactor operation could be

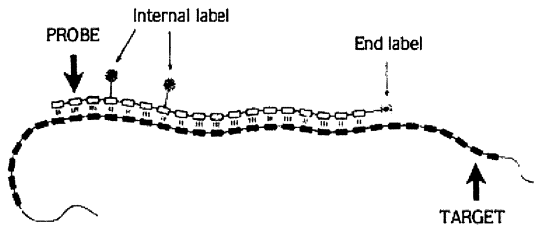


Fig. 2 Base pairing between a fluorescently labeled oligonucleotide probe and a target rRNA

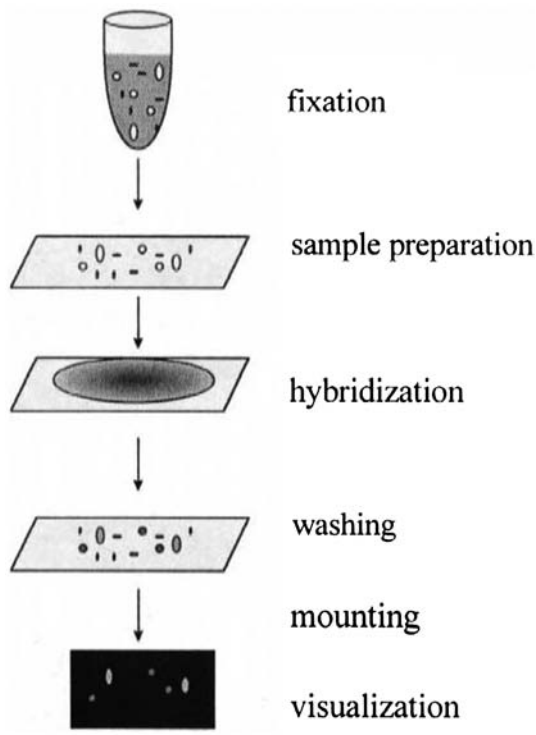


Fig. 3 Flow chart of a typical FISH procedure (Moter and Gobel 2000)

applied to engineered systems with direct monitoring using FISH (Aoi et al. 2000). During experiments carried out with freshwater aquaria, FISH analysis revealed that *Nitrosomonas marina*-like AOB may outcompete *Nitrosomonas tenius*-like AOB in the low-ammonia concentration environment of an aquarium, whereas *Nitrosomonas europea*-like AOB were found only in reactors with a history of high ammonia concentrations (Burrell et al. 2001). Community analysis using FISH during start-up of nitritation reactors showed that NOB bacteria of the genus *Nitrospira* were only active directly after inoculation with sewage sludge. The community of the AOB changed within the first 15 to 20 days from a more diverse set of populations in the inoculated sludge to a smaller subset in the reactors (Egli et al. 2003). Similarly, FISH was performed to analyze the nitrifying microbial communities in an activated sludge reactor (ASR) and a fixed biofilm reactor (FBR) for piggery wastewater treatment. Fish analysis showed that AOB (Nso1225) and denitrifying bacteria (RPP1088) were less abundant than other bacteria (EUB338) in ASR. Further analysis of the FBR showed that *Nitrosomonas* species (Nsm156) and *Nitrospira* species (NSR1156) were the dominant AOB and NOB respectively, in the piggery wastewater nitrification systems (Kim et al. 2004). It was also found that when coupled with oxygen uptake rate (OUR) data, FISH could provide valuable information about the nitrogen removal process in many practical wastewater treatment systems (Kim et al. 2001). The nitrifying microbial diversity and population structure of a sequencing biofilm batch reactor (SBBR) receiving sewage with high ammonia and salt concentrations (SBBR) was analyzed (Daims et al. 2001a). Quantification of AOB and NOB in the biofilm by FISH and Image analysis revealed that ammonia-oxidizers occurred in higher cell numbers and occupied a considerably larger share of the total biovolume than NOB. Ammonia oxidizers formed a dense layer of cell clusters in the upper part of the nitrifying biofilm from the trickling filter of an aquaculture water recirculation system, whereas the nitrite oxidizers showed less-dense aggregates in close vicinity to the *Nitrosomonas* clusters (Schramm et al. 1996). This distribution pattern was supported by Kim et al. 2003. It was reasoned out in the same publication that dissolved oxygen deficiency or limitation in the inner part of the nitrifying biofilm, where nitrite oxidizers exist, is responsible for the complete shut down of the nitrite oxidizers activity under the absence of FA inhibition. The tables below (Tables 3–5) give the list of probes that can be used to investigate the ecophysiology of the numerically important ammonia and nitrite oxidizer assemblages in any kind of environment or wastewater.

Table 3 Probe data for ammonia-oxidizing bacteria

Specificity	Probe	Sequence	Formamide (FISH)	Length and position	rRNA target	References
Most halophilic and halotolerant *Nitrosomonas* spp.	NEU	5′-CCC CTC TGC TGC ACT CTA-3′	40%	653–670 (18 bp)	16S	Wagner et al. (1995)
	Competitor	5′-TTC CAT CCC CCT CTG CCG-3′				
Nitrosomonas oligotropha-lineage	Nmo218	5′-CGG CCG CTC CAA AAG CAT-3′	35%	218–235 (18 bp)	16S	Gieseke et al. (2001)
Nitrosococcus mobilis (''*Nitrosomonas*'') lineage	NmV (Ncmob)	5′-TCC TCA GAG ACT ACG CGG-3′	35%	174–191 (18 bp)	16S	Pommerening-Röser et al. (1996)
Nitrosomonas europea, *N. halophila*, *N. eutropha*, Kraftisried-Isolat Nm103	Nse1472	5′-ACC CCA GTC ATG ACC CCC-3′	50%	1472–1489 (18 bp)	16S	Juretschko et al. (1998)
Nitrosomonas spp., *Nitrosococcus mobilis*	Nsm156	5′-TAT TAG CAC ATC TTT CGA T-3′	5%	156–174 (19 bp)	16S	Mobarry et.al. (1996)
Nitrosospira tenius-like ammonia-oxidizing bacteria	NSMR34	5′-TCC CCC ACT CGA AGA TAC G-3′	20%	131–149 (19 bp)	16S	Burrell et al. (2001)
Nitrosomonas marina-like ammonia-oxidizing bacteria	NSMR76	5′-CCC CCC TCT TCT GGA TAC-3′	20%	132–149 (18 bp)	16S	Burrell et al. (2001)
Betaproteobacterial ammonia-oxidizing bacteria	Nso1225	5′-CGC CAT TGT ATT ACG TGT GA-3′	35%	1224–1243 (20 bp)	16S	Mobarry et.al. (1996)
Betaproteobacterial ammonia-oxidizing bacteria	Nso190	5′-CGA TCC CCT GCT TTT CTC C-3′	55%	189–207 (19 bp)	16S	Mobarry et.al. (1996)
Nitrosospira spp.	Nsv443	5′-CCG TGA CCG TTT CGT TCC G-3′	30%	444–462 (19 bp)	16S	Mobarry et.al. (1996)

Table 4 Probe data for other broader bacterial groups

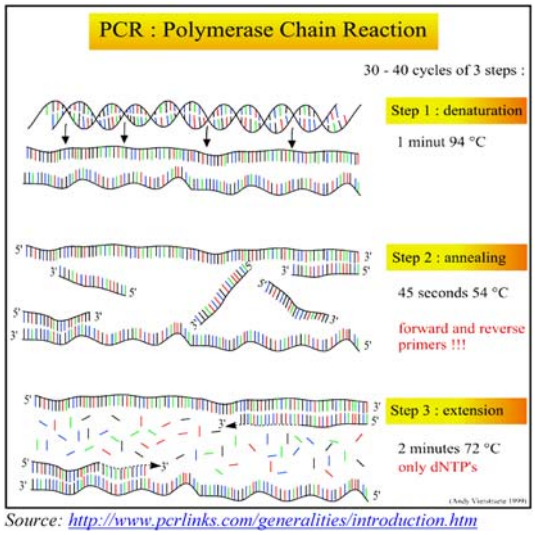
Specificity	Probe	Sequence	Formamide (FISH)	Length and position	rRNA target	References
Eubacteria	EUB 338	5′-GCT GCC TCC CGT AGG AGT-3′	0–50%	338–355 (18 bp)	16S	Amann et al. (1990)
α-subclass of *Proteobacteria*	ALF1b	5′-CGT TCG YTC TGA GCC AG-3′	20%	19–35 (17 bp)	16S	Manz et al. (1992)
β-subclass of *Proteobacteria*	BET42a	5′-GCC TTC CCA CTT CGT TT-3′	35%	1027–1043 (17 bp)	23S	Manz et al. (1992)
	Competitor	5′-GCC TTC CCA CAT CGT TT -3′				
γ-subclass of *Proteobacteria*	GAM42a	5′-GCC TTC CCA CAT CGT TT-3′	35%	1027–1043 (17 bp)	23S	Manz et al. (1992)
	Competitor	5′-GCC TTC CCA CTT CGT TT-3′				

Table 5 Probe data for nitrite-oxidizing bacteria

Specificity	Probe	Sequence	Formamide (FISH)	Length and position	rRNA target	References
Nitrobacter spp.	NIT3	5′-CCT GTG CTC CAT GCT CCG-3′	40%	1035–1052 (18 bp)	16S	Wagner et al. (1996)
	Competitor	5′-CCT GTG CTC CAG GCT CCG-3′				
Nitrospira moscoviensis, freshwater *Nitrospira* spp.	NSR1156	5′-CCC GTT CTC CTG GGC AGT-3′	30%	1156–1173 (18 bp)	16S	Schramm et al. (1998)
Nitrospira spp.	NSR447	5′-GGT TTC CCG TTC CAT CTT-3′	30%	447–464 (18 bp)	16S	Schramm et al. (1998)
Nitrospira moscoviensis, freshwater *Nitrospira* spp.	NSR826	5′-GTA ACC CGC CGA CAC TTA-3′	20%	826–843 (18 bp)	16S	Schramm et al. (1998)
Nitrospira moscoviensis, activated sludge clones A4 and A11	Ntspa1026	5′-AGC ACG CTG GTA TTG CTA-3′	20%	1026–1043 (18 bp)	16S	Juretschko et al. (1998)
Genus *Nitrospira*	Ntspa662	5′-GGA ATT CCG CGC TCC TCT-3′	35%	662–679 (18 bp)	16S	Daims et al. (2001b)
	Competitor	5′-GGA ATT CCG CTC TCC TCT-3′				
Most members of the phylum *Nitrospirae*	Ntspa712	5′-CGC CTT CGC CAC CGG CCT TCC-3′	50%	712–732 (21 bp)	16S	Daims et al. (2001b)
	Competitor	5′-CGC CTT CGC CAC CGG TGT TCC-3′				

5.2 Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)

Molecular biological techniques, which do not require isolation of bacterial strains have become increasingly popular, and are now more and more frequently used to detect and characterize bacteria in natural and man-made environments. Many molecular approaches start with the extraction of nucleic acids, DNA and RNA, from microbial communities. Different methods have been described for this purpose, (for an overview, see Akkermans et al. 1995, and references within). The extracted bacterial DNA is used as a target DNA in a polymerase chain reaction (Saiki et al. 1985) to amplify specific genes. RNA can be amplified using a so-called RT-PCR (Veers et al. 1987), whereby the RNA is first transcribed into cDNA by the enzyme reverse transcriptase (RT), and then amplified by the PCR (Fig. 4). In both cases the result will be a mixture of DNA fragments obtained from the different community members. A list of primers is given in Table 6.



Source: http://www.pcrlinks.com/generalities/introduction.htm

Fig. 4 Steps in polymerase chain reaction

Another promising molecular approach to analyse complex mixtures of microorganisms was presented by Muyzer et al. 1993. Instead of cloning the obtained PCR products into *E. coli* followed by a laborious sequencing of random

Table 6 List of some primers used for PCR amplification of universal bacteria or AOB

PCR primers name	Base pairs	Sequence (5′-3′)	Organisms	Reference
341 f-gc and 534r	~200 bp	cta cgg gag gca gca g att acc gcg gct gct gg	Eubacterial and Universal bacterial primers	Muyzer et.al. (1993)
968-gc-f and 1401-r	~530 bp	cgc ccg ggg cgc gcc ccg ggc ggg gcg ggg gca cgg ggg gaa cgc gaa gaa cct tac cgg tgt gta caa gac cc	Eubacterial 16S rDNA (V6-V8 region)	Pereira et al. (2002)
7-f and 1510-r	~1500 bp	aga gtt tga t(c/t)(a/c)t ggc tca g acg g(c/t)t acc ttg tta cga ctt	Complete bacterial 16S rDNA	Pereira et al. (2002)
27-f and 1492-r	~1460 bp	aga gtt tga tcm tgc tcag ggt tac ctt tgt tac gac tt	Universal bacterial primers	
Com1-f and Com2-r	~420 bp	cag cag ccg cgg taa tac	Universal bacterial primers	Lane et al. (1985)
519-f – 926-r		ccg tca att cct ttg agt tt		
21f and 958r	~937 bp	ttc cgg ttg atc cyg ccg amt cca att ycc ggc gtt gam tcc aat t	Archael and eubacterial 16S rRNA	Collins et al. 2003)
nitA and nitB	~1097 bp	ctt aag tgg gga ata acg cat cg tta cgt gtg aag ccc tac cca	AOB specific region of 16S rDNA	Voytek and Ward (1995)
CTO 189fA-GC and CTO 189fB-GC	~450 bp	ccg ccg cgc ggc ggg cgg ggc ggg ggc acg ggg gga gra aag cag ggg atc g	*β*-subdivision of ammonia-oxidising bacteria	Kowalchuk et al. (1997)
CTO 189f C-GC		cgc ccg ccg cgc ggc ggg cgg ggc ggg ggc acg ggg gga gga aag tag ggg atc g		
CTO 654r		cta gcy ttg tag ttt caa acg c		

clones, they separated PCR products from different organisms by a special kind of electrophoresis, i.e. DGGE. In DGGE, DNA fragments of the same length but with different base-pair sequences can be separated. The separation is based on the electrophoretic mobility of PCR-amplified DNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants (Fig. 5). Furthermore, the phylogenetic affiliation of the detected bacteria can be inferred after sequencing the individual bands of the DGGE gel and comparing the sequences to know 16S rRNA sequences (Muyzer and deWaal 1994; Muyzer and Ramsing 1995; Teske et al. 1996; Ferris et al. 1996; Rolleke et al. 1996).

Bacterial communities and betaproteobacterial ammonia-oxidizing bacteria (AOB) communities were evaluated seasonally in an intermittent-aeration sequencing batch process (SBR, plant A) and in 12 other livestock WWTPs. Sequencing of the DGGE bands indicated that *amoA* sequences belonging to the *Nitrosomonas europaea–eutropha* cluster were dominant in 11 plants,

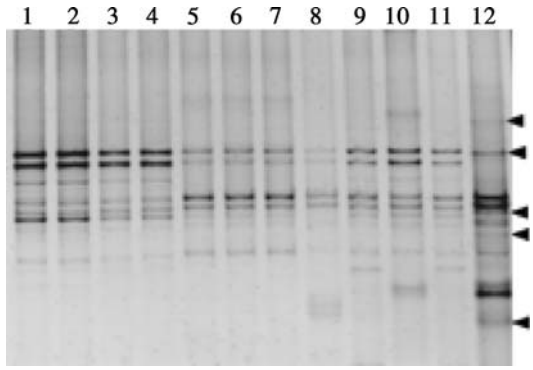


Fig. 5 DGGE band patterns of 16S rDNA. PCR amplification products obtained with the CTO_PCR primers and community DNA (Bruns et al. 1999)

where the ammonia-nitrogen concentration was high in the raw wastewater, whereas those belonging to the *Nitrosomonas ureae–oligotropha–marina* cluster were dominant in two plants where the concentration was relatively low (Otawa et al. 2006; Burrel et al. 2001). Uncultivated *Nitrospira*-like bacteria in different biofilm and activated-sludge samples were determined by

16S rRNA gene sequence analysis. This analysis revealed that the genus *Nitrospira* consists of at least four distinct sublineages (Daims et al. 2001b). The presence of bacteria from the *Nitrospira* phylum in wastewater treatment systems was first reported using PCR and cloning (Burrell et al. 1998). The number of *Nitrospira* sp. cells in the municipal WWTP was more than 62 times greater than the number of *Nitrosomonas oligotripha*-like cells, based on a competitive PCR analysis (Dionisi et al. 2002). Burrell and his group (2001) strongly suggested that start-up inocula for the establishment of nitrification in aquatic culture systems should optimally consist of *Nitrosomonas marina*-like AOB rather than *Nitrosomonas europaea–Nitrosococcus mobilis* cluster AOB. The diversity and community structure of the betaproteobacterial AOB in two full-scale treatment reactors—a biological aerated filter (BAF) and a trickling filter—receiving the same wastewater was compared using PCR of 16S ribosomal RNA (rRNA) gene fragments with AOB-selective primers combined with DGGE. It was found that the community structure of AOB was different in different sections of each of the reactors and differences were also noted between the reactors and all AOB-like sequences identified, grouped within the genus *Nitrosomonas*. A greater diversity of AOB was detected in the trickling filters than in the BAF though all samples analysed appeared to be dominated by AOB most closely related to *Nitrosococcus mobilis* (Rowan et al. 2003). Using real-time PCR quantification, members of the *Nitrosomonas oligotropha* cluster were found in all the samples collected from 12 different sewage treatment systems whose ammonia removal and treatment processes differed during three different seasons. Seasonal variation was observed in the total ammonia oxidizer numbers, but not in the ammonia-oxidizing bacterial communities (Limpiyakorn et al. 2005).

5.3 Quinone profile

Recently, the quinone profile, which is represented as the molar fraction of each quinone type in a mixed culture, has gained increased recognition as a simple tool for the analysis of microbial population dynamics for mixed cultures (Hedrick and White 1986; Hiraishi 1988; Fujie et al. 1994 1998; Hu et al. 1999). Quinones exist in almost all microbes and each bacterium has a coherent predominant quinone. Thus the fractional content of each quinone species in the microbial community, i.e. the quinone profile, can be used as an index to represent the change of microbial community (Nozawa et al. 1998). In *Proteobacteria*, Q-10 is found mostly in the α subclass, Q-8 in the β subclass, and Q-9 in the γ subclass (Hiraishi, 1988).

A study of the microbial community was made by Lim et al. 2002 in a full-scale biological nitrification/denitrification process of domestic wastewater based on quinone profile method. The process studied consisted of four reactors: anaerobic tank, anoxic tank, aeration tanks-1 & 2. There was an observable difference in microbial community structure between suspended and attached microorganisms in the aeration tanks. The fraction of nitrifying bacteria in the attached microorganisms was considered higher than that in the suspended microorganisms. The experimental results showed that the condition of aeration tank-1 may be suitable for the growth of *Nitrosomonas* species (predominant quinone type: UQ-8), but aeration tank-2 may be suitable to *Nitrobacter* species (predominant quinone type: UQ-10). The quinone content and nitrification activity of attached microorganisms in aeration tank-1 were higher than that in aeration tank-2.

The microbial community structure in an intermittently aerated submerged membrane bioreactor treating domestic wastewater was investigated by Lim et al. (2004) using the respiratory quinone profiling method. The operating cycle consisted of feeding the domestic wastewater into a 90-min oxic condition and a 60-min anoxic condition. A slight difference was observed in the microbial community structure of the suspended microorganisms in anoxic and oxic conditions. The dominant quinone type of anoxic and oxic conditions was ubiquinone (UQ)-8 followed by UQ-10 and menaquinone (MK)-6. The quinone profiles of the suspended microorganisms in the intermittently aerated SMBR with anoxic and oxic cycling showed that *Nitrosomonas* species,

Alcaligenes species, and *Thiobacillus*, the dominant quinone of whose is UQ-8 actively contributed to the biological nitrification/denitrification.

6 Parameters influencing the partial nitrification

Successful operation of a wastewater treatment system depends upon knowledge of parameters affecting its performance. Many parameters have been suggested for influencing nitrite accumulation, either individually or in combination with other factors, with simultaneous inhibition of nitrate production. Abiotic factors like temperature, pH and nutrients have a strong influence on the growth and activity of microorganisms.

6.1 pH and free ammonia and nitrous acid concentrations

The pH optima for the overall nitrification reaction appear to be slightly on the alkaline side. According to the research by Anthonisen (1976), pH influences the equilibrium between nitrite and the unionized, free nitrous acid (FNA) besides the NH_4^+/NH_3 (FA) equilibrium. These non-ionized forms of the ammonium and nitrite have—as ammonia (NH_3) and nitrous acid (HNO_2)—an inhibition effect on both the *Nitrosomonas* and the *Nitrobacter*. *Nitrosomonas* sps. and *Nitrobacter* sps. both are sensitive to their own substrate and more so to the substrate of the other. The *Nitrobacter* react more sensitively so that concentration of NH_3 in relatively low range is sufficient for inhibition. Accordingly, many researches carried out with different FA concentrations showed that pH influences the concentration of FA and causes inhibition of the nitrite oxidizers.

Surmacz-Gorska et al. (1997) found in their experiments that the pH of the wastewater was the decisive parameter in NOB activity inhibition. Similarly, it was found that nitrite accumulation was achieved at high pH (Suthersan and Ganczarcczyk 1986), suggesting that it is possible to accumulate nitrite controlling the pH at 8. Most of the literature related to nitrite accumulation using pH as a decisive factor states that pH in the range of 7.5–8.5 is most suited to inhibit the nitrite-oxidizers. Similarly, Balmelle et al. 1992 in their experiments showed that the optimum pH value is around 8.5 which is similar to those reported by Wild (1971) and Jones et al. (1983). Jenicek et al. (2004) similarly reported from their experiments that the importance of pH value at nitritation process seems to be crucial which was supported by Tokutumi (2004). At steady state operation without pH adjustment the variation of pH was from 8.0–6.6 in 1 operational cycle of 6 h of the nitritation reactor (Tokutumi 2004). When the pH was adjusted to 8.5 by NaOH addition, the process efficiency was improved to almost complete nitritation.

Villaverde et al. 1997 while dealing with the influence of pH over nitrifying biofilm activity stated that under steady state conditions nitrite build-up is the result of the superior sensitivity of nitrite oxidizers to factors such as temperature, pH, DO and inhibition. Nitrite accumulation started above pH 7.5, increasing asymptotically to 85% for a pH value of 8.5. The nitrite accumulation is likely caused by the selective inhibition of nitrite oxidizers by FA which increased also exponentially above pH 7.5. The pH assigns the distribution of NH_4^+/NH_3 and $NO_2^-HNO_2$. After adjusting the pH to 8.5 it resulted in more than 10% share of NH_3 N. On the other hand decrease of pH to 7.0 implies that NH_3 is absent. Similar changes induce the change of pH in system NO_2^-/HNO_2. At pH 7.5, practically no nitrous acid is present. If the pH decreases to 6.0 the share of HNO_2 is rising to 0.2%. The results were in accordance with those of Abeling and Seyfried (1992) who reported that pH values above 7.5 should be maintained to selectively inhibit the nitrite oxidation and accumulate nitrite in the system. In nitrification Glass and Silverstein (1998) also 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).

Free ammonia as stated above is understood to mean non-dissociated or un-ionized ammonia.

$$NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O \quad (16)$$

$$[NH_3\text{-}N]_{free} = [TAN][10^{pH}]/[K_a/K_w] + 10^{pH} \text{ with}$$
$$K_aK_w = \exp[6334/(273 + t)] \quad (17)$$

TAN = Total ammoniacal nitrogen = ammonium + free ammonia K_a = Ionization constant for ammonium (e.g., K_a at 20°C = $10^{-9.24}$) K_w = Ionization constant for water (e.g., K_w at 20°C = 0.69×10^{-14}) t = temperature in °C

According to the nitrous acid equilibrium, the concentration of FNA will increase as the pH decreases:

$$H^+ + NO_2^- \leftrightarrow HNO_2 \quad (18)$$

$$[HNO_2\text{-}N]_{free} = [NO_2^-N]/K_n[10^{pH}] \text{ with}$$
$$K_n = \exp[-2300/(273 + t)] \quad (19)$$

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

Accordingly in the research of Turk and Mavinic (1986, 1989) nitrite build-up was achieved with intermittent contact to high FA levels of 95 mg NH_3-N l^{-1} in the first cell of a four-cell system. Wong-Chong and Loehr (1978) observed that the *Nitrobacter* acclimated to FA could tolerate concentrations as high as 40 mg NH_3-N l^{-1}, while unacclimated ones were inhibited at concentrations of 3.5 mg NH_3-N l^{-1}. Ford et al. (1980) reported total inhibition of nitrification activity at FA levels of 24 mg NH_3-N l^{-1}, but noted that system recovery was possible, even at levels as high as 56 mg NH_3-N l^{-1}. Cecen and Gonenc (1994) noted, in the batch start-up phase, the combined effect of high ammonia and high pH (8.5) inhibited *Nitrobacter* and led to accumulation of nitrite. Mauret et al. (1996) showed that high FA concentration inhibits *Nitrobacter*, in the range of 6.6 and 8.9 mg NH_3-N l^{-1}.

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). Based upon these, boundary conditions of zones of nitrification inhibition were determined (Fig. 6). 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 FA, 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 FA than of FNA 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} found by Anthonisen et al. (1976).

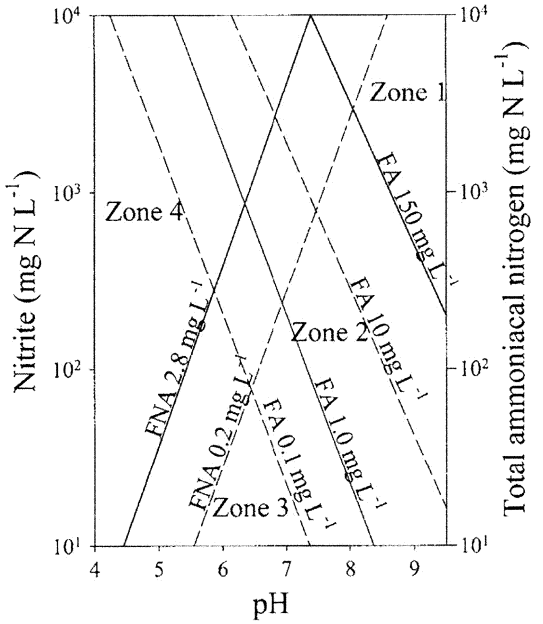


Fig. 6 Relationship between concentrations of free ammonia (FA) and free nitrous acid (FNA) and inhibition to nitrifiers. The dashed lines mark the lower limit and the solid lines mark the upper limit of the range of boundary conditions of zones of nitrification inhibition. Zone 1 = Inhibition of nitritation 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)

Although NH_4^+ can cause nitrite accumulation (e.g., Horan and Azimi 1992; Smith et al. 1997), the effect of free ammonia seems to be more

pronounced. FA is a competitive inhibitor of NOR activity, which is located on the cell membrane of NOB (Yang and Alleman 1992). Abeling and Seyfried (1992) stated that in order to attain the highest nitritation rate, it was decisive to prevent the inhibition of the *Nitrosomonas* caused by FA. The FA concentration necessary for the inhibition of *Nitrobacter* must be kept low enough to ensure that the inhibition of nitritation does not also take place. At pH = 8.5 and T = 20°C, the optimal FA concentration for maximum nitritation and minimum nitratation was found to be around 5 mg NH_3-N l^{-1} (Abeling and Seyfried 1992). On and after approximately 7 mg NH_3-N l^{-1} an inhibition on nitritation could be noticed. At concentrations of around 20 mg NH_3-N l^{-1} there was very little nitrification activity. Limits to prevent nitritation inhibition had been found out in batch tests which ranged between 10 and 150 mg NH_3-N l^{-1} (Anthonisen 1976). Also Neufeld et al.(1986) confirmed the beginning of nitritation inhibition at 10 mg NH_3-N l^{-1}.

Balmelle et al. 1992 stated that there was great sensitivity of *Nitrobacter* to free NH_3 for concentration as low as 1 mg N l^{-1}, with a percentage of inhibition reaching 90% for about 2 mg N l^{-1}. These observations confirmed the results from earlier articles under different batch reactor operating conditions. However, slightly different results were obtained in reactors with continuous and semi-continuous feeds, in which the acclimatization of the micro-organisms enables higher concentrations of free NH_3, of around 5 mg NH_3-N l^{-1}, to be tolerated. The main results of the research demonstrated the inhibiting effect of the free form of ammonium nitrogen on *Nitrobacter*, which may be the result of a combination of several factors like the initial ammonium nitrogen concentration, the pH and the temperature. Threshold concentrations for FA and nitrous acid, over which ammonium and nitrite oxidizers are partially or totally inhibited, have been reported for activated sludge. *Nitrobacter* is heavily inhibited by FA concentrations of 0.1–1.0 mg NH_3 l^{-1}, causing nitrite build-up (Villaverde et al. 1997 and Anthonisen et al. 1976). In all the seven methods investigated by Turk and Mavinic (1989) to overcome the effects of acclimation to FA, nitrite build-up could not be maintained for an extended period of time.

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 DO concentrations of 4–5 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 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. 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 FA 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 7. Notwithstanding the severe effects of high FA 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 and 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 operations of the reactor

Table 7 Comparison of literature values of free ammonia inhibition of nitrite oxidation in wastewater treatment

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)
	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)
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)
80	0.5	7.8		52% nitrite accumulation	Submerged biofilter	Fdz-Polanco et al. (1996)
	0.5–0.7			Th.c. inhibition nitrite-oxidation	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)

Th.c. = Threshold concentration, i.e. lowest concentration exhibiting some degree of inhibition

are factors altering the growth dynamics of NOB towards acclimatization 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. 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).

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 DO, suppressing *Nitrobacter*. Ruiz et al. 2003 found that temporal nitrite accumulation took place, especially after a change in the pH value, but due to biomass adaptation, a complete nitrification was again achieved in a few days. It was also stated that it is not possible to accumulate

nitrite on a long-term basis using pH as a key parameter. Hence the need of monitoring the effect of other operating parameters arises.

6.2 Dissolved oxygen

Oxygen is utilized in the oxidation reactions carried out by nitrifying bacteria. The stoichiometric quantities of oxygen required are: 3.43 mg for nitrification of 1 mg NH_3-N, and 1.14 mg for nitratation of 1 mg NO_2-N. The theoretical nitrogenous oxygen demand (NOD) is 4.57 mg per milligram of NH_3-N. When it comes to nitrification, the DO concentration is an utmost important parameter for both AOB as well as 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. 1990). Nitrite accumulation can be achieved by controlling the DO at low concentration since the oxygen saturation coefficients of Monod kinetics for nitritation and nitratation are known to be 0.3 and 1.1 mg l^{-1}, respectively (Wiesmann, 1994). 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 (Fig. 7) by Hanaki et al. (1990). When the reactor is operated at low DO (< 1.0 mg l^{-1}) condition, observed growth rate of ammonia oxidizer is 2.56 times faster than nitrite oxidizers (Tokutomi 2004). DO concentration below 1.0 mg l^{-1} is supposed to be sufficient to induce the dominance of the ammonia oxidizer. Oxygen limitation was selected as the main control parameter for inhibiting the growth of nitrite oxidizer and thus achieving only nitritation.

In a study by Hanaki et al. 1990 with a suspended growth reactor at 25°C, nitrite oxidation was strongly inhibited by low DO (< 0.5 mg l^{-1}). Their findings were that low DO did not affect ammonia oxidation as a whole in the pure nitrification system, the growth yield of ammonia oxidizers was doubled at low DO, and this positive effect of low DO compensated the

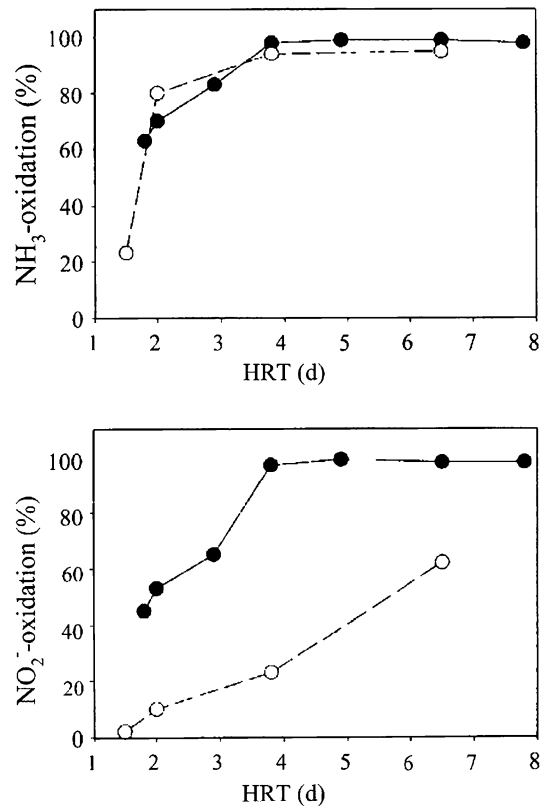


Fig. 7 Effect of O_2 concentration, sufficient O_2 (- • -) and 0.5 mg O_2 l^{-1} (- - o - -), on ammonia and nitrite oxidation in pure cultures of nitrifying bacteria after reaching steady state conditions for various hydraulic retention times (HRT) (after Hanaki et al. 1990)

reduced ammonia oxidation rate per unit mass of cell. Nitrite oxidation was strongly inhibited by low DO and the growth yield of nitrite oxidizers was unchanged by low DO. Thus, 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. Together with unaffected ammonia oxidation this resulted in accumulation of nitrite to 60 mg N l^{-1} at an HRT of 2.0–3.8 d^{-1} (Hanaki et al. 1990). Stenstrom and Poduska 1980 reported that it is obvious that there exists no clearly defined DO concentration for optimum nitrification. It appears that at higher MCRT, nitrification can be achieved at DO concentrations in the range of 0.5–1.0 mg l^{-1}, and at lower MCRT higher DO concentrations are needed. Based on the analysis of this study by

Stenstrom and Poduska 1980, the lowest DO concentration at which nitrification can occur appears to be approximately 0.3 mg l^{-1}, whereas Pollice et al. 2002 investigated the effects of sludge age and aeration on ammonium oxidation to nitrite. The results indicated that, at given temperature and pH, the sludge age was the critical parameter for partial nitrification when the oxygen supply was not limiting. Under limited oxygen supply, complete and stable conversion of ammonium into nitrite was obtained, independent of the sludge age. The latter parameter (sludge age) only showed some influence on the kinetics of ammonium oxidation under oxygen limitation.

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. 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 and Gerards 1993). By controlling the DO to low values, the oxidation of nitrite to nitrate can be controlled. This is possible owing to the higher affinity of the AOB for oxygen than the NOB (Garrido et al. 1997). 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. This was also supported by Bougard et al. (2006, b) and they concluded that the combined oxygen and ammonia control strategy is more appropriate since shift in the temperature setpoint strongly affects the composition of the microbial ecosystem present in the reactor whereas active control of oxygen and ammonia does not. Bernet et al. (2005) found that using O_2/NH_4^+-N ratio setpoints of 0.05 and 0.1, it was possible to oxidize up to 80% of the inflow ammonium into nitrite and hence concluded that the O_2/NH_4^+-N ratio in the bulk phase is the main parameter controlling nitrite accumulation. Also Joo et al. (2000) recorded an important connection between O_2 and $NH4^+$ load. Experiments with an upflow BAF (low superficial airflow = 0.21 cm s^{-1}) showed that NO_2^- accumulation increased for increasing NH_4^+ loads (Fig. 8). The figure 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.

6.3 Temperature

Nitrification reactions follow the van't Hoff-Arrhenius law up to 30°C (Levenspiel). Thus, nitrification proceeds better in warmer seasons or climates. Increased nitrite concentrations in WWTPs or even nitrogen-receiving surface waters have been found to vary with the seasons in the year. Tonkovic (1998) noted that nitrite

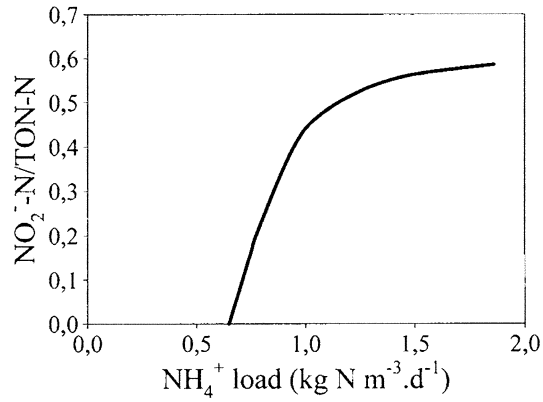


Fig. 8 Effect of NH_4^+ load on nitrite accumulation expressed as part of nitrite in the amount of total oxidized nitrogen (TON = $NO_2^- + NO_3^-$) (after Joo et al. 2000)

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). 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 water phase. An increase in temperature causes an increase in FA concentration, according to Eq. 17. 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).

Mathematical modeling with physiological parameters of *Nitrosomonas* and *Nitrobacter* was used to predict the success of nitritation (Hellinga et al. 1998, 1999), and it was proposed that mesophilic temperatures (between 35 and 40°C) and pHs between 7 and 8 are required to compete more effectively than nitrite oxidizers. Normal temperatures (5–20°C) and conditions in WWTPs favor growth of nitrite oxidizers; with the result that ammonium is completely oxidized to nitrate. The competitive disadvantage of nitrite oxidizers to ammonia oxidizers at 35°C has been experimentally established in sludge reactors (Hellinga et al. 1998; Logemann et al. 1998).

Balmelle et al. (1992) showed that in spite of concentration of FA (between 2 and 5 mg NH_3-N l^{-1}), normally inhibiting for the *Nitrobacter*, it was active over a range of temperature between about 10 and 20°C. Under these conditions, nitrite build-up remained low, since the effect of the *Nitrobacter* activation by temperature prevailed over its inhibition by FA. On the otherhand, beyond a temperature of 20–25°C, a slowing of the nitratating activity was observed together with an activation of the nitritating activity, which passes through a maximum at 25°C. With respect to *Nitrosomonas* under these conditions, the inhibiting effect of FA was preponderant for temperature higher than 25°C. This result confirmed that of Anthonisen (1976), but fairly disparate observations had been found in the literature, notably those of Ford et al. (1980) which suggested the optimal temperature range between 30°C and 36°C.

At the NH_3-N concentrations usually found in natural waters and domestic wastewaters, the NH_3-N oxidation rate has a strongly positive thermal sensitivity (Quinlan 1980). If the NO_2^--N oxidation rate has a negative thermal sensitivity, then as temperature rises nitrite production will dominate nitrite consumption more and more, and nitrite should accumulate to progressively higher concentrations. In batch culture experiments described by Braune and Uhlemann (1968) and Laudelout et al. (1974), nitrite accumulation appeared at higher temperatures. Complete nitrification was achieved in an inverse turbulent bed reactor with a granular floating solid which was operated at 30°C for 120 days. When the temperature was increased from 30 to 35°C in this biofilm reactor, an immediate and durable nitrite accumulation occurred in the system during 116 days and the system could be reversed back to complete nitrification by drastically decreasing the NLR alongwith a temperature decrease to 30°C (Bougard et al. 2006a, b). Nitrite accumulation has also been observed at 30°C in fluidized sand-bed nitrification reactors run in both batch and continuous modes (Tanaka et al. 1981; Tanaka and Dunn 1982; Denac et al. 1983). van Dongen et al. (2001) stated that according to their experiments the optimum temperature for nitrite accumulation was between 30 and 37°C, which was supported by Hellinga et al. (1998) and van Kempen et al. (2001). They stated that temperature ranges above 25°C and upto 35°C are best suited for SHARON type of reactor.

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). Knowles et al. (1965) determined that the ammonia and nitrite oxidation rates increase 2.6 and 1.8 times respectively per 10°C in a physiologically relevant temperature range. According to Knowles et al. (1965), the maximum specific growth rate of

Nitrobacter is significantly higher than that of *Nitrosomonas* at temperatures between 10 and 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. 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 and Wheaton 1991). 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 (Fig. 9). 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 (Jetten et al. 1997; Hellinga et al. 1998, 1999). A modeling 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. Quinlan 1980 concluded that nitrite accumulation may be a side-effect of the elevated temperatures that occur in some nitrifying systems during summertime and in thermal effluents containing high ammonia concentrations.

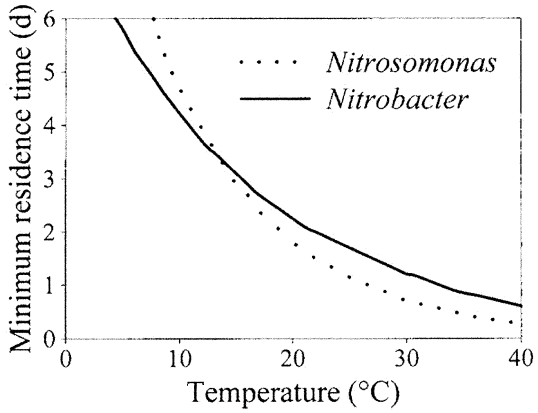


Fig. 9 Effect of temperature on the minimal required cell residence time for ammonia and nitrite oxidation. Above 14°C it is possible to wash out the NOB while maintaining the AOB (after Jetten et al. 1997, with temperature coefficients of Hunik et al. 1994)

6.4 Light

Light is inhibiting to both AOB and NOB, through the oxidation of cytochrome c caused by light in the presence of O_2. There is some suggestion in the literature that light might inhibit the activity of nitrifying bacteria. Warington (1954) found that nitrification proceeded more rapidly in cultures placed in a dark cupboard than on an open bench. Ulken (1970) determined that the ratio of oxygen uptake in the dark to that in light at 25°C was 1.22 for *Nitrosomonas* and 1.5 for *Nitrobacter* (cited by Painter 1970). Hooper and Terry (1983) observed complete inhibition of *Nitrosomonas* activity by a 200 W bulb.

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 = 2,490 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 and 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.

6.5 Inhibitory compounds

Phillips et al. (2002) stated that 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). Experiments carried out with $NaClO_3$ (sodium chlorate, 10 mM) showed that it inhibits the oxidation of NO_2^- to NO_3^- by *Nitrobacter* spp. but does not affect the oxidation of NH_4^+ to NO_2^- by *Nitrosomonas europea* (Belser and Mays 1980). Similarly, it was concluded by Lees and Simpson (1957) that chlorate is a specific inhibitor of nitrite-oxidizing bacteria. Low concentrations of chlorate (i.e., 10^{-5} M) inhibit autotrophic nitrite oxidizer growth, whereas it takes much higher concentrations (i.e., 10^{-3}–10^{-2} M) to completely inhibit nitrite oxidation. Hynes and Knowles (1983) found that the oxidation of NH_4^+ by *Nitrosomonas europea* was insensitive to 10 mM $NaClO_3$ (sodium chlorate) but was strongly inhibited by $NaClO_2$ (sodium chlorite; K_i, 2 μM), while the oxidation of NO_2^- by *Nitrobacter winogradskyi* was inhibited by both ClO_3^- and ClO_2^- which supports the above findings. 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 and 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 and 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 and 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 and 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°C ↔ 30°C), which could indicate a synergistic toxic effect between nickel and temperature (Randall and Buth 1984). 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). 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).

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 and 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 and 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-ionized or NH_2OH), and not necessarily with FA nor with low DO concentration. The pK_a value of ionized hydroxylamine at 25°C equals 7.99 (Yang and Alleman 1992).

7 Conclusions and discussions

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 diverse (Phillips et al. 2002).

It has been known over years that basically two groups of bacteria—the AOB and the NOB are involved in biological nitrification. The applications of molecular techniques have proven their value in the inference of phylogenetic relationships between microorganisms. It also broadens our understanding of the organization of microbial communities and unravels the role of the individual microorganisms in the cycling of chemical elements and the removal of toxic compounds from the environment. With the use of non-cultivation based molecular techniques like FISH, PCR-DGGE and Quinone profile as discussed in this review, the dominance of the ammonia-oxidizing group can be monitored in any kind of wastewater treatment system for proper functioning of the partial nitrification process.

The fact that, despite fragile balances nitrite accumulation is not the rule in aquatic systems is in view of the different characteristics of the nitrite-producing and -removing organisms. However recent technologies make use of the presence of nitrite in wastewater treatment or other domains as an intermediate. The proper manipulation of the operating parameters—maintaining high temperature (> 25°C), pH at 7.5–8.5°C, DO at < 0.5 mg l^{-1}—thereby affecting the FA concentration can help in accumulation of nitrite in the system. This accumulation of nitrite can be utilized through a nitrite shunt and can offer cost savings in aeration as well as in the form of lesser or no need for addition of organic carbon as compared to the conventional denitrification.

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