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REVIEW PAPER

Mechanism leading to N_2O production in wastewater treating biofilm systems

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Abstract Wastewater treatment plants are known to be important point sources for nitrous oxide (N_2O) in the anthropogenic N cycle. Biofilm based treatment systems have gained increasing popularity in the treatment of wastewater, but the mechanisms and controls of N_2O formation are not fully understood. Here, we review functional groups of microorganism involved in nitrogen (N) transformations during wastewater treatment, with emphasis on potential mechanism of N_2O production in biofilms. Biofilms used in wastewater treatment typically harbour aerobic and anaerobic zones, mediating close interactions between different groups of N transforming organisms. Current models of mass transfer and biomass interactions in biofilms are discussed to illustrate the complex regulation of N_2O production. Ammonia oxidizing bacteria (AOB) are the prime source for N_2O in aerobic zones, while heterotrophic denitrifiers dominate N_2O production in anoxic zones. Nitrosative stress ensuing from accumulation of NO_2^- during partial nitrification or denitrification seems to be one of the most critical factors for enhanced N_2O formation. In AOB, N_2O production is coupled to nitrifier denitrification triggered by nitrosative stress, low O_2 tension or low pH. Chemical N_2O production from AOB intermediates (NH_2OH, HNO, NO) released during high NH_3 turnover seems to be limited to surface-near AOB clusters, since diffusive mass transport resistance for O_2 slows down NH_3 oxidation rates in deeper biofilm layers. The proportion of N_2O among gaseous intermediates (NO, N_2O, N_2) in heterotrophic denitrification increases when NO or nitrous acid (HNO_2) accumulates because of increasing NO_2^-, or when transient oxygen intrusion impairs complete denitrification. Limited electron donor availability due to mass transport limitation of organic substrates into anoxic biofilm zones is another important factor supporting high N_2O/N_2 ratios in heterotrophic denitrifiers. Biofilms accommodating Anammox bacteria release less N_2O, because Anammox bacteria have no known N_2O producing metabolism and reduce NO_2^- to N_2, thereby lowering nitrosative stress to AOB and heterotrophs.

Keywords N_2O · NO · NO_2^- · Biofilm · AOB · NOB · Anammox · Heterotrophic denitrification

1 Introduction

In the last decades, wastewater treatment plants have received increasing attention for their role as point sources for N_2O emissions in the anthropogenic nitrogen cycle (Kampschreur et al. 2009b; Schreiber et al. 2012). N_2O is a strong greenhouse gas contributing to global warming and stratospheric ozone depletion (Ravishankara et al. 2009). Even

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though wastewater treatment only accounts for an estimated 1.3 % of the total anthropogenic N_2O emission, its contribution to the total greenhouse gas production associated with the human water supply and sanitation chain may be as high as 26 % (Kampschreur et al. 2009b). This includes production of drinking water as well as treatment of wastewater.

Numerous studies and review articles about N_2O emissions from wastewater treatment systems have been published (e.g. Kampschreur et al. 2009b; Law et al. 2012b; Tallec et al. 2006; Wunderlin et al. 2012). Most of these studies focus on suspended biomass (activated sludge), while biofilm systems have received little attention. At the same time, biofilm systems enjoy increasing popularity owing to their robustness and efficiency, and their ability to combine several microbial processes within one system (Khan et al. 2013; Morgenroth 2008b). However, the understanding of N_2O producing processes in biofilm systems is less developed than for suspended biomass cultures, because diffusion resistance and biomass competition across the biofilm need to be considered when studying N-transformation processes in biofilms (Morgenroth 2008a; Wanner and Gujer 1985). More knowledge is needed about the critical factors and biomass interactions controlling N_2O production in biofilms (Schreiber et al. 2012).

This review links known biomass interactions in wastewater treating biofilm systems to mechanisms and rates of microbial N transformation processes and evaluates their potential role in N_2O production. Chapter 2 introduces the biochemistry of N_2O production and consumption found among the main functional groups of organisms relevant to wastewater treatment systems. Chapter 3 combines the known biochemistry of N_2O production and consumption with current models of mass transfer and biomass interactions in biofilms and links them to environmental factors that are typically measured and controlled in wastewater treatment systems. Finally, Chapter 4 summarises N_2O emissions reported for different types of wastewater treating biofilm systems.

2 Key organisms and enzymatic processes involved in biological Nitrogen conversion

2.1 Ammonia oxidizing bacteria (AOB)

Ammonia oxidizing bacteria (AOB) are obligate chemolitoautotrophic, sometimes mixotrophic bacteria of the phylum Proteobacteria, which generate energy for growth and maintenance from the oxidation of ammonia (NH_3) to nitrite (NO_2^-). This group comprises several dozen species, which are adapted to different environments (e.g. Bothe et al. 2000). AOB are the key species mediating nitrification, i.e. the microbial conversion of NH_4^+ to NO_2^- in wastewater treatment systems and have therefore been at the core of interest as potential sources for N_2O emissions (Kampschreur et al. 2009b). Here, we focus on *Nitrosomonas sp.*, which is the most studied and most important AOB genus in wastewater treatment systems (Law et al. 2012a; Schreiber et al. 2009) and review metabolic pathways involved in N_2O production.

2.1.1 AOB metabolism

AOB oxidize ammonia (NH_3) to hydroxylamine (NH_2OH) and further to nitrite (NO_2^-). This two-step reaction is performed by the membrane bound enzyme ammonia monooxygenase (AMO) and the periplasmic enzyme hydroxylamine dehydrogenase (formerly hydroxylamine oxidoreductase) (HAO), respectively (Fig. 1). The HAO reaction releases one mole of NO_2^-, four moles of electrons and five moles of H^+ per mole NH_2OH oxidized (Wood 1986). Two of the electrons are shuttled back to AMO where they aid the oxidation of NH_3 to NH_2OH. The electron transport chain is mediated by the hemeproteins cytrochrome C554, cytochrome C552 and a quinone pool (Arp et al. 2002; Whittaker et al. 2000). The two other electrons are used to generate a proton motive force through reverse electron transport (Schmidt and Bock 1998; Whittaker et al. 2000; Wood 1986). Together, the nitrification of one mole of NH_3 releases three moles of protons resulting in the overall reaction:

$$NH_3 + 1.5\ O_2 \rightarrow NO_2^- + H^+ + H_2O \quad (1)$$

The design of wastewater treatment processes is often based on total ammonia nitrogen (TAN) (e.g. Ekama and Wentzel 2008; Gujer et al. 1999; Kaelin et al. 2009), while the true substrate for AMO is free NH_3 which is in equilibrium with undissociated NH_4^+ (Hyman and Arp 1995; Suzuki et al. 1974). Owing to the low acid dissociation of NH_4^+ in water (pKa NH_3/NH_4^+ 9.24) and its dependency on temperature and pH, the acid-base equilibrium between ammonium (NH_4^+) and the AOB substrate NH_3 is an important

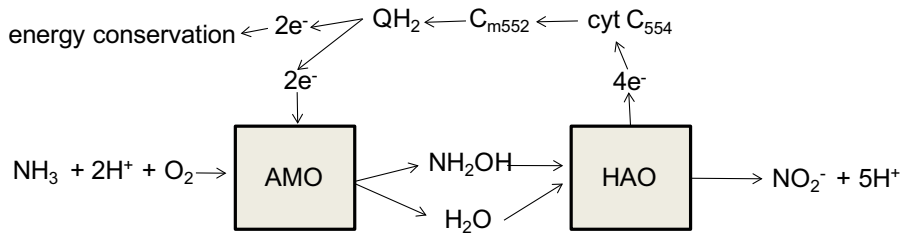

Fig. 1 Electron flow during oxidation of ammonia (NH_3) to nitrite (NO_2^-) in *Nitrosomonas sp.* driven by a two-step reaction involving the enzymes ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). Hydroxylamine oxidation generates 4 electrons which are channelled via the hemeproteins cytochrome C_{554} and cytochrome C_{m552} to a quinone pool (QH_2) where two of the four electrons are transferred to an electron chain for energy conservation (adapted from Arp et al. 2002)

factor, which needs to be taken into account when modelling nitrification based on TAN, especially in systems operating at low pH (Kaelin et al. 2009; Quinlan 1984).

The oxidation of ammonia to hydroxylamine by AMO is mediated by a complex reaction chain, which is not yet fully unravelled. A hypothesis put forward by Schmidt et al. (2001a) proposes a two-step reaction based on an internal recirculation of the oxidant dinitrogen tetroxide (N_2O_4) (Fig. 2). In this so-called NO_x cycle, NH_3 is oxidized with N_2O_4 releasing NH_2OH and nitric oxide (NO). The latter is reoxidized with O_2 at another active site of the enzyme to nitrogen dioxide (NO_2) which is in equilibrium with its dimeric form N_2O_4. Under aerobic conditions, NO or NO_2 were shown to significantly enhance the NH_3 oxidation rate (Beyer et al. 2009; Schmidt et al. 2001a; Zhang et al. 2010). However, in another study, the NO scavenger PTIO had little effect on the ammonia oxidation rate of *Nitrosospira multiformis* (Shen et al. 2013). This finding questions the significance of NO and of the proposed NO_x cycle for NH_3 oxidation in *N. multiformis*. An alternative hypothesis proposes that NO_2 or N_2O_4 act as direct oxidants in addition to O_2 (Zhang et al. 2010). The conversion of NH_2OH to NO_2^- by HAO is a complex reaction involving several intermediates (Fig. 2). NH_2OH binds to the active site of P_{460}, an iron containing multi-heme, where it is oxidized via the intermediates nitroxyl (HNO) and nitric oxide (NO) to nitrite (Kostera et al. 2008).

Nitrosomonas europaea and other AOB species have been shown to be equipped with respiratory enzymes which reduce NO_2^- to NO, N_2O or N_2. This process, denoted as "nitrifier denitrification" is considered to constitute a chemoorganotrophic survival metabolism under O_2 limiting conditions (Hooper 1968; Poth and Focht 1985; Schmidt and Bock 1997). Alternatively, nitrifier denitrification might be involved in protecting the organisms from high NO_2^- concentrations (Beaumont et al. 2002, 2005; Cua and Stein 2011). The latter has been questioned however, because nitrifier denitrification has relatively low reaction rates (Schmidt 2008). Nitrite reductase (NIR), the enzyme reducing NO_2^- to NO, was for a long time considered to be the central enzyme of nitrifier denitrification (Casciotti and Ward 2001; Hooper 1968). However, *N. europaea*

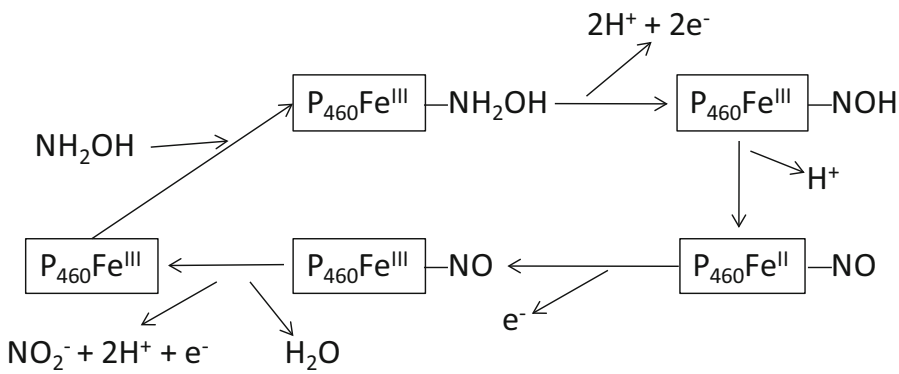

Fig. 2 Oxidation of NH_2OH to NO_2^- catalysed by hydroxylamine dehydrogenase involving the reduction and re-oxidation of iron in the multi-heme P_{460} (adapted from Kostera et al. 2008)

expresses NirK (one of the known NIR enzymes) under fully aerobic conditions (Beaumont et al. 2004b) and mutants lacking NirK were shown to reduce NO_2^- under anoxic conditions at rates comparable to the wild type (Beaumont et al. 2002). Hence, the first step in nitrifier denitrification is performed by another, so far unidentified nitrite reductase, while NirK seems to play an important role in the energy conservation during NH_2OH oxidation under oxic conditions (Kozlowski et al. 2014). The enzyme nitric oxide reductase (NOR) (Beaumont et al. 2004b) reduces NO to N_2O (Kostera et al. 2008; Schmidt et al. 2004a). The gene coding for NOR, *norB*, was shown to be essential for AOBs' ability to reduce NO_2^-, suggesting that NOR is the sole NO reductase in the nitrifier denitrification pathway (Kozlowski et al. 2014). Notwithstanding, *norB*-deficient *N. europaea* strains have been shown to reduce added NO to N_2O (Beaumont et al. 2004b). These findings point to two alternative NO reduction pathways: NO produced via reduction of NO_2^- by NOR coupled to energy conversation (Kozlowski et al. 2014; Schreiber et al. 2009) and NO released during ammonia oxidation (NO_x cycle, AMO intermediates, Fig. 2) being reduced by an alternative NO-reductase in the course of detoxification, likely connected to Cytochrome C_{554} (Upadhyay et al. 2006). *N. europaea* (Schmidt et al. 2004b), *Nitrosomonas eutropha* (Schmidt and Bock 1997, 1998) and likely also other AOB species (Beyer et al. 2009) are able to perform a complete reduction of NO_2^- to N_2 (via NO and N_2O), even though no homologue of N_2O reductase (N_2OR), the enzyme reducing N_2O to N_2 in canonical denitrification has been found in AOB (Chain et al. 2003). However, Nitrosocyanin (nsc), a protein similar to N_2OR in structure, could mediate this function, thus rendering AOB fully fledged denitrifiers (Arciero et al. 2002; Beyer et al. 2009; Schmidt 2009; Whittaker et al. 2000).

2.1.2 *Potential N_2O producing pathways in AOB*

Several studies suggest that the highly reactive intermediates NH_2OH, NOH or NO can be released from AOB during imbalanced NH_3 oxidation and that these intermediates serve as substrates for chemical N_2O formation under aerobic conditions (Law et al. 2012a; Wunderlin et al. 2013). This would explain the often times observed positive correlation between apparent NH_3 oxidation rates and N_2O emission rates (Law et al. 2011, 2012a; Yu et al. 2010). Proposed pathways for N_2O formation connected to the release of HAO intermediates are release of NOH and subsequent chemical decomposition to N_2O (Law et al. 2012a; Poughon et al. 2001) or release of NO and subsequent enzymatic reduction by cytochrome C_{554} to N_2O (Hooper and Terry 1979; Kostera et al. 2010, 2008; Upadhyay et al. 2006). Nitroxyl (NOH) is an early intermediate in the HAO pathway (Fig. 2), that can decompose abiotically to N_2O (Law et al. 2012a; Poughon et al. 2001; Stuven and Bock 2001). NH_2OH uptake by HAO is believed to be greater than electron transfer to cytochrome C_{554} (Kostera et al. 2010), which, at high NH_2OH oxidation rates, may result in build-up of excess reduction power. Stuven and Bock (2001) proposed that HAO transfers only two instead of four electrons to cytochrome C_{554} under high NH_2OH oxidation rates while releasing HNO. Yet, even under those conditions, the electron supply by AMO may exceed the reducing potential available for energy conservation, so that HNO is released from HAO and leads to chemical N_2O formation (Law et al. 2011, 2012a; Wunderlin et al. 2013). The latter may become as a self-accelerating process, which could be an explanation for the observed exponential relationship between NH_3 oxidation rates and N_2O emissions (Law et al. 2012a).

NO can be released from the $P_{460}Fe^{III}$-NO complex, during the final step of HAO metabolism (Kostera et al. 2008, see Fig. 3). Its further reduction to N_2O is likely performed by the protein cytochrome C_{554} (Upadhyay et al. 2006), which is believed to act as an immediate acceptor for electrons transferred by the HAO enzyme (Upadhyay et al. 2006; Yamanaka and Shinra 1974). Therefore, the putative NO reducing activity of cytochrome C_{554} is likely driven by electrons supplied by the HAO process. In presence of O_2, NO is oxidized abiotically to NO_2, which again may fuel the NO_x cycle and AMO activity (Schmidt et al. 2001b). However, this chemical reaction does probably not reach significant rates under normal growth conditions (Schmidt et al. 2001b; Udert et al. 2005). Upadhyay et al. (2006) proposed that the release of NO is a consequence of a decreased electron transfer rate from HAO to the cytochrome complex, which may be provoked when O_2 depletes to a level exceeding the capacity of AMO for receiving electrons from NH_2OH oxidation (Kostera et al. 2008; Upadhyay et al. 2006). Acidity was shown to be

another potential factor provoking incomplete HAO reaction (Jiang and Bakken 1999). Under strongly acidic conditions, abiotic N_2O formation by N-nitrosation of NH_2OH and NO_2^- has been proposed (Freeman 1973; Spott et al. 2011).

Chemical decomposition of the intermediate NH_2OH as a source of N_2O from NH_3 oxidation was proposed in the early literature (Bremner et al. 1980; Yoshinari 1990). NH_2OH may accumulate when the NH_2OH oxidation becomes slower than the NH_3 oxidation, for example in the case of limited availability of reducing equivalents (Wunderlin et al. 2013). Recent in vitro experiments showed that abiotic NH_2OH decomposition does not reach significant rates under normal growth conditions (Harper et al. 2009; Jason et al. 2007; Wunderlin et al. 2012). However, the latter abiotic reaction likely involves mandatory oxidation of NH_2OH to NOH with NO_2^- as electron acceptor. Hence, chemical decomposition of NH_2OH to N_2O may be promoted when NO_2^- is present at high concentrations (Tallec et al. 2006).

The exact biochemistry of NO_2^- reduction in nitrifier denitrification is not fully understood. In particular, the role of the copper-containing nitrite reductase NirK, a homologue of the dissimilatory nitrate reductase in canonical denitrification, remains unclear. Some studies found positive (Cua and Stein 2011; Kim et al. 2010), no (Beaumont et al. 2002) or even negative correlation (Jason et al. 2007) between the expression of NirK and N_2O formation in *N. europaea*. *In vitro* studies have shown that NirK-deficient strains of *N. europaea* released four times more N_2O than the wild type (Beaumont et al. 2002), while NOR-deficiency did not significantly affect N_2O production (Beaumont et al. 2004b). This may imply that nitrifier denitrification is not a major source of N_2O under normal growth conditions (Beaumont et al. 2002), which however is questioned by the recent hypothesis that NirK has a supportive function for NH_2OH oxidation by providing an additional electron sink for the HAO enzyme while another, so far unknown NIR homologue performs the NO_2^- reduction in nitrifier denitrification (Kozlowski et al. 2014).

2.1.3 *Regulation of N_2O production in nitrification*

2.1.3.1 Nitrite Nitrite accumulation is often pointed out to be a major factor leading to N_2O production by AOB, likely via incomplete nitrifier denitrification (Stein and Arp 1998; Beaumont et al. 2005; Yu and Chandran 2010; Cua and Stein 2011). Table 1 gives an overview over findings from AOB pure culture studies in which the proportion of N_2O originating from NO_2^- was determined with help of ^{15}N labelling. The study by Shaw et al. (2006) indicates that this proportion may vary among different AOB strains. Comparing the figures of Poth and Focht (1985) with those of Schmidt et al. (2004b) suggests that high NO_2^- levels increase the amount of N_2O produced relative to the amount of NH_3 oxidized, the proportion of N_2O having NO_2^- as N source and the N_2O/N_2 ratio. The latter might be due to incomplete nitrifier denitrification at high NO_2^- concentrations (Cua and Stein 2011; Poth and Focht 1985). This is supported by a pure culture study with *N. europaea* in which N_2O production increased substantially when NO_2^- concentrations exceeded 5 mM (Yu and Chandran 2010). NO_2^- concentrations >5 mM were also shown to inhibit AMO activity, especially at low pH, when HNO_2 is formed (Stein and Arp 1998; Yu and Chandran 2010). However, the effect of NO_2^- seems to be O_2 depended. In a recent mixed culture study, the trend of increasing N_2O production with increasing NO_2^- concentration was shown to be less obvious at high DO than at low DO levels (<1.5 mg L^{-1}) (Peng et al. 2015a). Increased N_2O production by nitrifier denitrification in response to high NO_2^- concentrations may reflect a detoxification reaction countervailing nitrosative stress (Stein and Arp 1998; Yu and Chandran 2010). However, detoxifying N_2O production has been challenged by a recent study with a mixed culture, in which increasing NO_2^- concentrations up to a level of 1 mM increased N_2O production while a further increase to 10 mM decreased N_2O production (Law et al. 2013). The authors attributed this to substrate inhibition of NIR above a certain NO_2^- threshold. However, since the mixed AOB culture was studied in a highly loaded partial nitrification reactor, continued high concentrations of NO_2^- may have altered the AOB community towards species that are less sensitive to NO_2^- and hence produce less N_2O (Castro-Barros et al. 2016; Chandran et al. 2011). A similar observation was made in a highly loaded wastewater reactor when introducing partial nitrification conditions by lowering DO and biomass retention time; biomass dominance shifted from *N. europaea* to *N. eutropha* and the transient N_2O peak faded out

Table 1 Results from ^{15}N labelling experiments investigating the source of N_2O emission in AOB

Study	Species	DO level mg O_2 L^{-1}	NO_2^- mg N L^{-1}	NH_4^+ mg N L^{-1}	N_2O % oxidized NH_3	N_2O % having NO_2^- as N source	Ratio N_2/N_2O
Poth and Focht (1985)	*N. europaea*	1	5–24	21	0.6–0.65	25	7–7.5
Schmidt et al. (2004b)	*N. europaea*	5	70–200	140	0.7	94	20
Shaw et al. (2006)	*N. europaea*	4–6	14–16	49	0.45	31	
	N. europaea	4–6	14–17	49	0.19	22	
	Nitrosospira briensis	4–6	14–18	49	0.03	14	
	Nitrosospira multiformis	4–6	14–19	49	0.08	12	
	Nitrosospira tenuis	4–6	14–20	49	0.7	50	

The ratios are calculated based on N equivalents

(Ahn et al. 2011). These findings point to a greater tolerance of *N. eutropha* to NO_2^- as compared with *N. europaea.* Another study with a mixed AOB culture in a wastewater reactor with low NH_3 load observed a shift from N_2O to NO emission when NO_2^- concentrations exceeded 3.6 mM (Castro-Barros et al. 2016). In contrast, a recent pure culture study with *N. europaea* found only temporary increase of NO production upon increasing NO_2^- (Yu et al. 2010). Clearly, more research is needed to elucidate the role of NO_2^-, NO and NO_2^-/HNO_2 partitioning for N_2O emissions from AOB activity associated with wastewater treatment.

2.1.3.2 Dissolved oxygen (DO) Comparing DO in the studies with *N. europaea* by Poth and Focht (1985) and Shaw et al. (2006) in Table 1 suggests that DO has little impact on N_2O production under aerobic conditions (DO $>$ 1 mg O_2 L^{-1}). This was also found in another pure culture study with *N. europaea* (Kester et al. 1997) and in a recent study with a mixed AOB culture (Peng et al. 2014), both of which did not find significant differences in the N_2O emission factor (relative to oxidised NH_3) at steady state when DO was above 1 mg O_2 L^{-1}. Isotope labelling indicated that N_2O originates from NH_2OH, likely produced via HAO intermediates under fully aerobic conditions (Peng et al. 2014). N_2O emissions by AOB increase dramatically when DO in the bulk liquid falls below 1 mg O_2 L^{-1} or fluctuates between aerobic and anaerobic conditions (Jia et al. 2013; Kester et al. 1997; Peng et al. 2014). Production of N_2O (and NO) during nitrification under limited O_2 availability is commonly attributed to nitrifier denitrification (Jia et al. 2013).

2.1.3.3 Inorganic carbon Another factor which influences N_2O production by AOB is the availability of inorganic carbon. In a recent study with a mixed biomass that was enriched in AOB and NOB (Peng et al. 2015b), N_2O emissions showed a positive relationship with inorganic carbon concentration. In intensively nitrifying reactor systems, autotrophic growth may result in a shortage of inorganic carbon, which may limit NH_3 oxidation activity by AOB. Increased availability of inorganic carbon will therefore lead to higher reaction rates of AOB and associated N_2O emissions (Peng et al. 2015b).

2.1.3.4 Acidity Acidic conditions (pH $<$ 5) may induce partial inhibition of the HAO with subsequent release of NO that might be further reduced to N_2O (Jiang and Bakken 1999). In addition, pH may indirectly affect N_2O production by AOB through controlling the partitioning of NO_2^- and HNO_2. The latter may induce the above discussed detoxification reactions and is

suspected to have an inhibitory effect on AMO (Stein and Arp 1998; Yu and Chandran 2010).

2.2 Nitrite oxidizing bacteria (NOB)

Nitrite oxidizing bacteria, NOB, refers to a group of gram-negative bacteria gaining their energy from oxidizing NO_2^- to NO_3^- (Spieck and Lipski 2011). Wastewater treatment systems are usually dominated by the two genera, *Nitrobacter sp.* and *Nitrospira sp.* (Blackburne et al. 2007). Which of these species dominates depends on the total ammonia (NH_3; NH_4^+) load, but also on O_2 availability and other environmental conditions (Okabe et al. 1999). The oxidation is catalysed by a complex multi-enzyme reaction chain (Spieck and Lipski 2011; Sundermeyer-Klinger et al. 1984). In brief, the membrane bound nitrite oxidoreductase (NXR) oxidizes NO_2^- to NO_3^-. This enzymatic reaction derives the oxygen needed from dissociation of H_2O and releases two protons and two electrons. The electrons are transferred to the heme-protein cytochrome-C oxidase, which reduces the final electron acceptor O_2 to O^{2-}, which in turn reacts with the protons released by NXR to H_2O (Sundermeyer-Klinger et al. 1984). This results in overall Eq. 2, which is also used for parameterizing O_2 consumption during NO_2^- oxidation (Kaelin et al. 2009) as well as for studying NOB kinetics (Ciudad et al. 2006).

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (2)$$

NOB are little studied owing to their poor growth in pure cultures (Spieck and Lipski 2011). Recent studies support the notion that NO_2^- rather than its protonated form nitrous acid (HNO_2) (*p*Ka NO_2^-/HNO_2 3.39 in water) is the true substrate for NXR (Park and Bae 2009; Poughon et al. 2001; Udert and Jenni 2013). However, *NOB* seem to be able to deprotonate HNO_2 prior to its oxidation (Park and Bae 2009). NOB have received little attention in the literature so far with respect to N_2O production. *Nitrobacter sp.* and *Nitrospira sp.* were shown to have an anoxic growth mode reducing NO_3^- with pyruvate or H_2 as electron donor (Colliver and Stephenson 2000; Maia and Moura 2014; Sundermeyer-Klinger et al. 1984). This metabolism is little studied but some authors assume that the terminal products are NO or N_2O (Starkenburg et al. 2008). In contrast to nitrifier denitrification by AOB, which was observed at variable O_2 concentrations (Beaumont et al. 2004a; Schmidt et al. 2004b), NO_3^- respiration by NOB is only active under anoxic conditions (Colliver and Stephenson 2000). A potential direct contribution of NOB to N_2O emissions seems therefore only relevant under oxygen limiting or anoxic conditions. However, NOB play a crucial role in controlling NO_2^- concentrations in waste water treatment systems, and may thus indirectly controlling N_2O emissions (Ch. 3).

It has been frequently stated that high NH_3 concentrations inhibit NOB (Anthonisen et al. 1976; Park and Bae 2009). However, NOB can adapt to high NH_3 and hence inhibition is species dependent (Yoo et al. 1999). Reported threshold concentrations vary from 0.04 to 0.08 mg NH_3–N L^{-1} for *Nitrospira sp.* to up to 30–50 mg NH_3–N L^{-1} for *Nitrobacter sp.* (Blackburne et al. 2007). HNO_2, the protonated form of NO_2^- is another frequently cited inhibitor of NOB (Hellinga et al. 1999; Park and Bae 2009; Zhou et al. 2011). In general, critical HNO_2 levels can be expected when NO_2^- accumulates at low pH. This typically occurs in weakly buffered systems with high nitrification rates owing to the proton release during AOB metabolism (Eq. 1) (Udert et al. 2005).

2.3 Heterotrophic denitrifiers

In wastewater treatment, heterotrophic denitrifiers refer to a large number of taxonomically unrelated chemoorganotrophic bacteria, which use the anoxyions NO_3^- and NO_2^- as terminal electron acceptors in the absence of oxygen and reduce them to gaseous NO, N_2O and N_2. Canonical denitrification of NO_3^- comprises four consecutive reduction steps in which the intermediates NO_2^-, NO and N_2O serve as substrates for the reductase enzymes nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and N_2O reductase (N_2OR), respectively (Zumft 1997). The overall chemical reaction is shown in Eq. 3 for the reduction of NO_3^- and Eq. 4 for the reduction of NO_2^-.

$$NO_3^- + 6H^+ \rightarrow 0.5\ N_2 + 3H_2O \quad (3)$$

$$NO_2^- + 4H^+ \rightarrow 0.5\ N_2 + 2H_2O \quad (4)$$

Denitrification is a facultative metabolic pathway, which enables denitrifiers to respire organic carbon anoxically. Denitrification is suppressed by O_2 in favour of oxic respiration. O_2 tension also regulates the relative activity of the denitrification enzymes, and

may thus determine the composition of the gaseous end products (Körner and Zumft 1989). In microaerobic zones having a DO in the range of 0.2–0.5 mg L^{-1}, the expression of N_2OR, the enzyme reducing N_2O to N_2, may be repressed, resulting in high N_2O emissions (Bergaust et al. 2012; Bonin and Raymond 1990; Morley et al. 2008). A second factor affecting the balance of the reduction steps is the pH. It has been shown that pH below 6.1 impairs N_2OR functioning, likely post-transcriptionally, i.e. no functional enzyme is synthesized at low pH unless acid tolerant denitrifiers proliferate. This was shown in pure culture studies of *Paracoccus denitrificans* (Bergaust et al. 2010) and in bacteria extracted from soils (Brenzinger et al. 2015; Dörsch et al. 2012; Liu et al. 2014).

Recent studies suggest that the reduction of N_2O to N_2 also depends on the availability of electron donors (Pan et al. 2013; Richardson et al. 2009). For instance, N_2O accounting for up to 20 % of biologically reduced N was attributed to low degradability of endogenous substrates in a biofilm (Itokawa et al. 2001), which limits electron availability for the reduction of N_2O to N_2 (Pan et al. 2013).

Similarly to its role in nitrification, accumulation of NO_2^- originating from NO_3^- reduction in anoxic wastewater reactor systems has been pointed out to be an important source for increased N_2O production in heterotrophic denitrification (e.g. Schreiber et al. 2012; Schulthess et al. 1995; Zhou et al. 2011). Campos et al. (2009) reported N_2O emission accounting for up to 55 % of totally reduced N when heterotrophic denitrification was fed with NO_2^- as an electron acceptor as compared to 0.8 % with NO_3^-. The exact mechanisms behind the enhanced N_2O production in the presence of NO_2^- are not known, but nitrosative stress response triggered by HNO_2 or NO (both of which can be formed chemically from NO_2^-) leading to enzymatic or abiotic reactions have been proposed. For example, NO may be formed chemically (abiotic) through dismutation of HNO_2 (Stein and Arp 1998). NO may also accumulate biotically as an intermediate of heterotrophic denitrification if NOR expression is delayed relative to NIR expression (Schulthess et al. 1995). HNO_2 is toxic and suspected to inhibit periplasmatic N_2OR, which would lead to incomplete denitrification and release of N_2O instead of N_2 (Schreiber et al. 2012; Zhou et al. 2008b). Inhibition of N_2OR by HNO_2 was observed in an anoxic activated sludge culture at concentrations as low as 0.2 μg HNO_2–N L^{-1}. At HNO_2 concentration ranging from 0.7 to 1.0 μg N L^{-1}, 50 % inhibition of N_2O reduction was observed whereas 4 μg HNO_2–N L^{-1} inhibited N_2O reduction completely. Based on these data, and the acid-base equilibrium constant for NO_2^-, a 50 % inhibition at pH 7 would take place at NO_2^- concentrations of 3–4 mg N L^{-1} and a total inhibition at 17 mg N L^{-1} or higher (Zhou et al. 2008b). The presence of free NO was shown to have an immediate, irreversible inhibitory effect on the N_2OR enzyme, while leading to enhanced NIR activity (Frunzke and Zumft 1986). Hence, regardless whether NO is formed biotically or abiotically during periods of high NO_2^- accumulation, it might result in a suppression of N_2O reduction in heterotrophic denitrification (Schulthess et al. 1995). Given the proposed irreversibility of the N_2OR inhibition, high N_2O production may be sustained over longer periods even if free NO only occurred as a temporary phenomenon.

2.4 Anaerobic ammonia oxidizing bacteria (Anammox)

A recently discovered group of obligate anaerobic, chemolithoautotrophic organisms gains energy for metabolism and growth from the anaerobic oxidation of NH_4^+ to N_2 with NO_2^- as electron acceptor (Jetten et al. 1998; Kuenen 2008; Mulder et al. 1995; van de Graaf et al. 1995). So far, only few Anammox species have been described, all of which belong to the class of *Planctomycetes*. The most studied ones are *Candidatus B. anammoxidans* (Strous et al. 1999a) and *Candidatus K. stuttgartiensis* (Schmid et al. 2000), which are also the main species used for wastewater treatment (Kuenen 2008). Anammox-bacteria were shown to grow in extreme dense colonies, preferably biofilms, at extremely low growth rates and are therefore difficult to purify or isolate (Strous et al. 1999a). The Anammox metabolism converts NH_4^+, NO_2^- and protons into N_2, NO_3^- and H_2O as shown in overall Eq. 5 (Kartal et al. 2013; Strous et al. 1999b).

$$1\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 0.066\ CH_2O_{0,5}N_{0,15} + 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O \quad (5)$$

The coefficients for Eq. 5 where experimentally derived by Strous et al. (1999b) and should be verified by pure culture experiments (Lotti et al. 2014). NO_2^-

is reduced to NO in a complex reaction chain (Eq. 6) which again reacts with NH_4^+ to N_2H_4 (Eq. 7). In a final step, N_2H_4 is oxidized to N_2 (Eq. 8). This synproportionation of NH_4^+ with NO_2^- results in an energy gain of −357 kJ mol^{-1} (Eq. 9) (Kartal et al. 2011).

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \left(E^\circ = +0.38\ V\right) \quad (6)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \left(E^\circ = +0.06\ V\right) \quad (7)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \left(E^\circ = -0.75\ V\right) \quad (8)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \left(\Delta G^\circ = -357\ kJ\ mol^{-1}\right) \quad (9)$$

This reaction chain, performed by a series of recently discovered enzymes, is not yet fully clarified (Kartal et al. 2013). The concurrent oxidation of NO_2^- to NO_3^- is assumed to generate reducing potentials for CO_2 fixation (Lotti et al. 2014). Anammox bacteria have a little investigated optional organotrophic metabolism, which can reduce organic acids (propionate, acetate) to CO_2 with NO_3^- as electron acceptor (Güven et al. 2005). ^{15}N labelling experiments indicated that these electron acceptors are reduced to NH_4^+ with NO_2^- as intermediate (Kartal et al. 2007a) rather than to N_2O and N_2. The purpose of this organotrophic metabolism may be energy conversation (Güven et al. 2005; Kartal et al. 2007b) or internal production of substrates (NH_4^+, NO_2^-) for the main metabolism (Kartal et al. 2007a). An assimilation of organic substrates into biomass was not observed (Güven et al. 2005; Kartal et al. 2007b). The organotrophic metabolism seems to be highly selective for volatile fatty acids (VFA). Other typical electron donors (e.g. glucose) had no significant effect or even inhibited (alcohols) Anammox metabolism (Güven et al. 2005).

Anammox bacteria use NH_4^+ as substrate (Kartal et al. 2013, 2011). This distinguishes them from AOB, which have NH_3 as substrate. However, since NH_4^+ and NH_3 are in equilibrium (pKa NH_3/NH_4^+ 9.24 in water), the availability of and competition for substrate depends on pH, which is controlled by proton release (AOB) or proton uptake (Anammox). The Anammox metabolism is inhibited in the presence of O_2, but the inhibition was shown to be reversible even after exposure to a DO as high as 8 mg O_2 L^{-1} (Hu et al. 2013). Also NO_2^- has a reversible inhibitory effect on the Anammox organism at higher concentrations (IC_{50} 400 mg L^{-1}) (Lotti et al. 2012). Anammox bacteria are at the same time extremely tolerant to NO, which they are able to reduce to N_2, most likely via the intermediate N_2H_4 with NH_4^+ as electron donor (Kartal et al. 2010).

According to present knowledge, Anammox bacteria do not produce N_2O (Hu et al. 2013). However, an earlier study showed that the heterotrophic organism *Pseudomonas aeruginosa* nitrosates the intermediate N_2H_4 and HNO_2 to N_2O (Kim and Hollocher 1984). It is possible that also Anammox bacteria perform a similar reaction under specific conditions. A recent pure culture study (Lotti et al. 2014) detected a small but measurable production of N_2O (0.2 % of removed N), which might have been produced by this pathway. Considerable N_2O emissions were reported from Anammox reactor systems, amounting to 0.4–1.3 % of the biologically transformed N (Hu et al. 2013). It was shown that emissions rates for NO and N_2O increased with increasing O_2 availability in the bulk liquid (Kampschreur et al. 2009a). However, these data were obtained from systems with mixed biomass cultures of both AOB and Anammox organisms and hence N_2O may have been produced by AOB. This would be consistent with a reported trend of decreased N_2O and NO production with increasing Anammox activity (Ni et al. 2013). More research is needed to clarify the N_2O production potential by Anammox.

3 Mechanism of N_2O production in biofilm systems

3.1 Biofilms in wastewater treatment application

This section summarizes the current understanding of biofilm functioning by using a mass balance model as a framework for the discussion of N_2O producing mechanism. A biofilm is defined as densely aggregated biomass which is typically attached to a solid surface, the so-called substratum (e.g. gravel, plastic media) (Morgenroth 2008a), but also unattached, spherical forms of biofilms, usually called granular sludge, exist (Khan et al. 2013). The mass flow of a

particular compound in a biofilm can be approached with help of a diffusive/reactive mass balance equation (Eq. 10, Morgenroth 2008a; Wanner and Gujer 1986). It describes the balance between accumulation, diffusive transport and reaction over the biofilm cross section where C_F is concentration, D_F the diffusion coefficient, x the distance from the biofilm surface and r_F the reaction rate for a particular compound in the biofilm.

$$\underbrace{\frac{\partial C_F}{\partial t}}_{accumulation} = \underbrace{D_F \frac{\partial^2 C_F}{\partial x^2}}_{diffusion} - \underbrace{r_F}_{reaction} \quad (10)$$

The simplest types of biofilms consist of only one functional group of organisms, inert biofilm mass and interstitial void spaces. However, wastewater usually provides a range of different substrates (e.g. organics, ammonia) which support multi-species biofilms (Wanner et al. 2006; Wanner and Gujer 1985). Most of the present models describe the biofilm as a rigid, stratified biomass with fast-growing organism in the outermost layers, which are in contact to the bulk liquid where substrate availability is highest and slow-growing organism in deeper biofilm layers, where substrate availability is limited due to diffusion constraints (Morgenroth 2008a; Wanner and Gujer 1985; Wanner and Reichert 1996). However, a one-dimensional, rigid biomass distribution only partially reflects true spatial biomass dynamics. Especially biomass detachment, which typically takes place during periodical events, may impact the three-dimensional biofilm structure. Another important aspect is biomass competition that needs to be better characterized in future biofilm studies (Derlon et al. 2013; Elenter et al. 2007; Horn et al. 2003; Morgenroth and Wilderer 2000).

In systems loaded with organic substrate and ammonia, as typical for domestic wastewater, the outermost biofilm layer is dominated by fast growing heterotrophic bacteria. In the presence of abundant organic substrates, all O_2 is consumed by heterotrophic activity and little nitrifying biomass establishes (Wanner and Gujer 1985). When the availability of organic substrate becomes limiting for heterotrophic activity, the biofilm is substrate limited and oxygen is not completely consumed by heterotrophs. Under these conditions, O_2 is still present in deeper layers where slow growing nitrifying organisms (AOB, NOB) can establish. Biofilms accommodating both heterotrophic and autotrophic organisms are often called heterogeneous biofilms. In biofilm systems receiving little organic substrate, heterotrophs are not able to compensate biomass decay by growth and will therefore only represent a minor fraction of the biomass. This results in so-called autotrophic or nitrifying biofilms which are typically dominated by nitrifiers in aerobic zones (Morgenroth 2008a; Wanner and Gujer 1985) and Anammox in anoxic zones (Jin et al. 2012; Lackner and Horn 2012; Wang et al. 2010). Recently, a novel type of engineered biofilm has been introduced based on membrane aeration, a so-called counter-diffusional biofilm, which is supplied by gaseous substrates (e.g. O_2, CH_4) via a membrane across the substratum (growing surface), resulting in a counter-flow with corresponding gradients of gaseous and liquid components. So far, such systems have only been applied at the experimental scale and are therefore beyond the scope of this review.

3.2 Exploring critical factors leading to N_2O production in biofilms and mathematical modelling

Mathematical modelling is frequently used to explore mass transport limitation resulting in substrate gradients and biomass competition in biofilms (e.g. Elenter et al. 2007; Hao et al. 2001; Wanner and Gujer 1986). In recent time, several attempts have been made to explore N_2O emitting processes within the complex multi-organismal environment of biofilms with help of mathematical modelling (Table 2). All of the models are based on the general mass balance equation (Eq. 10), but they differ considerably in the way metabolic reaction chains are represented and parameterized. Table 2 gives an overview over recent model studies, which are further discussed below.

Model A (Schreiber et al. 2009) focuses on NO and N_2O and considers transformation and transport processes directly related to these N species. Model B (Ni and Yuan 2013; Peng et al. 2016a) describes N conversion in four different groups of organism: AOB, NOB, heterotrophs and Anammox bacteria. AOB and heterotrophs are considered as potential sources for N_2O. Model C (Sabba et al. 2015) focuses on AOB dominated biofilm, assuming AOB being the sole organism in the system. The authors adopted a novel

Table 2 Modelling studies exploring N_2O producing pathways in co-diffusional biofilms

Model reference	Biofilm and mass transport model (software)	Involved organism	N_2O producing pathways	Validation with experimental data
Model A (Schreiber et al. 2009)	1D-biofilm model with static biomass; mass transport adopted from (Wanner and Gujer 1986) (MATLAB)	AOB	Nitrifier denitrification with low O_2 or high NO_2^-	Flow-cell experiments over 1–2 days including profiling of key N-species with micro sensors
			Temporary NO release by HAO with enhanced NO_2^-	
		Heterotrophs (incomplete)	Activation of partial denitrification (NIR, NOR) below 1 μM O_2	
		NOB	None	
Model B (Ni and Yuan 2013b; Peng et al. 2016a)	1D-biofilm model with dynamic biomass mass transport adopted from (Wanner and Gujer 1986) (Aquasim 2.1)	AOB	Nitrifier denitrification under micro-aerobic conditions in the presence of NO_2^-	Pure modelling study based on parameters determined in suspended biomass cultures (Ni et al. 2011). So far no validation with measured data from real biofilm system
		Heterotrophs	Incomplete denitrification on micro-aerobic conditions	
		NOB, Anammox	None	
Model C (Sabba et al. 2015)	1D biofilm model with static biomass (AOB only) (COMSOL)	AOB	Nitrifier denitrification with NO_2^- and reducing equivalents from HAO reaction	Validation with data from a partial nitrification reactor (Pijuan et al. 2014)
			Intermediate (NO) release from HAO with O_2 tension	

model of AOB metabolism recently developed by Ni et al. (2014), in which the particular enzymatic reaction steps are connected via different operational electron carriers. Only Model B includes heterotrophic denitrification as a complete process. Model A only encompasses partial denitrification, i.e. the reduction of NO_2^- via NO to N_2O, which is activated when O_2 falls below 0.3 mg L^{-1}. All modeling studies listed in Table 2 identified AOB as the major source of N_2O, albeit taking different modelling approaches. To gain more insight into these approaches, we implemented the different AOB reaction terms into Aquasim 2.1 (Reichert 1994) and tested them using combinations of different steady-state conditions. NH_3 was kept constant at non-limiting concentration (50 mg L^{-1}), while O_2 was kept constant at three different concentration levels: non-limiting (4 mg O_2 L^{-1}), slightly rate-limiting (1 mg O_2 L^{-1}; given AMO/HAO affinity constants of 126–600 μg O_2 L^{-1}) and rate-limiting (0.2 mg O_2 L^{-1}) (Kester et al. 1997). NO_2^- was set to either 0.2 mg N L^{-1} (background), 5 mg N L^{-1}, simulating NO_2^- peak concentrations in complete nitrifying wastewater treatment system, or 50 mg N L^{-1} as typical for partial nitrification systems.

When NO_2^- was set to low (0.2 mg N L^{-1}), all AOB models predicted greatest N_2O production (relative to NH_3 oxidation) under micro-aerobic conditions (Fig. 3), which is in accordance with findings from pure culture studies (Ch. 2.1.3) and a recent study on mixed AOB biomass taken from a wastewater treatment plant (Peng et al. 2014). However, when increasing O_2 from 1 to 4 mg L^{-1}, Model A predicted a larger decrease in N_2O emission than Models B and C. This is in conflict with incubation studies with *N. europaea,* which did not

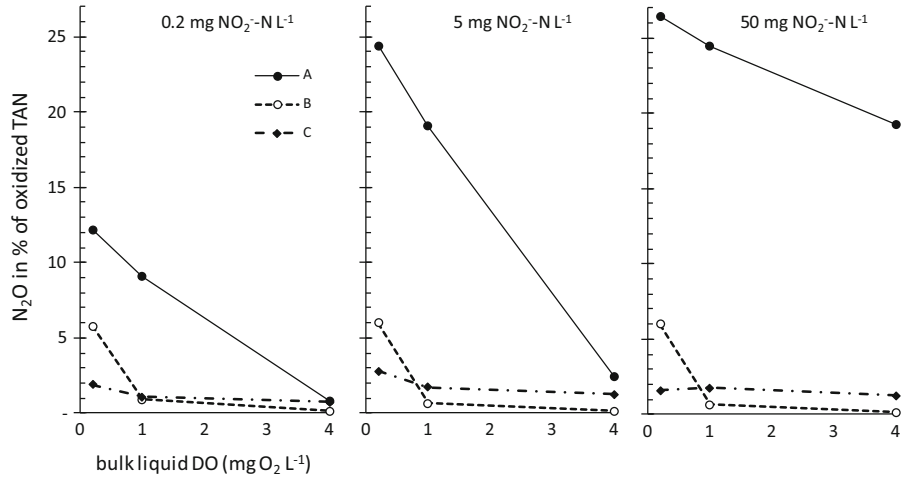

Fig. 3 Production of N_2O relative to NH_3 (TAN) oxidation of an AOB culture on different O_2 and NO_2^- concentration levels simulated with tree metabolic models (A, B, C)

show a significant change in the steady state N_2O emission factor in a DO range of 0.6–4.0 mg O_2 L^{-1} (Kester et al. 1997). Model A may therefore overestimate the impact of O_2 increase above 1 mg O_2 L^{-1} on N_2O emissions. Increasing NO_2^- concentration resulted in distinct, contradictory behavior among the three AOB models, especially under fully oxic conditions (DO > 1 mg L^{-1}). Model A showed a remarkable increase in N_2O production with increasing NO_2^-, which was most pronounced at a DO of 4 mg L^{-1}. Here, an increase of NO_2^- from 0.2 to 50 mg N L^{-1} resulted in a more than one order of magnitude higher N_2O emission factor (Fig. 3). These findings are in accordance with measured N_2O emissions from a fully aerated biofilm where DO was measured to be 1.6 mg O_2 L^{-1} at the substratum and around 5 mg L^{-1} close to the biofilm surface (Schreiber et al. 2009). Model C predicted a decrease of the N_2O emission factor with increasing NO_2^- (Fig. 3), which is a result of the Haldane-type substrate inhibition for NIR implemented in this model (Sabba et al. 2015). Model B calculated a slight increase of the N_2O emission factor when NO_2^- increased from 0.2 to 5 mg N L^{-1}, especially under micro-aerobic conditions, but there was little change in N_2O upon further increase of NO_2^- (Fig. 3). The different responses to NO_2^- shown by the three models reflect the variable effects of NO_2^- on N_2O production found in different AOB cultures (Ch. 2.3) and underlines the need to clarify the mechanism underlying N_2O production by AOB in response to NO_2^-.

None of the models captured the exponential increase of N_2O production with increasing NH_3 oxidation rates as observed in certain wastewater systems (Law et al. 2011). N_2O production and NH_3 oxidation rates were weakly linearly related in Model B ($R^2 = 0.4$) and in Model A ($R^2 = 0.1$), while slightly negatively related in Model C ($R^2 = 0.7$). Model C accumulated large amounts of NH_2OH at a DO of 4 mg L^{-1}, accounting for 27 % of the oxidized NH_3 (molar ratio) at high oxidation rates (Fig. 3), which is accordance to what was found for Model C in the surface-near layer by Sabba et al. (2015). NH_2OH release to medium was reported in a recent study with *N. europaea* (Yu and Chandran 2010) and did not exceeded 0.1 % of the NH_3 consumption. This suggests that model C probably overestimates NH_2OH release.

The applicability of Model A in its present form is limited to explore NO and N_2O production under the specific in vitro conditions used by its developers (Schreiber et al. 2009). Especially the lack of a complete heterotrophic denitrification reaction chain is a serious shortcoming given the frequently reported occurrence of anoxic zones in biofilms, where a reduction of N_2O to N_2 by N_2OR may occur if sufficient electron donors are present (Ch. 2.3). For exploring N_2O production in a wastewater treating biofilm system, Model B and C seem to be more adequate. Model C needs to be expanded to encompass all four main groups of organism as already suggested by its authors (Sabba et al. 2015). Model C is more

complex and therefore more difficult to parameterize, especially with respect to the numerous metabolic intermediates and electron flows that are difficult to determine in a real system. The published parameterisation captures N_2O production well under certain conditions but seems to overestimate NH_2OH accumulation. More work is needed to determine and validate the parameters for a wider set of conditions, ideally in pure culture studies with relevant AOB organism. Model B is based on the relatively well-established activated sludge model ASM (Henze et al. 1987), in which the basic metabolic parameters are well established and validated. Model B seems therefore easier to parameterize and may be at present to most applicable model to explore N conversions associated with N_2O production in biofilms, which is also reflected in the outcome of our model testing (Fig. 3).

3.2.1 *O_2 and its impact on N_2O emissions in different biofilm zones*

As a result of O_2 consumption by microbial activity and diffusive mass transport resistance, aerated biofilms have pronounced O_2 gradients, supporting hyp- or anoxia in deeper layers. These zones support N_2O formation in the presence of NO_2^- or other oxidized N-compounds by AOB (Ch. 2.1.3) and heterotrophic denitrifiers (Ch. 2.3). As shown by microprobes, hypoxic zones (<0.2 mg O_2 L^{-1}) in well aerated biofilms (bulk liquid DO > 4 mg O_2 L^{-1}) are relatively small, accounting for less than 10 % of the aerated biofilm (Okabe et al. 1999; Schramm et al. 1999). Hence, under high aeration conditions, hypoxic N_2O formation is likely small, so that biofilm systems do not a priori have greater N_2O emission factors than suspended biomass cultures. This is also well supported by several biofilm studies listed in Table 3, reporting N_2O emission factors comparable to those of well-functioning suspended biomass systems (Kampschreur et al. 2008). However, the hypoxic fraction of the aerated biofilm increases with decreasing bulk liquid DO. Modelled O_2 profiles for biofilms with a bulk liquid DO of 1 mg O_2 L^{-1} (Sabba et al. 2015) and 0.3 mg O_2 L^{-1} (Ni and Yuan 2013) showed hypoxic biofilm fractions of 50 and 75 %, respectively. Moreover, at low DO (<0.5 mg L^{-1}), the stimulating effect of NO_2^- accumulation on N_2O production by AOB seems to be most pronounced (Peng et al. 2015a). Recent studies using Models B and C (Ni and Yuan 2013; Sabba et al. 2015) clearly indicate that poorly aerated biofilms tend to produce more N_2O, especially in the presence of NO_2^- or NH_2OH. The latter is a result of complex interactions between different organisms, which in turn depend on O_2 availability. This is discussed in more detail in chapter 3.2.2. Together with NO_2^- and NH_2OH accumulation, bulk liquid DO is a key factor for controlling N_2O emissions in biofilm-based wastewater treatment plants (e.g. Lochmatter et al. 2013; Peng et al. 2016a; Sabba et al. 2015).

A recent in vitro study (Schreiber et al. 2009) showed that decreasing the O_2 concentration across a biofilm induces remarkable transient N_2O accumulation, especially in the boundary zones between hypoxia and anoxia. With help of Model A, the authors attributed these N_2O emission peaks to a temporary metabolic imbalance of AOB, which resulted in N_2O production from NO release by HAO (Ch. 2.1.2). In praxis, such N_2O peaks occur when the composition of the wastewater changes, but also under batch operation conditions. Frequent changes in the DO profile may also be an explanation for the observed inverse relationship between N_2O production and RPM in a rotating disk reactor (Rezić et al. 2007).

Microprobe studies (Okabe et al. 1999; Schramm et al. 1999; Schreiber et al. 2009) of biofilms in an O_2 saturated bulk liquid (DO 7.5–8.5 mg O_2 L^{-1}) showed that the O_2 concentration at the biofilm surface is notably lower (1.9–5.7 mg L^{-1}) than in the bulk liquid due to the external boundary layer mass transport resistance. Given Monod terms with a typical O_2 affinity constant of 0.6 mg O_2 L^{-1} (Brockmann et al. 2008), O_2 availability in the biofilm surface limits NH_3 oxidation rates to 75–90 % of the maximum reaction rates for suspended biomass. The modelling studies listed in Table 3 captured the rate-limiting effect caused by O_2 mass transport resistance within the biofilm. However, they do not take into account the additional mass transport resistance across the boundary layer, which seems to be partially controlled by TAN flux (Fig. 4). This negative feedback interaction between O_2 consumption by AOB and O_2 availability in the biofilm likely limits the maximum possible NH_3 oxidation rate in a biofilm. Therefore, we believe that the frequently cited N_2O formation from AOB intermediates released during high reaction rates (Ch. 2.1.3) plays only a minor role

for N2O production in biofilms. This is supported by a recent study (Lochmatter et al. 2013) which found highest N_2O production rates in a biofilm at low DO (1 mg O_2 L^{-1}), coinciding with accumulation of NO_2^-, but not at high DO (5 mg O_2 L^{-1}) and accordingly higher NH_4^+ flux with almost quantitative conversion of NH_3 to NO_3^-.

3.2.2 *Accumulation of NO_2^-*

Nitrite, as a highly reactive and toxic N oxyanion, which is central to the metabolism of both AOB (Ch. 2.1.3) and denitrifiers (Ch. 2.3), plays an important role in N_2O formation by biofilms. A biofilm controls NO_2^- accumulation, dependent on biomass composition, substrate load and O_2 availability. In a balanced suspended biomass reactor supporting both AOB and NOB, NO_2^- accumulation is usually small, in the range of 0–0.2 mg N L^{-1} (Peng et al. 2014). However, NO_2^- has been reported to accumulate in nitrifying reactors when DO in bulk liquid falls below 2 mg O_2 L^{-1} (Brockmann et al. 2008; Fux et al. 2004a; Sliekers et al. 2005). This is commonly attributed to an imbalance between NO_2^- production by AOB and consumption by NOB owing the higher affinity of AMO for O_2 as compared with NXR. This imbalance is utilized in partial nitrification reactors for producing NO_2^- for Anammox based N removal (Ciudad et al. 2006; Hellinga et al. 1998; Sliekers et al. 2005). Recent cross-section studies of biomass distribution in nitrifying biofilm systems (Okabe et al. 1999; Schramm et al. 1999) found the highest NOB abundance in deeper zones with less O_2 availability. The study of Okabe et al. (1999) showed that these NOB clusters were dominated by *Nitrospira sp.*, while the faster growing NOB species *Nitrobacter sp.* were almost absent. Hence, *Nitrospira sp.* seems to be better adapted to oxygen limiting conditions than *Nitrobacter sp*. This may be related to the presence of *Nitrospira*-like "complete nitrifiers" in deeper biofilm layers, which have been recently shown to thrive in substrate-influx, oxygen limited zones (Daims et al. 2015). These newly discovered, slow-growing *Nitrospira* species are able to oxidize NH_4^+ all the way to NO_3^- at oxygen concentrations as low as 0.1 mg O_2 L^{-1}, thereby counteracting NO_2^- accumulation in deeper biofilm layers (van Kessel et al. 2015). Hence, decreased O_2 availability does not necessarily lead to critical NO_2^- accumulation under steady state conditions, especially when other NOB inhibiting factors such as high NH_3 concentrations are absent. This is also illustrated by reported difficulties to limit the occurrence of NOB in one-stage partial-nitrification-Anammox reactors via DO as the only controlling parameter (Fux et al. 2004a).

Anoxic biofilm zones can act as a sink for NO_2^- through activity of heterotrophic denitrifies or Anammox bacteria. In Anammox systems, NO_2^- production by AOB and NO_2^- consumption by Anammox can be kept in balance by controlling DO and ammonia loading rates (Hao et al. 2001; Peng et al. 2016b). In such well-balanced Anammox systems (Table 3), NO_2^- in the bulk liquid has been reported to be below 0.5 mg N L^{-1} (e.g.Christensson et al. 2013; Yang et al. 2013). In systems without Anammox, heterotrophic denitrifieres may reduce NO_2^- in anoxic zones of nitrifying biofilms. However, the activity of denitrifiers is strongly limited by the electron donor availability (Kindaichi et al. 2004a; Rittmann et al. 1994). Hence, the reported co-existence of heterotrophs in nitrifying biofilms (Okabe et al. 1999; Schramm et al. 1999) plays likely only a minor role in NO_2^- accumulation.

3.2.3 *pH and inorganic carbon*

In biofilms, low pH may lead to enhanced N_2O production by causing nitrosative stress for AOB (Ch. 2.1.3) and heterotrophic denitrifiers (Ch. 2.3) through protonation of NO_2^- to HNO_2. HNO_2 is also a strong inhibitor for NOB, which again enhances accumulation of NO_2^- (Ch. 2.1.3). Metabolic activity consumes or produces protons, which results in marked pH gradients in biofilms. In aerated zones, protons are produced by nitrification (Eq. 1, Ch. 2.1.1) and during dissolution of CO_2 produced by respiration. In anoxic biofilm zones, acidification by CO_2 dissolution may be driven by heterotrophic denitrification. In aerated suspended biomass cultures, CO_2 does usually not affect the pH significantly, as it is continuously stripped out. In biofilm systems however, CO_2 accumulates due to mass transport limitation, which can lead to measurable pH decrease (Xiao et al. 2013). Heterotrophic denitrification (Eqs. 3, 4, Ch. 2.3), but also Anammox (Eq. 5, Ch. 2.4) consume protons, thus counteracting pH decrease in anoxic zones. Yet, micro-probe studies have demonstrated strong pH gradients towards acidic conditions in deeper biofilm

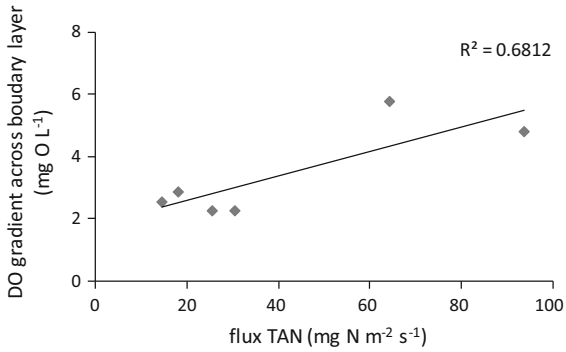

Fig. 4 Difference between O_2 concentration in bulk liquid and biofilm surface expressed as boundary layer gradient in relation to the flux of total ammonia nitrogen (TAN) determined by microprobe studies. The bulk liquid DO was in a range of 7.5–8.5 mg L^{-1} in all of the studies (Okabe et al. 1999; Schramm et al. 1999; Schreiber et al. 2009)

zones in completely anoxic biofilms with high heterotrophic activity (Xiao et al. 2013). This suggests that the proton release from the dissolution of CO_2 exceeds proton consumption by dissimilatory NO_3^- and NO_2^- reduction. In biofilms dominated by autotrophic organism, CO_2 may be consumed at high rates and become limiting, especially for Anammox bacteria which have a lower affinity for inorganic carbon than AOB or NOB (Ma et al. 2015). Hence, depletion of inorganic carbon in autotrophic biofilms would favour AOB and NOB and thus increase N_2O production from increasing NO_2^- due to lower Anammox activity. In suspended biomass cultures, CO_2 limitation was shown to reduce N_2O emissions from intermediates released by AOB at high reaction rates (Peng et al. 2015b). In biofilms, this effect is unlikely to occur because of O_2 limitation, which impedes AOB reaction rates regardless of the availability of inorganic carbon (Ch. 3.2.2).

3.2.4 Accumulation of AOB intermediates

Several studied have suggested that high AOB activity goes along with release of intermediates (NO, HN_2OH) conductive to N_2O formation (Ch. 2.2.2). In biofilms, transient NO accumulation was detected in hypoxic and anoxic zones upon decrease of O_2 or increase of NO_2^- (Schreiber et al. 2009). NH_2OH on the other hand, was proposed to accumulate in aerated zones as a result of higher AOB reaction rates (Sabba et al. 2015). By applying Model C (Table 2) to a well-aerated nitrifying biofilm, Sabba et al. (2015) simulated N_2O production by nitrifier denitrification driven by excessive NH_2OH release into hypoxic or anoxic zones and proposed that this could be one of the major N_2O producing pathways in a well aerated, AOB dominated biofilms. However, as discussed earlier, the O_2 mass transport limitation likely limits AOB activity (Ch. 3.2.1) so that extracellular NH_2OH accumulation in a ratio of 1:2 in relation to produced NO_2^- as proposed by Model C (Ch. 3.2) seems unlikely.

The presence of extracellular NH_2OH (>3 mg N L^{-1}) may further induce biomass detachment from biofilms as indicated by recent studies with *Nitrosonomas sp.*, which observed a change in growth pattern from dense, spherical micro-colonies to single scattered cells (Harper et al. 2009; Kindaichi et al. 2004b). However, the NH_2OH dosage applied by these studies (3.5–40 mg N L^{-1}) is several orders of magnitudes higher than bulk liquid concentration simulated by Sabba et al. (2015) or measured by a recent study (Yu and Chandran 2010). NH_2OH is difficult to measure and is therefore little investigated. Irrespective the uncertainties about magnitude and fate of NH_2OH released by AOB, N_2O production from extracellular NH_2OH needs further investigation.

4 Reported N_2O emissions in different types of wastewater treating biofilm systems

To give an overview of published N_2O emission data from biofilm systems, a search was performed with the keywords "N_2O" and "biofilm" in Web of Science (Thomson Reuters, New York). By December 2015, the search yielded 59 studies comprising both natural and artificial biofilm systems. The results were screened for studies of wastewater treating biofilm systems that provide data on N_2O emissions under different operational conditions (Table 3). The N_2O emission factor (N_2O–N in % of biologically transformed N) was calculated from the data if not provided by the authors. Studies for which a calculation of the emission factor was not possible were omitted.

The results of the literature survey are summarized in Table 3. N_2O emission factors vary greatly from 0.2 to 57 % N_2O–N emitted per unit biologically transformed N. The main findings from this compilation can be summarized as follows: (a) both aerobic and anaerobic reactor conditions allow for high N_2O

Table 3 Reported N_2O emission from biofilm systems under different loading regimes and system parameters

Reference	Type of system	Dominating organisms	Aeration and DO	Nitrite (mg N L^{-1})	N_2O emission factor
Todt and Dörsch (2015)	MBBR	AOB, NOB, heterotrophs	Continuous (3–5 mg O_2 L^{-1})	0.1–220	0.7–8.5 % (N_2O peaks coincided with NO_2^- accumulation peaks)
Ma et al. (2015)	MBBR	AOB, Anammox	Continuous (1.7 mg O_2 L^{-1})	1.5	0.5–1.5 %
Eldyasti et al. (2014)	Fluidized bed	Heterotrophs	None (anoxic reactor)	0.1–0.4 (with COD/N 5)	0.6–1.1 % (with COD/N = 5)
				0.7 with COD/N 3.5	1.9 % (with COD/N = 3.5)
Lochmatter et al. (2013)	Granular sludge	AOB, NOB, heterotrophs	Intermittent (short cycles) (5.5 mg O_2 L^{-1})	1–4	1–9 % (inverse relationship to COD load)
Yang et al. (2013)	MBBR	AOB Anammox	Intermittent (1.5 mg O_2 L^{-1})	6–7	0.5–1.3 %
			Continuous (1.5 mg O_2 L^{-1})	4–7	0.7–1.3 %
Kong et al. (2013)	MBBR	AOB	Intermittent (1–2 mg O_2 L^{-1})	200–250	2.7 %
Christensson et al. (2013)	MBBR	AOB Anammox	Continuous (0.5–1.5 mg O_2 L^{-1})	0–5	0.1–0.8 % at DO < 1 mg O_2 L^{-1}
					0.8–2.2 % at DO > 1 mg O_2 L^{-1}
Lo et al. (2010)	IFAS (MBBR)	AOB, NOB, heterotrophs	Intermittent (2–5 mg O_2 L^{-1})	<1	27 % (21 % of N load)
Campos et al. (2009)	BAS	AOB, NOB, heterotrophs	Anoxic batch incubation of biofilm from aerated reactor	0 (e.a. NO_3^-)	0.3–1.2 % (with NO_3^- as e.a.)
				100	52–57 % (with NO_2^- as e.a.)
Zhou et al. (2008a)	Granular sludge	AOB, NOB, heterotrophs (PAO)	Anoxic batch incubation from aerated reactor	0.5 (low)	1.2–1.4 % at low NO_2^-
				30 (high)	40–55 % at high NO_2^-
Rezić et al. (2007)	Rotating tube	Heterotrophs (pure culture study with *P. denitrificans*)	Rotation on different speeds (RPM) (DO n.a.)	n.a.	3–25 % with inverse relationship to RPM
Bougard et al. (2006)	Fluidized bed	AOB, NOB	Continuous (DO n.a.)	1600	6 % (with high NO_2^-)
				0–500	9 % (with low NO_2^-)
Park et al. (2000)	Aerated biofilm filter	AOB, NOB, heterotrophs	Intermitted DO during aerobic phase 0.2 mg O_2 L^{-1}	<0.1	3 % without addition of e.d. substrate
					0.2 % with addition of e.d. substrate

The N_2O emission factor refers to the emitted N_2O in pct. of biologically transformed N (in N equivalents). Some studies provide the emission factor in pct. of inlet load, or removed N (which is not considering transformed N remaining in the reactor liquid, e.g. NO_2^-). For these studies, the emission factor in pct. of transformed N was calculated or estimated based on the provided data

MBBR moving bed biofilm reactor, *IFAS* integrated fixed-film activated sludge, *BAS* biologically aerated filter, *n.a.* not available, *e.a.* electron acceptor

emission factors; (b) heterogeneous biofilm systems fed with both organics and ammonia substrates tend to have higher N_2O emission factors than autotrophic biofilms fed with ammonia alone; (c) under anoxic conditions, large N_2O emissions are clearly linked to elevated NO_2^- concentrations, which is not the case for aerobic conditions. No clear tendency was found for different aeration regimes and DO levels, but there is some indication that intermitting aeration or batch operation results in greater N_2O emission factors than operation at constant DO.

4.1 N_2O production in aerated, heterogeneous biofilms loaded with organic substrates and ammonia

Table 3 suggests that N_2O emissions are greater in systems with heterogeneous biofilms receiving both organic substrates and ammonia than in nitrifying biofilms loaded with ammonia alone. This notion is supported by three studies which explored the effect of organic load on heterogeneous biofilms in more detail and showed that the addition of organic substrate increased N_2O emission especially at high DO levels (>1 mg L^{-1}) (Campos et al. 2009; Lochmatter et al. 2013; Todt and Dörsch 2015). So far, N_2O producing mechanisms within the complex framework of interacting heterotrophic and autotrophic biomass in aerated heterogeneous biofilms have been little investigated. In stratified biomass, readily degradable organic substrates control the O_2 consumption by heterotrophic activity and thus O_2 availability in deeper biofilm zones, were nitrifiers are located (Ch. 3.1). Increase in organic load will therefore decrease O_2 availability for nitrifiers and reduce the nitrification activity, but at the same time may increase NO_2^- accumulation due to the lower O_2 affinity of NOB (Ch. 3.2.2). Transient shifts in O_2 availability across the biofilm (Ch. 3.2.1) are more frequent in systems loaded with organic substrate.

Another aspect affecting N_2O emissions in heterogeneous biofilms is the size-dependent penetration depths and degradability of organic substrates. A typical wastewater contains both slowly and readily degradable organics, which to some extent correlate with molecule size (Dulekgurgen et al. 2006; Henze and Comeau 2008). Readily degradable substrates usually refer to low-molecular organic matter (e.g. volatile fatty acids), while slow-degradable substrates refer to high-molecular, colloidal or particulate organic matter. In some types of wastewater, slow-degradable organic substrates may also consist of low-molecular compounds such as humic acids (Dulekgurgen et al. 2006). With these slow-degradable substrates, the growth rate of heterotrophic organisms is too small to outcompete nitrifiers (Jin et al. 2012; Lackner and Horn 2012; Wang et al. 2010). The co-occurrence of substrate limitation and nitrification therefore depends on the presence of readily degradable compounds. If present at high concentrations, slow-degradable, low molecular substrates penetrate deeper into anoxic biofilm zones where they may fuel incomplete heterotrophic denitrification and produce N_2O (Todt and Dörsch 2015). Heterogeneous biofilms are little studied for N_2O producing processes and more research is needed to assess the effect of organic molecule size and distribution on N_2O production.

4.2 N_2O production in aerobic biofilms predominately loaded with ammonia

A combination of partial nitrification (also called nitritation) and anaerobic ammonia oxidation by Anammox bacteria has gained popularity for biological N-removal, especially for treatment of wastewaters with high ammonia content (e.g. Fux et al. 2004b; Jetten et al. 1998; Kampschreur et al. 2006; Yang et al. 2013). In biofilm systems, nitritation and Anammox can be run in a single reactor by combining AOB activity in aerobic zones with Anammox bacteria in anoxic zones (Christensson et al. 2013; Fux et al. 2004b; Ma et al. 2015; Yang et al. 2013). N removal based on one-stage partial-nitrification-Anammox seems to produce less N_2O than traditional nitrification/denitrification-based N-removal (Table 3). This is due to the efficient conversion of nitrite to N_2 in systems harbouring Anammox, which keeps NO_2^- concentrations low. This can be achieved by keeping the NO_2^- production rate of AOB below the NO_2^- consumption rate of *Anammox* by actively controlling DO (Cema et al. 2011; Christensson et al. 2013; Peng et al. 2016a) and/or the TAN bulk concentration (Peng et al. 2016a). Modelling studies indicate that high N removal efficiency with low N_2O emissions can be achieved at a DO around 0.5 mg L^{-1} and TAN < 50 mg N L^{-1}. A higher DO up to 1 mg L^{-1} together with a larger TAN concentration result in greater AOB activity and NO_2^- accumulation. If DO

exceeds 2 mg L^{-1}, N_2O production declines, however N removal efficiency declines as well due to increased presence of NOB and production of NO_3^- (Peng et al. 2016a).

Another critical factor for N_2O emissions in autotrophic biofilms may be the temporal presence of organic substrates fuelling heterotrophic denitrifiers. Organic substrates can originate from the inlet, but also from endogenous biomass decay (Ma et al. 2015). Table 3 suggests that the occurrence of heterotrophic denitrifiers in anoxic biofilm zones increases N_2O production, especially in the presence of NO_2^- and/or slowly degradable substrates (Ch. 3.4). The competition between heterotrophs and Anammox bacteria in anoxic biofilm zones is determined by the concentration of organic substrates relative to the substrate affinity constant of heterotrophs, but also by the ratio of volatile fatty acids (VFA) to N. Anammox bacteria use VFA as electron donors for their organotrophic metabolism (Güven et al. 2005; Winkler et al. 2012a) and it is assumed that Anammox bacteria outcompete heterotrophs for VFA as long as NH_4^+ is present in ample amounts (Kartal et al. 2007b), With VFA/N ratios (expressed as COD/N) lower than 0.5 g O_2 g N^{-1}, Anammox bacteria were shown to outcompete heterotrophic bacteria (Winkler et al. 2012b). The extent of N_2O production by heterotrophic denitrifiers in Anammox systems may therefore not only depend on the availability, but also on the type of organic substrates present. In autotrophic biofilms, VFA are likely short-lived, since continuous presence of these readily degradable substrates would support heterotrophic organisms in the aerobic surface-near biofilm layers and transform the biofilm into a heterogeneous biofilm.

4.3 N_2O emissions from anoxic biofilms loaded with organic matter and NO_2^- or NO_3^-

Two research groups studied N_2O emissions from heterotrophic denitrifying biofilms under anoxic conditions (Campos et al. 2009; Zhou et al. 2011, see also Table 3). Heterotrophic degradation of organic matter produces CO_2 regardless whether O_2 or NO_x^- is used as electron acceptor. CO_2 may accumulate, resulting in a measurable pH drop as shown by a recent microprobe study (Ch. 3.2.3). As the pH in the inner, anoxic biofilm layers fell below 6.8, heterotrophic denitrification appeared to become incomplete resulting in greater N_2O concentrations within the biofilm (Xiao et al. 2013). This can be attributed to direct impairment of N_2OR by low pH itself and/or HNO_2 (Ch. 2.3). A critical concentration of HNO_2 was likely reached in the study of Campos et al. (2009) when supplying the biofilm system with NO_2^- as electron acceptor. This would explain the more than one order of magnitude greater N_2O emissions compared to a system fed with NO_3^- (Campos et al. 2009). Thus, N_2O formation in anoxic biofilm systems seems to be mainly controlled by NO_2^- concentration levels, acidification by CO_2 production and associated HNO_2 formation.

5 Summary

The following key controls for N_2O production in biofilms were identified:

- N_2O formation occurs mainly in hypoxic or anaerobic zones where low O_2 tensions trigger production via nitrifier denitrification or incomplete heterotrophic denitrification, especially in the presence of NO_2^- and other intermediates released from nitrification activity in aerated zones.
- NO_2^- accumulation is a key factor for N_2O formation in biofilms. It is controlled by complex metabolic interactions between different types of organisms, which are controlled by O_2 and substrate availability along the biofilm cross section.
- Heterogeneous biofilm systems loaded with mixed wastewater seem to be particularly conductive to N_2O formation, as frequent shifts in the O_2 profile occur with varying loads of organic substrate and TAN which affect O_2 consumption by heterotrophs and AOB.
- Mathematical models are useful tools to explore N_2O emissions in biofilms, but there are knowledge gaps about the N_2O producing metabolism, especially of AOB, and consequently about the factors controlling N_2O formation by AOB.

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