The pH dependency of N-converting enzymatic processes, pathways and microbes: effect on net N2O production

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The pH dependency of N-converting enzymatic processes, pathways and microbes: effect on net-N₂O production

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Summary
Nitrous oxide (N₂O) is emitted during microbiological nitrogen (N) conversion processes, when N₂O production exceeds N₂O consumption. The magnitude of N₂O production vs consumption varies with pH and controlling net-N₂O production might be feasible by choice of system pH. This article reviews how pH affects enzymes, pathways and microorganisms that are involved in N-conversions in water engineering applications. At a molecular level, pH affects activity of co-factors and structural elements of relevant enzymes, by protonation or deprotonation of amino acid residues or solvent ligands causing steric changes in catalytic sites or proton/electron transfer routes that alter catalytic activity. Augmenting molecular information with, e.g. nitritation or denitrification rates, yields explanations of net-N₂O production with pH changes. Ammonia oxidizing
bacteria are of highest relevance for N₂O production, while heterotrophic denitrifiers are relevant for N₂O consumption at pH > 7.5. Net-N₂O production in N-cycling water engineering systems is predicted to display a “bell-shaped” curve in the range of pH 6.0-9.0 with a maximum at pH 7.0-7.5. Net N₂O production at acidic pH is dominated by N₂O production, whereas N₂O consumption can outweigh production at alkaline pH. Thus, pH 8.0 may be a favorable pH set-point for water treatment applications regarding net N₂O production.
1. Introduction

Emissions of nitrous oxide (N₂O) to the atmosphere are of concern, as N₂O is a greenhouse gas with a large global warming potential and ozone depleting properties (Ravishankara et al., 2009; IPCC, 2013). As a result of an increased load of reactive nitrogen (N) to the biological N-cycle by anthropogenic activities, emissions of N₂O are increasing (Canfield et al., 2010; Steffen et al., 2015). Especially agricultural soils and engineered nitrogen removal systems, like constructed wetlands or urban wastewater treatment plants (WWTPs), constitute hubs of N₂O emissions (Canfield et al., 2010; Law et al., 2012). Nitrogen is introduced to these systems in its most reduced form, i.e. as ammonium (NH₄⁺) or organic nitrogen (which is readily converted to ammonium via ammonification). Whilst in agriculture the assimilation of NH₄⁺-N into biomass is the primary objective, water treatment applications seek to convert NH₄⁺ into environmentally inert dinitrogen gas (N₂). In all systems the loss of nitrogen in the form of N₂O is significant and strategies to mitigate N₂O emissions are sought after (Hénault et al., 2012; Law et al., 2012).

Various prokaryotes harbor the energy associated with conversion of N-species between different redox states. The biological N-network hosts a multitude of conversion reactions, which are catalyzed by a variety of enzymes (Fig. 1). N₂O constitutes the end product of a number of metabolic pathways or is an intermediate towards N₂. As, in principle, both N₂O production and consumption reactions exist, net-N₂O production occurs as the result of non-ideal flow of N-species through the N-network (Stein, 2010). Hence, the key to lower N₂O emissions are overall pathways that prevent the accumulation of N₂O. pH is one of the parameters that affect conversion rates of enzymes (Illanes et al., 2008). The enzymes involved in the N-network have different pH optima and pH may cause imbalances.
between enzymatic reaction steps that lead to the accumulation of intermediates, such as hydroxylamine (NH$_2$OH), nitrite (NO$_2$), nitric oxide (NO) or N$_2$O. Conversely, setting pH may offer an opportunity to synchronize N-conversion rates to reduce accumulation of N$_2$O. A better understanding of the effect of pH on the N-network can support decision making on pH set-point management to lower N$_2$O emissions from soils and engineered N-removal systems.

**Figure 1**

This article reviews how the pH affects enzymes, pathways and microbes that are involved in the N-network and which are relevant for net-N$_2$O production. It further explores, if pH optima of individual enzymes hold sufficient information to hypothesize favorable pH set-points for lower net-N$_2$O production. Written with the background of biological N-removal during wastewater treatment processes, most examples are taken from WWTPs. However, as the same microbial pathways are active in other environments, findings may be relevant for fields outside of wastewater treatment. Microorganisms in WWTPs are usually not exposed to environmental pH values outside the range pH 6.0-9.0 (Henze and Comeau, 2008). Therefore this article focuses on effects of pH in this range.

Summarizing, the effect of pH is complex, partly because of the sheer number of enzymes, pathways and organisms involved in N-conversion, but also because pH affects various central processes in cells, signaling or transcriptional and post-transcriptional phenomena. Accordingly, the attempt to infer optimal pH set-points for complex microbial communities and their N-conversion processes with respect to N$_2$O is ambitious. Yet, the demand for N$_2$O mitigation strategies and guidelines, especially in water engineering applications, justifies an effort, even though simplifications and constraints are unavoidable. Because
such applications often involve well-controlled systems with relatively steady conditions (bioreactors), compared to rapidly changing environments of natural systems, it is plausible that pH control strategies may constitute a feasible tool to manage N₂O net production.

1.1. The effect of pH on enzymatic conversion rates

The conversion rate of an enzyme \(v = \text{k}_{\text{cat}}[E]_0[S]/(\text{K}_M + [S])\) is affected by pH either by changes of the turnover number \(\text{k}_{\text{cat}}\), the substrate concentration \([S]\) or both (Illanes et al., 2008). During nitrogen removal, pH governs the speciation of the acid/base pairs \(\text{NH}_4^+/\text{NH}_3\) (pKa=9.3) and \(\text{HNO}_2/\text{NO}_2^-\) (pKa=3.4) (Nelson and Cox, 2005). Substrate speciation becomes relevant, when it changes the availability of substrate for enzymatic reactions or causes inhibition. \(\text{k}_{\text{cat}}\) is affected by pH, when changes of the enzymatic structure reduce the catalytic activity of enzymes (Illanes et al., 2008). Based on the interplay of \(\text{k}_{\text{cat}}\) and the substrate concentration, pH set-points may be used to manage one or the other in order to control enzymatic conversion rates.

1.2. The relevance of the cellular location of enzymes for an effect of changes in ambient pH: periplasmic pH and cytoplasmic pH homeostasis in bacteria and archaea

To manage \(\text{k}_{\text{cat}}\) by pH set-points, the enzymes need to be accessible for changes of ambient pH. However, changes of pH in the environment of a cell do not directly transfer into changes of pH throughout the cell, but affect cellular compartments differently (Slonczewski et al., 2009). Hence, the effect of ambient pH on the activity of an enzyme depends on its cellular location (Fig. 2). Bacteria and archaea depend on a stable pH in their cytoplasm for their metabolic machinery to operate efficiently and the proton motive force to generate chemical energy reliably (Booth, 1985). Microorganisms have developed
regulation mechanisms to maintain a near-constant cytoplasmic pH in environments with substantially different ambient pH values (Booth, 1985; Slonczewski, 2009; Krulwich, 2011). Most neutrophilic bacteria maintain their cytoplasmic pH stably at 7.5-7.7 against a pH in the environment ranging from 5.5 to 9.0 (Pedan, 2005; Slonczewski, 2009). Accordingly, a change of ambient pH in the range of pH 6.0-9.0, as present in WWTPs, would not transfer into changes of pH in the cytoplasm. Consequently, enzymes that are localized in the cytoplasm or other intra-cell compartments would not be directly affected by changes of pH in the environment (Slonczewski et al., 2009; van der Star et al., 2010). In contrast, the pH of the periplasm of gram-negative bacteria is not maintained and follows the pH of the environment (Wilks, 2007).

**Figure 2**

**2. Physiological pH values of relevant prokaryotes**

Physiological pH optima of microorganism combine various effects of pH on the organisms, e.g. the effect of pH on individual enzymes, the speciation of substrates and gene expression. Considerations of potential pH set-points for e.g. water treatment systems are confined by physiologically feasible pH values of microorganisms. In the following, relevant microorganisms of the N-network are introduced and physiological pH optima are summarized.

**Figure 3**

**Aerobic ammonia oxidizing bacteria**

Aerobic ammonia oxidizing bacteria (AOB) are chemolithoautotrophs that oxidize NH$_3$ to NO$_2^-$ via NH$_2$OH, as well as NO, which has recently been recognized as another obligate
intermediate during nitritation (Caranto and Lancaster, 2017). AOB are also able to reduce 
NO$_2^-$ to N$_2$O by the nitrifier denitrification pathway (Wrage et al., 2001). The enzymes 
involved are ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), 
nitrite reductase (NIR) and nitric oxide reductase (NOR) (Schreiber et al., 2012). Nitritation 
and nitrifier denitrification play central roles in WWTPs, also in respect to N$_2$O production 
(Stein, 2011). AOB found in WWTP mainly affiliate with *Nitrosomonas*, *Nitrosospira*, 
*Nitrosovibrio* and *Nitrosococcus* genera, with *Nitrosomonas* and *Nitrosospira* as the most 
dominant AOB (Kowalchuk and Stephen, 2001). In general, AOB in WWTPs can grow 
across a wide range of pH with optima at pH 7.4-8.2 (Grunditz and Dalhammar, 2001; 
Park et al., 2007; Claros et al., 2013; Daalkhaijav and Nemati, 2014). Bacterial amoA, 
which is commonly used as a molecular marker for AOB, showed low abundance under 
acidic conditions (pH < 5.5) (De Boer and Kowalchuk, 2001) and increased with increasing 
pH (Leininger et al., 2006; Nicol et al., 2008).

**Ammonia oxidizing archaea**

Ammonia oxidizing archaea (AOA) perform autotrophic ammonia oxidation similar, but not 
identical to AOB (Kozlowski et al., 2016); no HAO or NOR genes have been identified in 
pure or enrichment cultures of AOA, indicating that they might be unable to produce N$_2$O 
enzymatically through side reactions of ammonia oxidation or nitrifier denitrification 
(Walker et al., 2010; Tourna et al., 2011; Spang et al., 2012; Stieglmeier et al., 2014). AOA 
are widespread in nitrifying bioreactors in WWTPs or biofilters for drinking water 
production and are also found in soil and sediments (Park et al., 2006; Zhang et al., 2009; 
Gülay et al., 2014). Optimal growth of AOA has been reported in the range of pH 7-7.5 
(French et al., 2012; Spang et al., 2012). Nicol et al. (2008) observed that transcriptional 
abundance and expression of archaeal amoA, a widely used molecular marker of AOA,
decreased with increasing pH. Yet, archaeal amoA was found in environments with a wide range of pH (pH 3.7 to 8.65) (Erguder et al., 2009). AOA have been frequently found to outnumber AOB and dominate in abundance and ammonia oxidizing activity, especially in acidic soils (Nicol et al., 2008; Gubry-Rangin et al., 2010; Zhang et al., 2012; Li et al., 2018).

Nitrite oxidizing bacteria

Nitrite oxidizing bacteria (NOB) are aerobic chemolithoautotrophs that oxidize NO$_2^-$ to nitrate (NO$_3^-$). The reaction is catalyzed by the enzyme nitrite oxidoreductase (NXR) (Schreiber et al., 2012). NOB have been found in several genera distributed among different bacterial lineages, i.e. *Nitrobacter*, *Nitricoccus*, *Nitrispina*, *Nitrispira* and *Nitrotoga*. Even though the genus *Nitrospira* is often the numerically dominant NOB in WWTPs (Daims et al., 2001), virtually all knowledge of the physiology and biochemistry of NO$_2^-$ oxidation has been derived from studies of a limited number of strains of *Nitrobacter* species (Daims et al., 2016). The activity of NOB in WWTPs was found to be pH-dependent with optima at pH 7.9 ± 0.4 (Park et al., 2007). More specifically, the optimum growth of isolated pure cultures of *Nitrospira* and *Nitrobacter* species occurred at pH 7.6-8.0 (Ehrich et al., 1995) and 7.9 (Grunditz and Dalhammar, 2001), respectively.

Complete ammonia oxidizing bacteria

Complete ammonia oxidizing bacteria (Comammox) are able to oxidize NH$_3$ to NO$_3^-$ (Daims et al., 2015; van Kessel et al., 2015). Besides NXR, *Nitrospira* sp. harbor the enzymes needed to oxidize ammonia to nitrite (AMO and HAO), which differ from the canonical enzymes utilized by proteobacterial AOB (e.g., Palomo et al (2016), reported only 56% similarity between amino acid sequence for amoA from *Nitrosomonas* and
Nitrospira sp.). AMO and NXR are membrane bound, whilst HAO is located in the periplasm (Daims et al., 2015). It is still to be determined, if an effect of pH on these enzymes is similar to its effect on canonical AOB enzymes. Nevertheless, some studies already demonstrate that pH has an impact on niche differentiation between different microorganisms capable of ammonia oxidation in soils (Hu and He, 2017). NirK, the gene for Cu-NIR, is also present in comammox, but no evidence of NO accumulation in comammox enrichments cultures suggests that nitrite reduction may not be particularly robust (Daims et al., 2015). Comammox have been found in different water engineered systems, including WWTPs (Chao et al., 2016; Gonzalez-Martinez et al., 2016; Pjevac et al., 2017). However, their abundance in wastewater treatment plants is lower than AOB, which is likely due to the competitive advantage of AOB in systems with high substrate concentrations (Costa et al., 2006; Kits et al., 2017). In general, further research is needed to understand the role of comammox in the nitrification process in full scale WWTPs.

Anaerobic ammonia oxidizing bacteria

Anaerobic ammonia oxidation (anammox) is performed by anaerobic ammonia oxidizing bacteria (AnAOB). Anammox is catalyzed by the sequential activity of the enzymes NIR, hydrazine synthase (HZS) and hydrazine dehydrogenase (HDH) (Kartal et al., 2013). All three enzymes are located in the anammoxosome, an intra-ribosomal compartment (Niftrik et al., 2004; Kartal et al., 2011; Bagchi et al., 2016; Bhattacharjee et al., 2017). The lumen of the anammoxosome is separated by three single bilayer membranes from the environment (van Niftrik et al., 2008). Additionally, the high density of the anammoxosome membrane reduces passive proton diffusion (Sinninghe Damsté et al., 2002, 2005). The pH of the anammoxosome and the riboplasm of anammox cells is slightly acidic (pH 6.3)
and close to neutral (pH 7.3), respectively (van der Star et al., 2010). AnAOB seem to be able to maintain the pH in their compartments up to ambient pH values of 7.8. Above pH 8.4 inhibition was observed, likely due to the collapse of proton motive force for ATP synthesis and a decrease in enzymatic activity (van der Star et al., 2010; Puyol et al., 2014). An ambient pH of 7.2-7.6 has been suggested as the optimum for AnAOB activity (Puyol et al., 2014).

**Heterotrophic denitrifying bacteria**

Denitrification is a stepwise reduction pathway with several free intermediates (\(2\text{NO}_3^- \rightarrow 2\text{NO}_2^- \rightarrow 2\text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2\)), and only when the pathway proceeds all the way to \(\text{N}_2\) does it meet the strict definition of denitrification (Zumft, 1997). The four reductive steps are catalyzed by nitrate reductase (NAR), NIR, NOR and nitrous oxide reductase (NOS), respectively. This respiratory process is primarily carried out by heterotrophic denitrifiers (HD) under anoxic or suboxic conditions (Zumft, 1997), which are widely distributed in both soils and aquatic habitats (Knowles, 1982). HD in WWTPs are often closely affiliated with Proteobacteria and Bacteroidetes (Lu et al., 2014). Denitrification rates are commonly measured as \(\text{NO}_3^-\) reduction rates and have been reported to increase with increasing pH with an optimum in the range of pH 7.0 to 8.0 (Van Cleemput and Patrick, 1974; Knowles, 1982). Recently, Šimek and coworkers suggested that the expression ‘optimum pH for denitrification’ should be avoided, as the results for pH optima obtained by different experimental methodology and denitrifier communities were incongruent (Šimek and Cooper, 2002; Šimek et al., 2002). Studies have shown, that denitrification in activated sludge from WWTPs decreased at low pH values (pH < 6-6.5), generating \(\text{N}_2\text{O}\) as the major product, which suggests a non-uniform effect of pH on the different enzymatic
reactions during denitrification (Hanaki et al., 1992; Thomsen et al., 1994; Thörn and Sörensson, 1996). Recently discovered obligate N₂O reducers, that only possess the genetic potential to reduce N₂O to N₂, could be of interest in terms of N₂O mitigation strategies (Jones et al., 2013; Graf et al., 2014). Managing their activity in microbial communities could reduce net-N₂O production by increasing N₂O consumption.

**Denitrifying anaerobic methane oxidizing bacteria and other bacteria hosting nitric oxide dismutase**

A novel N conversion pathway has recently been discovered, in which microbes reduce NO₂⁻ to NO and then dismutate the NO to N₂ (and O₂) in a single enzymatic conversion step catalyzed by nitric oxide dismutase (NOD). This process is usually coupled with alkane oxidation by a monooxygenase (Ettwig et al., 2012). Bacteria that harbor NOD in WWTPs are diverse and are especially abundant in systems with transient occurrence of NO₂⁻/NO₃⁻ or hypoxia (Zhu et al., 2017). They also have been detected in activated sludge systems and anaerobic digesters from municipal WWTP (Ho et al., 2013). Here we focus on *Methylomirabilis oxyfera* and related denitrifying anaerobic methane oxidizing bacteria (DAMO), which host NOD. *M. oxyfera* and related DAMO bacteria have been used for simultaneous nitrite and methane removal from effluents of anaerobic digestion processes (Cai et al., 2015; López et al., 2016). Other relevant bacteria and archaea exist in DAMO driven systems, but they do not host NOD and are therefore not subject of discussion here.

NOD produces oxygen and thus exerts a control of the denitrifying pathway of DAMO. Once oxygen concentrations increase, the expression of NIR – that supplies NOD with its substrate NO – is downregulated, which results in a decrease of denitrification activity...
(Luesken et al., 2012). However, the enzyme that is responsible for aerobic methane oxidation (pMMO) in DAMO bacteria can scavenge the oxygen produced by NOD, thus avoiding the downregulation of NIR. pMMO is located in the cell membrane and is in contact with the periplasm (Wu et al., 2011). The balance between pMMO and NOD enables DAMO bacteria to simultaneously oxidize methane aerobically and to denitrify \( \text{NO}_2^- \) anaerobically via NOD.

Little is known about a potential effect of pH on NOD and NOD hosting bacteria. Generally, further research is needed to identify the microbes that perform nitric oxide dismutation with NOD and to understand a potential impact of pH on them. Learning how to engineer systems that rely on NO dismutation could potentially decrease \( \text{N}_2\text{O} \) emissions from water treatment systems.

In summary, a wide range of growth pH optima have been reported for different microorganisms involved in the N-network (Fig. 3). Based on these optima, it is not feasible to deduce favorable pH set-points for wastewater treatment processes, as pH alone is likely insufficient to engineer the microbial community and select for or against functional microbial guilds to an extent that would reduce net-\( \text{N}_2\text{O} \) production in these systems. Instead, pH may be a suitable tool to manage fluxes through different enzymatic conversion routes, when a pH control at the enzymatic level proves successful.

### 3. The effect of pH on individual enzymes involved in N-conversion

Outside the pH optimum of an enzyme, a reduction in catalytic activity is mainly caused by changes in the enzyme’s structural integrity (Illanes et al., 2008). Changes of structure are caused by the disruption of covalent and non-covalent bonds in the polymers.
Furthermore, changes of the ionization state of amino acid residues in the active site of an enzyme can affect catalytic activity (Illanes et al., 2008). Information about the ionization state of amino acid residues is relevant to understand how the activity of an enzyme may be affected by pH (Nelson and Cox, 2005). The isoelectric point (pI) defines the pH at which the occurrence of an amino acid in its protonated and de-protonated form is balanced; the pI is used in this review to inform the reader about the ionization states of amino acids. Amino acid residues stabilize metal ions and solvents, cause the secondary, tertiary and quaternary structure of an enzyme and form electron and proton transport routes within enzymatic complexes (Nelson and Cox, 2005).

The molecular structure has been solved for a reference of most of the enzymes discussed in this article (Table 1). The high-resolution structures reveal catalytically active sites, intramolecular electron/proton transfer pathways and electron acceptor/donor binding sites. All enzymes involved in the discussed N-conversion processes possess at least one of the following inorganic co-factors: iron-sulfur clusters (Fe-S), Molybdenum (as part of a molybdopterin cofactor) or Cu or Fe (often in the form of heme groups), all of which play relevant functional roles in the enzymes. The effect of pH on each of these structures appears to be consistent across the enzymes.
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<tr>
<th>Enzyme</th>
<th>Full name</th>
<th>Reaction</th>
<th>pH optimum</th>
<th>Cellular location</th>
<th>Structure available</th>
<th>Catalytic atom</th>
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<tr>
<td>AMO</td>
<td>ammonia monooxygenase</td>
<td>( \text{NH}_3 + \text{O}_2 + 2\text{H}^+ + 2e^- \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O} )</td>
<td>Not reported (7.5 for related particulate methane monooxygenase (pMMO)) (Prior and Dalton, 1985; Zahn and DiSpirito, 1996)</td>
<td>Cytoplasmic membrane (Fiencke and Bock, 2006; Simon and Klotz, 2013)</td>
<td>Not available, refer to the structure of pMMO (Lieberman and Rosenzweig, 2005)</td>
<td>Likely Cu</td>
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<td>HAO</td>
<td>hydroxylamine dehydrogenase</td>
<td>( \text{NH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{HNO}_2 + 4\text{H}^+ + 4e^- ) or ( 2\text{NH}_2\text{OH} + 1.5\text{O}_2 \rightarrow 2\text{NO} + 3\text{H}_2\text{O} )</td>
<td>8.5 (Hooper et al., 1984)</td>
<td>Periplasm (Igarashi et al., 1997)</td>
<td>Yes (Igarashi et al., 1997)</td>
<td>Fe (heme)</td>
</tr>
<tr>
<td>NXR <em>Nitrobacter</em></td>
<td>nitrite oxidoreductase</td>
<td>( \text{NO}_2^- + 0.5\text{O}_2 \rightarrow \text{NO}_3^- )</td>
<td>Not reported</td>
<td>Cytoplasmic membrane (Coble, 1976)</td>
<td>No</td>
<td>Mo</td>
</tr>
<tr>
<td>NXR <em>Nitrospira</em></td>
<td>nitrite oxidoreductase</td>
<td>( \text{NO}_2^- + 0.5\text{O}_2 \rightarrow \text{NO}_3^- )</td>
<td>Not reported</td>
<td>Periplasm (Koch et al., 2015)</td>
<td>No</td>
<td>Mo</td>
</tr>
<tr>
<td>NAR</td>
<td>nitrate reductase</td>
<td>( \text{NO}_3^- + 2\text{H}^+ + 2e^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O} )</td>
<td>7 ± 0.5 (Carlson et al., 1982)</td>
<td>Cytoplasm (Berks et al., 1995; Bertero et al., 2003)</td>
<td>Yes (Moura et al., 2004)</td>
<td>Mo</td>
</tr>
<tr>
<td>NAP</td>
<td>nitrate reductase</td>
<td>( \text{NO}_3^- + 2\text{H}^+ + 2e^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O} )</td>
<td>8 (Radcliffe and Nicholas, 1970); 6.5-7.5 (Carlson et al., 1982)</td>
<td>Periplasm (Tavares et al., 2006)</td>
<td>Yes (Jepson et al., 2007)</td>
<td>Mo</td>
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<tr>
<td>Cu-NIR</td>
<td>nitrite reductase</td>
<td>( \text{NO}_2^- + 2\text{H}^+ + e^- \rightarrow \text{NO} + \text{H}_2\text{O} )</td>
<td>&lt;6.5 (Kim et al., 2009); 5.6-6 (Wijma et al., 2006)</td>
<td>Periplasm (Silvestrini et al., 1994)</td>
<td>Yes (Fukuda et al., 2016)</td>
<td>Cu</td>
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<tr>
<td>cd-NIR</td>
<td>nitrite reductase</td>
<td>( \text{NO}_2^- + 2\text{H}^+ + e^- \rightarrow \text{NO} + \text{H}_2\text{O} )</td>
<td>5.8 (Richter et al., 2002); 6.7 (Lam and Nicholas, 1969)</td>
<td>Periplasm (Silvestrini et al., 1994)</td>
<td>Yes (Farver et al., 2009)</td>
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<td>cNOR</td>
<td>nitric oxide reductase</td>
<td>( 2\text{NO} + 2\text{H}^+ + 2e^- \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} )</td>
<td>6 (Field et al., 2002); 5 (Flock et al., 2005)</td>
<td>Cytoplasmic membrane (Hino et al., 2010)</td>
<td>Yes (Hino et al., 2010)</td>
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<td>NOS</td>
<td>nitrous oxide reductase</td>
<td>( \text{N}_2\text{O} + 2e^- + 2\text{H}^+ \rightarrow \text{N}_2 + \text{H}_2\text{O} )</td>
<td>7 (Fujita and Dooley, 2007); 8 (Johnston et al., 2017)</td>
<td>Periplasm (Pauleta et al., 2013)</td>
<td>Yes (Pauleta et al., 2013)</td>
<td>Cu</td>
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<td>HZS</td>
<td>hydrazine synthase</td>
<td>( \text{NH}_4^+ + \text{NO} + 2\text{H}^+ + 3e^- \rightarrow \text{N}_2\text{H}_4 + \text{H}_2\text{O} )</td>
<td>Not reported</td>
<td>Anammoxosome (Kartal et al., 2011)</td>
<td>Yes (Dietl et al., 2015)</td>
<td>Fe (heme)</td>
</tr>
<tr>
<td>HDH</td>
<td>hydrazine dehydrogenase</td>
<td>( \text{N}_2\text{H}_4 \rightarrow \text{N}_2 + 4\text{H}^+ + 4e^- )</td>
<td>8 (Schalk et al., 2000); 8-8.5 (Shimamura et al., 2008)</td>
<td>Anammoxosome (Kartal et al., 2011)</td>
<td>Yes (Maalcke et al., 2016)</td>
<td>Fe (heme)</td>
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</table>
3.1. The effect of pH on catalytic metal atoms

All enzymes discussed in this article contain metal ions as co-factors (Table 1). Variations of pH alter the protonation states of amino acid residues in proximity of these co-factors and affect the steric organization of the metal ions, which in turn affects the catalytic activity of the enzymes (Nelson and Cox, 2005).

Copper

Copper atoms are part of the catalytic sites of Cu-Nitrite reductase (Cu-NIR) and nitrous oxide reductase (NOS) (Table 1). Cu-atoms are likely also part of the catalytic site of ammonia monooxygenase (AMO), although a molecular structure of the enzyme has so far not been obtained (Zahn and DiSpirito, 1996).

Cu-NIR contains two Cu-sites per homotrimer, type-1 and type-2, each of which contains one Cu-atom (Jacobson et al., 2007). The type-1 copper site is located close to the surface of Cu-NIR and accepts an electron from electron donors like pseudoazurin (Kukimoto et al., 1996). The electron from the type-1 copper site is transferred to the type-2 copper in the active site to reduce a NO₂⁻ molecule (Suzuki, 1994). The type-2 copper site is located between two monomers of Cu-NIR and is directly involved in catalysis – NO₂⁻ binds to the copper ion (Godden et al., 1991). High pH (pH 8.4) leads to the deprotonation of amino acid residues in the proximity of the type-2 Cu-ion and also results in a loss of protons of the solvent ligand in proximity of the type 2 Cu-ion (Jacobson et al., 2007). The solvent ligand has been suggested, depending on the pH, to be either a water
molecule or hydroxide ion that contributes with the presence or absence of its proton to the active site geometry. With the absence of protons from amino acid residues and the solvent ligand at pH 8.4 structural transformations of the type-2 copper site geometry were reported, which change the site geometry from a nearly regular tetrahedron into a more distorted tetrahedral geometry (Godden et al., 1991; Jacobson et al., 2007). It was hypothesized that the type-2 copper site is unable to accept an electron from the type-1 copper site in the distorted geometry, which may explain the rapid decrease of enzyme activity with increasing pH (measured in the range pH 6.0 to 9.0)(Jacobson et al., 2007).

NOS also contains two Cu-centers, CuA and CuZ (Pauleta et al., 2013; Carreira et al., 2017). CuA is located close to the surface of the enzyme and accepts electrons from the physiological electron donors cytochrome c550 and pseudoazurin, before passing them on to CuZ (Mattila and Haltia, 2005; Gorelsky et al., 2006). CuZ constitutes the catalytic site and contains four Cu-atoms, which are bridged by two sulfur atoms (Pauleta et al., 2013). The intramolecular electron transfer between CuA and CuZ appears to be rate limiting for N₂O reduction in the range of 4<pH<8, when the electron transfer rate to CuZ is hampered due to conformational changes of CuA (Gorelsky et al., 2006). However, at more alkaline pH (pH > 8.9) a loss of catalytic activity was also observed, likely due to the deprotonation of a lysine residue (pl = 9.7) in CuZ, which in its deprotonated form cannot stabilize the CuZ⁺ intermediate anymore (Johnston et al., 2017). Therefore, the protonation of the lys397 residue appears to be essential for a successful reduction of N₂O via CuZ⁺. The balance between both effects of pH, either the effect of pH on the electron transfer from CuA to CuZ or the protonation state of lys397, may contribute to an observed pH optimum of pH 7-8 (Fujita and Dooley, 2007; Johnston et al., 2017).
Iron

Iron atoms play a central role in almost all enzymes involved in the N-network and act either as catalysts in the active sites or mediate electron transfer within an enzymatic complex. The iron atoms in the active sites are often (e.g. HAO, cd-NIR, HZS, HDH, cNOR), but not always (e.g. cNOR; cNOR contains one heme and one non-heme Fe) part of heme groups. The Fe-atoms in heme groups commonly interact with proximal histidine (his) residues ($pI=7.6$) (Igarashi et al., 1997; Maalcke et al., 2016). Protonated his-residues move the Fe-atom out of the heme plane and cause the Fe-atom to change its spin state (Perutz et al., 1998). The switch of spin state R to T results in a change of free energy that eventually results in a decrease of substrate affinity and catalytic rates (details in (Perutz et al., 1998)). In order to successfully stabilize heme Fe-atoms, his-residues apparently need to be present in their deprotonated form. However, the role of his-residues is not limited to the stabilization of Fe-atoms. For instance, in cd-NIR his-residues are directly involved in the binding of substrate, when a his-residue binds one of the oxygen atoms of the substrate $NO_2^-$ by hydrogen bonds (Tavares et al., 2006). cd-NIR is highly reactive in vitro at pH<7, but poorly active at alkaline pH, when his-residues are deprotonated (Tavares et al., 2006).

Molybdenum

The molybdenum containing molybdo-bis (pyranopterin guanine dinucleotide) co-factor is characteristic for NXR, NAR and NAP and catalyzes either oxidative hydroxylation or reductive dehydroxylation, in which the hydroxyl group is derived from water (Rajagopalan and Johnson, 1992). In principle, one Mo-atom is coordinated with two pyranopterins and interacts with cys- (NAP) or ser- or asp-residues (NAR)($pI=5.1, 5.7$ and $2.8$, respectively)(Moura et al., 2004). Two conserved his-residues in the proximity of the
pyranopterin rings appear to be of significance for the catalytic activity of NAR (Bertero et al., 2003; Wu et al., 2015). In their protonated form the his-residues stabilize the pyranopterin rings and the Mo(V) intermediate during catalysis. Upon loss of an overall positive charge, the midterm potential of the molybdenum ion decreases, which is directly correlated with enzyme activity (Wu et al., 2015). Therefore, an increase of pH appears to directly transfer into a loss of catalytic turnover of NAR.

For NAP the exact mechanism of how pH affects the catalytic activity is unknown. The ionization state of thiol ligands appears to be relevant for the activity of the Mo-ion (Axley et al., 1991; Jepson et al., 2007) and similar causalities as for NAR may apply. However, it needs to be verified, if the findings on the effect of pH on NAR are also valid for NAP.

3.2. The effect of pH on intramolecular proton and electron transport

The effect of pH on enzymes is not limited to amino acid residues in proximity of catalytic metal ions. The transfer of protons and electrons towards and away from the catalytic sites is also affected by pH. Proton transport usually occurs through channels in the three-dimensional structure and is mediated by initially deprotonated amino acid residues. For example, in cNOR the transfer of protons from the periplasm to the active site is crucial for NO reduction activity (Flock et al., 2008). Protons are channeled by a series of glu-residues (pI=3.2) from the surface of the enzyme to the catalytic heme centers. The exact mechanism of proton transport is unknown, but the transfer is strongly affected by pH and decreases with increasing pH from \( k_{\text{max}} = 250 \text{ s}^{-1} \) at pH 5 to \( k_{\text{obs}} = 10 \text{ s}^{-1} \) at pH 8 (Flock et al., 2005). The transfer of protons likely becomes the rate limiting step of cNOR at neutral and alkaline pH (Flock et al., 2005).
In HZS it has been proposed that a cluster of polar amino acid residues (γAsp112, pI=2.8; γArg143 and γArg167, pI=10.8) between γAsp168 and the surface mediates proton transfer to the active site (Dietl et al., 2015). However, the exact mechanism is unknown. The effect of changing pH on the proton transfer of HZS has not been studied and may in fact be of low relevance, as the pH inside the anammoxosome has been reported to be stable (approx. pH 6.3) (van der Star et al., 2010).

Electron transfer is usually mediated by series of heme or Fe-S clusters (HAO, NXR, NAR, NAP, cd-NIR, cNOR) that accept electrons from electron donors, like cytochromes, at the surface of the enzymatic complexes and transfer them sequentially to the active sites. The edge-to-edge distance between heme or Fe-S clusters has to be short enough for direct electron transfer, e.g. 15 Å in HZS or 7 Å and 11 Å in NAR (Bertero et al., 2003; Dietl et al., 2015). Larger distances can be bridged by amino acid residues (Bertero et al., 2003; Dietl et al., 2015). For instance, in HZS it has been proposed that three electrons from the redox partner kuste2854 enter the enzymatic complex via a haem (γII) close to the surface of HZS (Dietl et al., 2015). The electrons may then be transferred to another haem (γI) that is situated in the active site. A his-residue (γHis144) that is located between γI and γII may play an important role in the electron transfer between both haems and its protonation state may affect electron transfer rates. However, in general, it is not well understood how pH affects these electron transport paths in detail, although effects of pH have been described in some cases: In NAR for example the electron transfer to the Mo-center occurs via [Fe-S] clusters and is affected by pH (Bertero et al., 2003; Rothery et al., 2004). The protonation state of Arg-residues (pI=10.8) may play a role in altering the midterm potential of the [Fe-S] clusters, but the exact mechanism is unknown (Bertero et al., 2003).
In summary, molecular structures with their detailed information about the position of amino acid residues, ligands and metal atoms give valuable insights in how variations in pH cause changes in catalytic sites, ligand affinity or electron and proton transport routes. The insights on the molecular level may also facilitate the interpretation of changes of overall enzyme kinetics with pH. However, findings on the molecular level have to be augmented with kinetic data in order to quantify the effect of pH on enzymatic conversion rates.

3.3. pH optima of enzyme activity

3.3.1. The discrepancy between in vitro and in vivo enzyme kinetics. pH optima of enzyme activity are commonly obtained by in vitro activity assays, when purified enzymes, substrates and often non-physiological activation agents and electron acceptors are introduced to an assay medium. Usually, the conditions applied are optimized for good signal-to-noise ratios and differ from conditions in the cellular environment (García-Contreras et al., 2012). Also, when kinetics of different enzymes, that interact in the cell in the same environment, are obtained under different assay conditions, uncertainty about the properties of enzymatic cascades is introduced. In recent years attempts have been made to obtain enzyme kinetics that represent in vivo conditions by setting with physiologically relevant assay conditions (van Eunen and Bakker, 2014), including the buffer capacity and anion composition, macromolecular crowding and pH. However, most pH optima listed in databases to date were not obtained by in vivo-like assays. Accordingly, their validity for physiological conditions needs to be handled with care. Until in vivo-like enzyme kinetics are more widely reported, pH optima of enzyme
activity from *in vitro*-assays may serve as a starting point for considerations about favorable pH set-points.

### 3.3.2. pH optima of NIR, NOR and NOS in respect to N₂O net-production

When studying the pH optima of the enzymes that are metabolically up- and downstream of N₂O in the N-network, the enzymes NIR and NOR and the enzyme NOS with acidic and alkaline pH optima, respectively, stand out (Fig. 4). The difference suggests that pH may regulate the rates between N₂O production and consumption.

**Figure 4**

NOR and NIR play an important role in N₂O production, as they either produce N₂O directly (NOR) or produce NO (NIR), a precursor of N₂O. NO is a free radical with cytostatic or –toxic properties. In order to keep intracellular NO concentrations low, microorganisms have developed rapid detoxification mechanisms that involve reduction of NO to N₂O, e.g. by NOR or cytP₄₅₀ (Stein, 2011; Caranto *et al.*, 2016). Oxidation rate constants for NOR are pH dependent and decrease with increasing pH (oxidation rate constants of NOR at pH 6.0 = 100 s⁻¹ and at pH 7.5 = 12 s⁻¹ (Lachmann *et al.*, 2010)). Therefore, N₂O production by NOR decreases at alkaline pH.

NIR catalyzes the reduction of NO₂⁻ to NO, a reaction that is reversible. In fact, it has been reported that the directionality of Cu-NIR of *Alcaligenes faecalis* is strongly dependent on pH (Wijma *et al.*, 2004). Kᵦ for nitrite reduction was twelve times higher at pH 6.0, than at pH 8.0. The catalytic bias of NIR towards oxidation of NO to NO₂⁻ at pH 8, rather than reduction of NO₂⁻, is caused by an altered reduction potential of the electron donor pseudoazurin and hydroxyl inhibition above pH 7 (Wijma *et al.*, 2004). However, as the
reduction of NO$_2^-$ to NO clearly occurs during denitrification at pH 7-8 in natural systems, the effect of pH on the enzyme is unlikely the only factor that determines the catalytic directionality of NIR. The findings are particularly interesting in the context of the recently proposed role of Cu-NIR during nitritation (Caranto and Lancaster, 2017). Further research will inform if the bidirectionality of Cu-NIR determines the fate of NO during nitrification and denitrification and thus acts as a source or sink of NO as a precursor of N$_2$O.

In contrast to NIR and NOR, the activity of NOS determines, whether N$_2$O or N$_2$ is the final product of denitrification (as comprehensively reviewed in (Carreira et al., 2017)). High activity of NOS increases N$_2$O consumption and has the potential to decrease net N$_2$O production. The N$_2$O:N$_2$ product ratio of denitrification increases with decreasing pH and conversely decreases with increasing pH (Šimek and Cooper, 2002; Bergaust et al., 2010). However, an increase of N$_2$O:N$_2$ at acidic pH is likely not caused by lower gene copy numbers of nosZ, nor lower transcription rates of nosZ vs. nirS that may explain an imbalance between N$_2$O production and consumption rates; on the contrary, the ratio of nosZ/nirS transcripts is larger at lower pH (pH = 6.1), than at higher pH (pH = 8.0)(Liu et al., 2010). pH seems to affect the N$_2$O:N$_2$ ratio mainly on the post-transcriptional level, when higher pH determines the successful assembly of functional NOS at pH >7, whereas at pH ≤6.1 the assembly of functional NOS seems to be disturbed (Liu et al., 2014).

Does the effect of pH on NIR, NOR and NOS mean that for lower N$_2$O net-production, N-removal processes should simply be operated at more alkaline pH to promote N$_2$O consumption over production? A direct correlation between pH optima of individual enzymes and the N$_2$O net-production of entire N-removal processes is unlikely, as enzymes cannot be treated as independent entities. They depend on electrons and
substrates from other enzymatic conversion steps. Therefore, the comparison of individual enzyme activities as a function of pH alone does not necessarily hold sufficient information to evaluate the effect of pH on transformation rates of entire pathways.

4. The effect of pH on pathway conversion rates and net-$\text{N}_2\text{O}$ production

Here the effects of pH on the pathway level are reviewed (Fig. 5). Five pathways are considered: a) Nitritation, b) Nitrifier denitrification, c) Anaerobic ammonia oxidation, d) Heterotrophic denitrification and e) Oxygenic denitrification of nitrite.

Figure 5

4.1. Nitritation

_Nitritation (AOB)_

Nitritation has been understood as the sequential oxidation of $\text{NH}_3$ to $\text{NO}_2^-$ via $\text{NH}_2\text{OH}$, in which the oxidation of $\text{NH}_3$ to $\text{NO}_2^-$ is catalyzed by AMO and HAO (Kostera _et al._, 2010). Recently, _in vitro_ experiments showed that HAO reduces $\text{NH}_2\text{OH}$ in a three electron oxidation to NO, instead of a four electron oxidation to $\text{NO}_2^-$. The occurrence of $\text{NO}_2^-$, as observed in previous $\text{NH}_2\text{OH}$ oxidation experiments, would then either be attributed to a subsequent non-enzymatic oxidation of NO to $\text{NO}_2^-$ by $\text{O}_2$ or an enzymatic oxidation step (potentially via Cu-NIR). An enzymatic oxidation of NO to $\text{NO}_2^-$ would preserve the fourth electron from $\text{NH}_2\text{OH}$ oxidation in the electron pool to contribute to the generation of the proton motive force (Caranto and Lancaster, 2017).

From a thermodynamic perspective, nitritation is favored at alkaline pH, when the Gibbs free energy for $\text{NH}_3$ oxidation is larger at neutral and alkaline pH, than at acidic pH.
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(\Delta G^{\circ}_{25^\circ C, \text{pH } 7} = -274.7 \text{ kJ/kmol N vs. } \Delta G^{\circ}_{25^\circ C, \text{pH } 4} = -240.5 \text{ kJ/kmol N})(\text{Wrage et al., 2001}).

pH also influences the bioavailability of cytochromes as reaction partners of e.g. HAO and thus may affect nitritation rates. At pH 10 cytochrome \(c_{554}\) was shown to be three times more available in the periplasm in its required soluble form, than at pH 8, when it was adhered to the membrane (McTavish et al., 1995). Nitritation rates may therefore not only be limited by the activity of enzymes, but also the availability of downstream electron carriers.

The current model of electron flow in AOB during nitritation describes that the four electrons released by HAO are transferred to electron acceptors, either cytochrome 554 (c554) or directly to membrane bound \(c_{m}552\). The electrons then enter the ubiquinone pool possibly via a designated “Hydroxylamine Ubiquinone Redox Module” (HURM) (Hooper et al., 1997; Simon and Klotz, 2013; Perez-Garcia et al., 2014). Two electrons are channeled back to AMO for the reduction of \(O_2\), while the remaining electrons are used to establish the proton motive force. However, the exact pathways of electron transport in AOB during nitritation remain to be resolved and may involve other redox mediators, such as c552.

Additionally, direct interactions between the cytochromes that alter the electron flow cannot be excluded, e.g. c554 with \(c_{m}552\) (Simon and Klotz, 2013; Stein et al., 2013; Kozlowski et al., 2016). The model needs to be refined in respect to the findings of \(\text{NH}_2\text{OH}\) oxidation to NO (Caranto and Lancaster, 2017). However, the proposed three electron oxidation of \(\text{NH}_2\text{OH}\) to NO by HAO may be compatible with the current model. The flow of one electron to NirK for the reduction of \(\text{NO}_2^-\) to NO during denitrification, which is commonly mediated by cytochromes c550-553 or (pseudo) azurins or cupredoxins (Simon and Klotz, 2013), may be reversed and that way contribute an electron to the generation of the proton motive force. It has been demonstrated that the reduction of \(\text{NO}_2^-\) to NO is
reversible and that the catalytic equilibrium is dependent on pH: at acidic pH (pH 6.2) $\text{NO}_2^-$ reduction occurred at faster rates, than NO oxidation, while at pH 8.0 NO oxidation to $\text{NO}_2^-$ was faster (Wijma et al., 2004). Therefore, the pH may be relevant for the accumulation of NO during nitritation.

In the proposed nitritation pathway by Caranto and Lancaster, i.e. $\text{NH}_3 \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO} \rightarrow \text{NO}_2^-$, the balance between HAO and NO oxidation rates, potentially by NirK, are relevant: a low oxidation rate of NirK would result in the accumulation of NO and potentially to increased $\text{N}_2\text{O}$ production due to reduction of accumulated NO to $\text{N}_2\text{O}$. Both HAO and NirK are located in the periplasm and are affected by ambient pH. The pH optima of both enzymes are substantially different (pH 8.5 for HAO vs. 6.0 for NirK). Lower pH would result in relatively higher NO oxidation rates by NirK, compared to $\text{NH}_2\text{OH}$ oxidation rates of HAO, which would prevent accumulation of NO. In contrast, at alkaline pH NO oxidation rates of NirK may be lower, than $\text{NH}_2\text{OH}$ oxidation rates by HAO, which may lead to accumulation of NO and potentially increased $\text{N}_2\text{O}$ production rates.

Observations from nitrifying AOB enrichment cultures and cultures of $N. \text{europaea}$ support the stimulating effect of pH on nitritation and $\text{N}_2\text{O}$ production rates (Shammas, 1986). pH optima and minima for $\text{N}_2\text{O}$ production rates were identified at pH 8.0-8.5 and below pH 7.0, respectively (Hynes and Knowles, 1984; Law et al., 2011; Rathnayake et al., 2015). In summary, alkaline pH appears to favor higher nitritation rates and higher $\text{N}_2\text{O}$ production rates by AOB. The role of the different enzymes involved in nitritation and potential imbalances of metabolic fluxes between them will be elucidated in future studies.

Nitritation (AOA)
N₂O production during ammonia oxidation has been detected in pure and enrichment cultures of AOA (Jung et al., 2014; Stieglmeier et al., 2014). Compared to the better elucidated N₂O production pathways of AOB, the mechanisms of N₂O production by AOA are not well understood, due to the lack of physiological and genomic evidence for enzymatic processes for N₂O production (Walker et al., 2010; Tourna et al., 2011; Spang et al., 2012). Recently, NO and NH₂OH have been identified as essential intermediates during archaeal ammonia oxidation, however both intermediates are probably reduced to N₂O non-enzymatically (Vajrala et al., 2013; Zhu-Barker et al., 2015; Kozlowski et al., 2016). This contrasts to the enzymatic N₂O production in bacteria (Walker et al., 2010; Tourna et al., 2011; Spang et al., 2012; Stieglmeier et al., 2014). Hence, the production of N₂O during ammonia oxidation in soil was suggested to be lower for AOA, than AOB (Hink et al., 2017). pH selects for different bacterial and archaeal communities and therefore determines the relative contribution of AOA and AOB to overall N₂O production (Nicol et al., 2008). In fact, AOA become the dominant contributors to nitritation and respective N₂O production rates in low pH environments, e.g. soils (Li et al., 2018). The production of N₂O by AOA in wastewater treatment systems has, to our knowledge, not been documented as relevant.

4.2. Nitrifier denitrification

Nitrifier denitrification (ND) describes the reduction of NO₂⁻ to N₂O by NIR and NOR in AOB (Wrage et al., 2001). Rates of ND increase at low oxygen tension. In the current model a bottleneck of electron flow occurs at low oxygen tension between HAO and the terminal oxidase, likely between HAO and c₅₅₂ or in the c₅₅₂ pool between the cytochrome complexes C-III and C-IV, as a result of insufficient availability of oxygen as an electron acceptor (Simon and Klotz, 2013; Kozlowski et al., 2016). In order to relieve the
bottleneck NIR and NOR may be activated and accept electrons from c552 to reduce \( \text{NO}_2^- \) and NO to \( \text{N}_2\text{O} \), thus constituting the nitrifier denitrification pathway (Kozlowski et al., 2016). However, the regulation of electron flow in AOB does not follow a single principle and different phylotypes appear to apply different regulatory features, which likely depend on the genomic history and environment (Stein et al., 2013).

A relevant role of regulation may be assigned to the small red-copper protein nitrosocyanin. Nitrosocyanin may regulate whether electrons from HAO are transferred to c554 or \( c_{M} \), thus determining their fate in the electron flow cascade of AOB (Stein et al., 2013). Electrons loaded to \( c_{M} \) would directly enter the quinone pool to be distributed to AMO, whereas electrons loaded to c554 would be available for further distribution to other electron carriers, e.g. c552, and to NIR and NOR. Therefore, nitrosocyanin may act as a regulator of electron flow in order to obtain redox balance in environments with sufficiently high substrate concentrations (Stein et al., 2013).

It is challenging to study the rate of nitrifier denitrification isolated. Accordingly, a pH optimum is difficult to obtain. Based on the pH optima of NIR and NOR, it may be hypothesized that elevated rates of ND occur at slightly acidic pH (Table 1). However, as mentioned above, ND occurs in a complex interaction with the cellular electron pool and different electron carriers and a deduction of a pH optimum only from NIR and NOR is likely too simple. It may be speculated that, similar to \( c_{554} \) (McTavish et al., 1995), an effect of pH also on \( c_{552} \) or nitrosocyanin may play a role for the conversion rates of NIR and NOR. However, it is currently unknown, if the pH affects the concentration of \( c_{552} \) or nitrosocyanin directly or indirectly or how pH affects nitrifier denitrification rates in general.
4.3. Anaerobic ammonia oxidation

The anaerobic ammonia oxidation (anammox) pathway comprises three sequential steps in which the substrates NH$_4^+$ and NO$_2^-$ are converted into N$_2$ (Strous et al., 2006; Kartal et al., 2011, 2013). Initially, NO$_2^-$ is reduced to NO by NIR. Then, NH$_4^+$ is oxidized with NO to hydrazine (N$_2$H$_4$) by HZS. Finally, N$_2$H$_4$ is oxidized to N$_2$ by HDH. All enzymes involved are located in the anammoxosome. The transfer of electrons and protons between the three enzymatic reactions has been proposed to be mediated by the quinol::cytochrome c oxidoreductase system (complex III, bc1 complex) in the membrane of the anammoxosome (Kartal et al., 2011). The anammox pathway does not include N$_2$O as an intermediate (Strous et al., 2006; Kartal et al., 2013) and is generally not considered to yield N$_2$O. Although the enzymes involved in anammox are located in the anammoxosome, and therefore a change of ambient pH is unlikely to affect the enzymes directly, anammox rates increase with pH and are highest between pH 7.5-8.3. An explanation for this paradox may be found in an inhibitory effect of pH on other cellular processes, than the enzymatic conversion reactions themselves, which results in changes of anammox rates with pH. Substrate transporters have been proposed to play a significant role in substrate trafficking of AnAOB cells and an effect of pH on these proteins is likely, yet largely unknown (Kartal et al., 2013).

4.4. Heterotrophic denitrification

Heterotrophic denitrification (HD) refers to the reduction of NO$_3^-$ or NO$_2^-$ to gaseous products (NO, N$_2$O and N$_2$), in which NO$_3^-$ or NO$_2^-$ serve as terminal electron acceptors. HD involves the enzymes NAR, NIR, NOR and NOS. HD comprises both N$_2$O production and consumption reactions. The ratio of complete (N$_2$) and incomplete (N$_2$O) denitrification
as a function of pH is relevant in respect to net-$\text{N}_2\text{O}$ production. Various studies, especially from agricultural soils, report an increased $\text{N}_2\text{O}/(\text{N}_2+\text{N}_2\text{O})$ ratio with decreasing soils (Šimek and Cooper, 2002; Bergaust et al., 2010; Bakken et al., 2012), which was assigned to an impaired post-transcriptional assembly of NOS at pH < 7.0 (Liu et al., 2014). The effect of a relatively higher $\text{N}_2\text{O}/(\text{N}_2+\text{N}_2\text{O})$ ratio at low pH may however be compensated by a decline in overall denitrification rates with decreasing pH ($\text{NO}_3^-$ reduction rates increase with pH with an optimum between pH 7.0 and 8.0 (Van Cleemput and Patrick, 1974; Knowles, 1982)). Absolute $\text{N}_2\text{O}$ production rates from denitrification can therefore be expected to be highest in the range pH 7.0-7.5, before $\text{N}_2\text{O}$ reduction by NOS becomes effective at pH > 7.5 (Liu et al., 2014).

4.5. Oxygenic denitrification of nitrite

The oxygenic denitrification of nitrite is a two steps process, in which nitrite is first reduced by cd-NIR to NO. NO is then further dismutated to $\text{N}_2$ and $\text{O}_2$ by NOD. The process bypasses $\text{N}_2\text{O}$ as an intermediate towards $\text{N}_2$. An effect of pH on process rates of oxygenic denitrification of nitrite has, to our knowledge, not been reported yet. NIR and NOD are usually located in the periplasm and are likely exposed to changes of ambient pH. It may therefore be hypothesized that denitrification rates decrease, when pH diverges from the pH optimum of NIR (pH 5.8-6.7) and NOD (pH optimum not reported). The pH optimum of pMMO (pH 7.5; (Zahn and DiSpirito, 1996)) may also play a role, as reduced activity of pMMO may lead to accumulation of oxygen that in turn inhibits the denitrification process. However, with the current knowledge no reasonable pH optimum for oxygenic denitrification of nitrite via NOD can be identified.
In summary, slightly alkaline pH results in higher rates of nitritation, anammox and complete denitrification. Although changes of nitrifier denitrification and nitratation rates with pH have not been assessed in detail, rates of ND and nitratation are expected to decrease with increasing pH based on information available for the enzymes involved in these pathways (Fig. 5). The link between enzymatic pH optima and pathway conversion rates may enable predictions of net-N\textsubscript{2}O production rates.

5. Exploring favorable pH set-points for lower net-N\textsubscript{2}O production in wastewater bio-reactors.

Based on the findings of the previous sections, this last section hypothesizes favorable pH set-points (in respect to lower net-N\textsubscript{2}O production rates) for nitrogen removing bio-reactors in wastewater treatment applications. The microbial communities in these systems commonly comprise AOB, AnAOB, NOB and HD. In respect to N\textsubscript{2}O production AOB appear to be of most significance, whereas HD play a dominant role for N\textsubscript{2}O consumption. As both functional guilds, i.e. AOB and HD, are present in essentially all nitrogen removing wastewater systems like nitrification-denitrification, partial nitrification or partial nitritation-anammox, considerations may be transferable between applications. The effect of pH on net N\textsubscript{2}O production rates in these systems is hypothesized in three scenarios: pH 6.0-6.5, pH 7.0-7.5 and pH 8.0-9.0.

Slightly acidic pH 6.0-6.5

At slightly acidic pH, e.g. pH 6.5, nitration rates are not at maximum (see 4.1), as the pH optimum of HAO lies at approx. 8.0-8.5. The proximity to the pH optimum of Cu-NIR (approx. pH 6, see Fig. 4) would result in an efficient removal of NO. Both N\textsubscript{2}O production
and consumption rates are expected to be low at slightly acidic pH, which results in low net N$_2$O production rates. Therefore, lower net N$_2$O production rates can potentially be achieved in N-removing bioreactors by operation at slightly acidic pH, yet at the cost of reduced nitritation rates. This mode of operation may be interesting for nitrifying and partially nitrifying applications.

**Neutral pH 7.0-7.5**

At neutral pH, e.g. pH 7.0-7.5, nitritation rates increase, as reaction conditions are closer to the pH optimum of HAO. NH$_2$OH oxidation rates by HAO would be larger than NO oxidation rates by Cu-NIR and accumulated NO would be reduced to N$_2$O by either NOR or other detoxification mechanisms. N$_2$O production rates are therefore expected to be higher at pH 7.0-7.5, than at pH 6.5. Net N$_2$O production rates are also expected to be high, as the pH is not high enough to allow an efficient reduction of N$_2$O to N$_2$ by NOS. Out of all three pH scenarios net-N$_2$O production rates are expected to be highest in the range pH 7.0-7.5. This pH range appears as unfavorable for nitrogen removing applications, e.g. nitrification-denitrification, partial nitrification and partial nitrification-anammox, in respect to net N$_2$O production.

**Alkaline pH 8.0-9.0**

NH$_2$OH oxidation rates would be maximal at pH 8.0-9.0 and NO oxidation rates by Cu-NIR would be elevated. N$_2$O production rates are expected to peak at alkaline pH. However, other than at pH 7.0-7.5, high N$_2$O consumption rates may counterbalance production rates at alkaline pH, thus causing low net-N$_2$O production rates (see Fig 5). Therefore, alkaline pH of approx. 8.0 appears to be favorable for wastewater treatment applications, as net N$_2$O production rates may be low, while ammonia removal rates can be maintained.
In summary, the net $\text{N}_2\text{O}$ production curve with pH would be “bell-shaped” with low net $\text{N}_2\text{O}$ production rates at pH $\leq 6.5$, high rates at pH $7.0$-$7.5$ and low rates at pH $\geq 8.0$.

6. Conclusion

When $\text{N}_2\text{O}$ production exceeds $\text{N}_2\text{O}$ consumption microbial communities constitute sources of $\text{N}_2\text{O}$. The microbial guilds that drive nitrogen conversion in wastewater treatment processes (ammonia oxidizing bacteria or archaea, complete or anaerobic ammonia oxidizing bacteria, nitrite oxidizing bacteria and heterotrophic denitrifying bacteria) all have physiological growth optima in the range pH 7.0-8.0. Selecting specific functional microbial guilds based on pH does, therefore, not appear feasible for the mitigation of net $\text{N}_2\text{O}$ production in complex microbial communities.

All enzymes that are of particular relevance for $\text{N}_2\text{O}$ production and consumption are either located in (i.e. HAO, NIR and NOS) or in contact with the periplasm, when embedded in the cytoplasmic membrane (i.e. NOR). Accordingly, they are exposed to changes in ambient pH. In general, pH alters the catalytic activities of enzymes involved in nitrogen conversion, mainly by affecting their structural integrity. However, its effects vary: in Cu-NIR and NOR, protonation or deprotonation of amino acid residues alter the steric organization of catalytic sites (Cu-NIR, cNOR) or the substrate affinity of catalytic metal ions (Cu-NIR, cd-NIR), which leads to sub-optimal conversion rates at pH $> 7.0$. Electron and proton transfer within enzymatic complexes is also affected by pH. In HZS and NAR electron transport becomes rate limiting with increasing pH, and hampered proton transport reduces the turnover of cNOR 25-fold from pH 5 to 8. Finally, pH also exerts a post-translational effect and prevents the assembly of NOS to a functional enzyme at pH $< 7.0$. The result is a decrease of $\text{N}_2\text{O}$ consumption rates at acidic pH.
The different effects of pH on the molecular level are consistent with rate measurements of different bacterial N-conversion reactions, e.g. nitritation and denitrification, or the N₂O/N₂ product ratio of denitrification. Nitritation and N₂O production rates by AOB increase with pH in the range pH 6.0-8.0, while N₂O consumption by heterotrophic denitrifiers increases with pH > 7.0. Overall, net N₂O production in bacterial communities of wastewater engineering applications at low pH < 7 appears to be dominated by N₂O production, whereas at pH > 7.5 N₂O consumption counteracts production and leads to reduced net N₂O production rates. Therefore, lower net N₂O production rates can be achieved at acidic pH < 7.0, however at the cost of lower NH₄⁺ removal rates. Low net N₂O production at simultaneously high NH₄⁺ removal rates is possible at alkaline pH > 7.5 due to N₂O consumption in microbial communities that host denitrifying bacteria. Highest net N₂O production rates would occur at pH 7.0-7.5, when N₂O consumption does not outweigh increased production.

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Originality-Significance Statement

The presented work contributes to research in environmental microbiology by identifying the effects of pH on the enzymatic level of nitrogen conversion reactions, by identifying trends of conversion rates and relative N₂O production of relevant pathways upon changes
of pH, by connecting the effects of pH on the enzyme level with resulting changes on the pathway level and by hypothesizing favorable pH set-points for wastewater treatment applications with the scope of lower net-N$_2$O production.

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Figure 1 - Web of nitrogen conversion reactions. Enzymes involved in catalysis are ammonia monoxygenase (AMO), hydroxylamine dehydrogenase (HAO), nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite oxidoreductase (NXR), nitrite reductase (NIR), nitric oxide reductase (NOR), nitrous oxide reductase (NOS), hydrazine synthase (HZS), hydrazine dehydrogenase (HDH) and nitric oxide dismutase (NOD).
Figure 2 - Location of functional enzymes. The catalytic site of ammonia monooxygenase (AMO) and nitrite oxidoreductase (NXR) *Nitrobacter*, and nitrate reductase (NAR) are located in the cytoplasm. Hydrazine synthase (HZS), hydrazine dehydrogenase (HDH) and nitrite reductase (NIR) of anammox are located in the anammoxosome. Hydroxylamine dehydrogenase (HAO), nitrite oxidoreductase (NXR) *Nitrospira*, nitrate reductase (NAP), nitrite reductase (NIR), nitrous oxide reductase (NOS), nitric oxide dismutase (NOD) and the catalytic site of nitric oxide reductase (NOR) are located in the periplasm.
Figure 3 - Physiological pH optima of organisms reported in literature. Organisms: AOB, aerobic ammonia oxidizing bacteria; AOA, ammonia oxidizing archaea; NOB, nitrite oxidizing bacteria; AnAOB, anaerobic ammonia oxidizing bacteria; HD, heterotrophic denitrifiers. References are given in Table S1.
Figure 4 - pH optima of enzymes involved in N-conversion. Enzymes: ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), nitrite oxidoreductase (NXR), nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR), nitrous oxide reductase (NOS), hydrazine synthase (HZS), hydrazine dehydrogenase (HDH), and nitric oxide dismutase (NOD). Enzymes in gray shade (AMO, NXR *Nitrospira*, NXR *Nitrospira*, HZS and NOD) represent a lack of reported optimum pH. The optimum pH of AMO was referred to the particulate methane monooxygenase (pMMO). References are given in Table 1.
Figure 5 - Qualitative changes of N-conversion and N$_2$O production rates of different pathways with varying pH.