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

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

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15 **Summary**

16 Nitrous oxide (N₂O) is emitted during microbiological nitrogen (N) conversion processes,
17 when N₂O production exceeds N₂O consumption. The magnitude of N₂O production vs
18 consumption varies with pH and controlling net-N₂O production might be feasible by
19 choice of system pH. This article reviews how pH affects enzymes, pathways and
20 microorganisms that are involved in N-conversions in water engineering applications. At a
21 molecular level, pH affects activity of co-factors and structural elements of relevant
22 enzymes, by protonation or deprotonation of amino acid residues or solvent ligands
23 causing steric changes in catalytic sites or proton/electron transfer routes that alter
24 catalytic activity. Augmenting molecular information with, e.g. nitritation or denitrification
25 rates, yields explanations of net-N₂O production with pH changes. Ammonia oxidizing

26 bacteria are of highest relevance for N₂O production, while heterotrophic denitrifiers are
27 relevant for N₂O consumption at pH > 7.5. Net-N₂O production in N-cycling water
28 engineering systems is predicted to display a “bell-shaped” curve in the range of pH 6.0-
29 9.0 with a maximum at pH 7.0-7.5. Net N₂O production at acidic pH is dominated by N₂O
30 production, whereas N₂O consumption can outweigh production at alkaline pH. Thus, pH
31 8.0 may be a favorable pH set-point for water treatment applications regarding net N₂O
32 production.

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37 1. Introduction

38 Emissions of nitrous oxide (N_2O) to the atmosphere are of concern, as N_2O is a
39 greenhouse gas with a large global warming potential and ozone depleting properties
40 (Ravishankara *et al.*, 2009; IPCC, 2013). As a result of an increased load of reactive
41 nitrogen (N) to the biological N-cycle by anthropogenic activities, emissions of N_2O are
42 increasing (Canfield *et al.*, 2010; Steffen *et al.*, 2015). Especially agricultural soils and
43 engineered nitrogen removal systems, like constructed wetlands or urban wastewater
44 treatment plants (WWTPs), constitute hubs of N_2O emissions (Canfield *et al.*, 2010; Law *et al.*,
45 2012). Nitrogen is introduced to these systems in its most reduced form, i.e. as
46 ammonium (NH_4^+) or organic nitrogen (which is readily converted to ammonium via
47 ammonification). Whilst in agriculture the assimilation of NH_4^+ -N into biomass is the
48 primary objective, water treatment applications seek to convert NH_4^+ into environmentally
49 inert dinitrogen gas (N_2). In all systems the loss of nitrogen in the form of N_2O is significant
50 and strategies to mitigate N_2O emissions are sought after (Hénault *et al.*, 2012; Law *et al.*,
51 2012).

52 Various prokaryotes harbor the energy associated with conversion of N-species between
53 different redox states. The biological N-network hosts a multitude of conversion reactions,
54 which are catalyzed by a variety of enzymes (Fig. 1). N_2O constitutes the end product of a
55 number of metabolic pathways or is an intermediate towards N_2 . As, in principle, both N_2O
56 production and consumption reactions exist, net- N_2O production occurs as the result of
57 non-ideal flow of N-species through the N-network (Stein, 2010). Hence, the key to lower
58 N_2O emissions are overall pathways that prevent the accumulation of N_2O . pH is one of
59 the parameters that affect conversion rates of enzymes (Illanes *et al.*, 2008). The enzymes
60 involved in the N-network have different pH optima and pH may cause imbalances

61 between enzymatic reaction steps that lead to the accumulation of intermediates, such as
62 hydroxylamine (NH_2OH), nitrite (NO_2^-), nitric oxide (NO) or N_2O . Conversely, setting pH
63 may offer an opportunity to synchronize N-conversion rates to reduce accumulation of
64 N_2O . A better understanding of the effect of pH on the N-network can support decision
65 making on pH set-point management to lower N_2O emissions from soils and engineered
66 N-removal systems.

67 **Figure 1**

68 This article reviews how the pH affects enzymes, pathways and microbes that are involved
69 in the N-network and which are relevant for net- N_2O production. It further explores, if pH
70 optima of individual enzymes hold sufficient information to hypothesize favorable pH set-
71 points for lower net- N_2O production. Written with the background of biological N-removal
72 during wastewater treatment processes, most examples are taken from WWTPs.

73 However, as the same microbial pathways are active in other environments, findings may
74 be relevant for fields outside of wastewater treatment. Microorganisms in WWTPs are
75 usually not exposed to environmental pH values outside the range pH 6.0-9.0 (Henze and
76 Comeau, 2008). Therefore this article focuses on effects of pH in this range.

77 Summarizing, the effect of pH is complex, partly because of the sheer number of enzymes,
78 pathways and organisms involved in N-conversion, but also because pH affects various
79 central processes in cells, signaling or transcriptional and post-transcriptional phenomena.

80 Accordingly, the attempt to infer optimal pH set-points for complex microbial communities
81 and their N- conversion processes with respect to N_2O is ambitious. Yet, the demand for
82 N_2O mitigation strategies and guidelines, especially in water engineering applications,
83 justifies an effort, even though simplifications and constraints are unavoidable. Because

84 such applications often involve well-controlled systems with relatively steady conditions
85 (bioreactors), compared to rapidly changing environments of natural systems, it is
86 plausible that pH control strategies may constitute a feasible tool to manage N₂O net
87 production.

88 **1.1. The effect of pH on enzymatic conversion rates**

89 The conversion rate of an enzyme ($v = k_{\text{cat}} \cdot [E]_0 \cdot [S] / (K_M + [S])$) is affected by pH either by
90 changes of the turnover number (k_{cat}), the substrate concentration [S] or both (Illanes *et*
91 *al.*, 2008). During nitrogen removal, pH governs the speciation of the acid/base pairs
92 NH₄⁺/NH₃ (pKa=9.3) and HNO₂/NO₂⁻ (pKa=3.4) (Nelson and Cox, 2005). Substrate
93 speciation becomes relevant, when it changes the availability of substrate for enzymatic
94 reactions or causes inhibition. k_{cat} is affected by pH, when changes of the enzymatic
95 structure reduce the catalytic activity of enzymes (Illanes *et al.*, 2008). Based on the
96 interplay of k_{cat} and the substrate concentration, pH set-points may be used to manage
97 one or the other in order to control enzymatic conversion rates.

98 **1.2. The relevance of the cellular location of enzymes for an effect of** 99 **changes in ambient pH: periplasmic pH and cytoplasmic pH** 100 **homeostasis in bacteria and archaea**

101 To manage k_{cat} by pH set-points, the enzymes need to be accessible for changes of
102 ambient pH. However, changes of pH in the environment of a cell do not directly transfer
103 into changes of pH throughout the cell, but affect cellular compartments differently
104 (Slonczewski *et al.*, 2009). Hence, the effect of ambient pH on the activity of an enzyme
105 depends on its cellular location (Fig. 2). Bacteria and archaea depend on a stable pH in
106 their cytoplasm for their metabolic machinery to operate efficiently and the proton motive
107 force to generate chemical energy reliably (Booth, 1985). Microorganisms have developed

108 regulation mechanisms to maintain a near-constant cytoplasmic pH in environments with
109 substantially different ambient pH values (Booth, 1985; Slonczewski, 2009; Krulwich,
110 2011). Most neutrophilic bacteria maintain their cytoplasmic pH stably at 7.5-7.7 against a
111 pH in the environment ranging from 5.5 to 9.0 (Pedan, 2005; Slonczewski, 2009).
112 Accordingly, a change of ambient pH in the range of pH 6.0-9.0, as present in WWTPs,
113 would not transfer into changes of pH in the cytoplasm. Consequently, enzymes that are
114 localized in the cytoplasm or other intra-cell compartments would not be directly affected
115 by changes of pH in the environment (Slonczewski *et al.*, 2009; van der Star *et al.*, 2010).
116 In contrast, the pH of the periplasm of gram-negative bacteria is not maintained and
117 follows the pH of the environment (Wilks, 2007).

118 **Figure 2**

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

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

126 **Figure 3**

127 **Aerobic ammonia oxidizing bacteria**

128 Aerobic ammonia oxidizing bacteria (AOB) are chemolithoautotrophs that oxidize NH_3 to
129 NO_2^- via NH_2OH , as well as NO , which has recently been recognized as another obligate

130 intermediate during nitrification (Caranto and Lancaster, 2017). AOB are also able to reduce
131 NO_2^- to N_2O by the nitrifier denitrification pathway (Wrage *et al.*, 2001). The enzymes
132 involved are ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO),
133 nitrite reductase (NIR) and nitric oxide reductase (NOR) (Schreiber *et al.*, 2012). Nitrification
134 and nitrifier denitrification play central roles in WWTPs, also in respect to N_2O production
135 (Stein, 2011). AOB found in WWTP mainly affiliate with *Nitrosomonas*, *Nitrosospira*,
136 *Nitrosovibrio* and *Nitrosococcus* genera, with *Nitrosomonas* and *Nitrosospira* as the most
137 dominant AOB (Kowalchuk and Stephen, 2001). In general, AOB in WWTPs can grow
138 across a wide range of pH with optima at pH 7.4-8.2 (Grunditz and Dalhammar, 2001;
139 Park *et al.*, 2007; Claros *et al.*, 2013; Daalkhajav and Nemati, 2014). Bacterial *amoA*,
140 which is commonly used as a molecular marker for AOB, showed low abundance under
141 acidic conditions (pH < 5.5) (De Boer and Kowalchuk, 2001) and increased with increasing
142 pH (Leininger *et al.*, 2006; Nicol *et al.*, 2008).

143 **Ammonia oxidizing archaea**

144 Ammonia oxidizing archaea (AOA) perform autotrophic ammonia oxidation similar, but not
145 identical to AOB (Kozlowski *et al.*, 2016); no HAO or NOR genes have been identified in
146 pure or enrichment cultures of AOA, indicating that they might be unable to produce N_2O
147 enzymatically through side reactions of ammonia oxidation or nitrifier denitrification
148 (Walker *et al.*, 2010; Tourna *et al.*, 2011; Spang *et al.*, 2012; Stieglmeier *et al.*, 2014). AOA
149 are widespread in nitrifying bioreactors in WWTPs or biofilters for drinking water
150 production and are also found in soil and sediments (Park *et al.*, 2006; Zhang *et al.*, 2009;
151 Gülay *et al.*, 2014). Optimal growth of AOA has been reported in the range of pH 7-7.5
152 (French *et al.*, 2012; Spang *et al.*, 2012). Nicol *et al.* (2008) observed that transcriptional
153 abundance and expression of archaeal *amoA*, a widely used molecular marker of AOA,

154 decreased with increasing pH. Yet, archaeal *amoA* was found in environments with a wide
155 range of pH (pH 3.7 to 8.65) (Erguder *et al.*, 2009). AOA have been frequently found to
156 outnumber AOB and dominate in abundance and ammonia oxidizing activity, especially in
157 acidic soils (Nicol *et al.*, 2008; Gubry-Rangin *et al.*, 2010; Zhang *et al.*, 2012; Li *et al.*,
158 2018).

159 **Nitrite oxidizing bacteria**

160 Nitrite oxidizing bacteria (NOB) are aerobic chemolithoautotrophs that oxidize NO_2^- to
161 nitrate (NO_3^-). The reaction is catalyzed by the enzyme nitrite oxidoreductase (NXR)
162 (Schreiber *et al.*, 2012). NOB have been found in several genera distributed among
163 different bacterial lineages, i.e. *Nitrobacter*, *Nitricoccus*, *Nitrospina*, *Nitrospira* and *Nitrotoga*.
164 Even though the genus *Nitrospira* is often the numerically dominant NOB in WWTPs
165 (Daims *et al.*, 2001), virtually all knowledge of the physiology and biochemistry of NO_2^-
166 oxidation has been derived from studies of a limited number of strains of *Nitrobacter*
167 species (Daims *et al.*, 2016). The activity of NOB in WWTPs was found to be pH-
168 dependent with optima at $\text{pH } 7.9 \pm 0.4$ (Park *et al.*, 2007). More specifically, the optimum
169 growth of isolated pure cultures of *Nitrospira* and *Nitrobacter* species occurred at pH 7.6-
170 8.0 (Ehrich *et al.*, 1995) and 7.9 (Grunditz and Dalhammar, 2001), respectively.

171 **Complete ammonia oxidizing bacteria**

172 Complete ammonia oxidizing bacteria (Comammox) are able to oxidize NH_3 to NO_3^-
173 (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Besides NXR, *Nitrospira* sp. harbor the
174 enzymes needed to oxidize ammonia to nitrite (AMO and HAO), which differ from the
175 canonical enzymes utilized by proteobacterial AOB (e.g., Palomo *et al.* (2016), reported
176 only 56% similarity between amino acid sequence for *amoA* from *Nitrosomonas* and

177 *Nitrospira* sp.). AMO and NXR are membrane bound, whilst HAO is located in the
178 periplasm (Daims *et al.*, 2015). It is still to be determined, if an effect of pH on these
179 enzymes is similar to its effect on canonical AOB enzymes. Nevertheless, some studies
180 already demonstrate that pH has an impact on niche differentiation between different
181 microorganisms capable of ammonia oxidation in soils (Hu and He, 2017). *NirK*, the gene
182 for Cu-NIR, is also present in comammox, but no evidence of NO accumulation in
183 comammox enrichments cultures suggests that nitrite reduction may not be particularly
184 robust (Daims *et al.*, 2015). Comammox have been found in different water engineered
185 systems, including WWTPs (Chao *et al.*, 2016; Gonzalez-Martinez *et al.*, 2016; Pjevac *et*
186 *al.*, 2017). However, their abundance in wastewater treatment plants is lower than AOB,
187 which is likely due to the competitive advantage of AOB in systems with high substrate
188 concentrations (Costa *et al.*, 2006; Kits *et al.*, 2017). In general, further research is needed
189 to understand the role of comammox in the nitrification process in full scale WWTPs.

190 **Anaerobic ammonia oxidizing bacteria**

191 Anaerobic ammonia oxidation (anammox) is performed by anaerobic ammonia oxidizing
192 bacteria (AnAOB). Anammox is catalyzed by the sequential activity of the enzymes NIR,
193 hydrazine synthase (HZS) and hydrazine dehydrogenase (HDH) (Kartal *et al.*, 2013). All
194 three enzymes are located in the anammoxosome, an intra-riboplasmic compartment
195 (Niftrik *et al.*, 2004; Kartal *et al.*, 2011; Bagchi *et al.*, 2016; Bhattacharjee *et al.*, 2017). The
196 lumen of the anammoxosome is separated by three single bilayer membranes from the
197 environment (van Niftrik *et al.*, 2008). Additionally, the high density of the anammoxosome
198 membrane reduces passive proton diffusion (Sinninghe Damsté *et al.*, 2002, 2005). The
199 pH of the anammoxosome and the riboplasm of anammox cells is slightly acidic (pH 6.3)

200 and close to neutral (pH 7.3), respectively (van der Star *et al.*, 2010). AnAOB seem to be
201 able to maintain the pH in their compartments up to ambient pH values of 7.8. Above pH
202 8.4 inhibition was observed, likely due to the collapse of proton motive force for ATP
203 synthesis and a decrease in enzymatic activity (van der Star *et al.*, 2010; Puyol *et al.*,
204 2014). An ambient pH of 7.2-7.6 has been suggested as the optimum for AnAOB activity
205 (Puyol *et al.*, 2014).

206 **Heterotrophic denitrifying bacteria**

207 Denitrification is a stepwise reduction pathway with several free intermediates ($2\text{NO}_3^- \rightarrow$
208 $2\text{NO}_2^- \rightarrow 2\text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$), and only when the pathway proceeds all the way to N_2 does
209 it meet the strict definition of denitrification (Zumft, 1997). The four reductive steps are
210 catalyzed by nitrate reductase (NAR), NIR, NOR and nitrous oxide reductase (NOS),
211 respectively. This respiratory process is primarily carried out by heterotrophic denitrifiers
212 (HD) under anoxic or suboxic conditions (Zumft, 1997), which are widely distributed in both
213 soils and aquatic habitats (Knowles, 1982). HD in WWTPs are often closely affiliated with
214 Proteobacteria and Bacteroidetes (Lu *et al.*, 2014). Denitrification rates are commonly
215 measured as NO_3^- reduction rates and have been reported to increase with increasing pH
216 with an optimum in the range of pH 7.0 to 8.0 (Van Cleemput and Patrick, 1974; Knowles,
217 1982). Recently, Šimek and coworkers suggested that the expression 'optimum pH for
218 denitrification' should be avoided, as the results for pH optima obtained by different
219 experimental methodology and denitrifier communities were incongruent (Šimek and
220 Cooper, 2002; Šimek *et al.*, 2002). Studies have shown, that denitrification in activated
221 sludge from WWTPs decreased at low pH values (pH < 6-6.5), generating N_2O as the
222 major product, which suggests a non-uniform effect of pH on the different enzymatic

223 reactions during denitrification (Hanaki *et al.*, 1992; Thomsen *et al.*, 1994; Thörn and
224 Sörensson, 1996). Recently discovered obligate N₂O reducers, that only possess the
225 genetic potential to reduce N₂O to N₂, could be of interest in terms of N₂O mitigation
226 strategies (Jones *et al.*, 2013; Graf *et al.*, 2014). Managing their activity in microbial
227 communities could reduce net-N₂O production by increasing N₂O consumption.

228 **Denitrifying anaerobic methane oxidizing bacteria and other bacteria hosting nitric** 229 **oxide dismutase**

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

243 NOD produces oxygen and thus exerts a control of the denitrifying pathway of DAMO.
244 Once oxygen concentrations increase, the expression of NIR – that supplies NOD with its
245 substrate NO – is downregulated, which results in a decrease of denitrification activity

246 (Luesken *et al.*, 2012). However, the enzyme that is responsible for aerobic methane
247 oxidation (pMMO) in DAMO bacteria can scavenge the oxygen produced by NOD, thus
248 avoiding the downregulation of NIR. pMMO is located in the cell membrane and is in
249 contact with the periplasm (Wu *et al.*, 2011). The balance between pMMO and NOD
250 enables DAMO bacteria to simultaneously oxidize methane aerobically and to denitrify
251 NO_2^- anaerobically via NOD.

252 Little is known about a potential effect of pH on NOD and NOD hosting bacteria. Generally,
253 further research is needed to identify the microbes that perform nitric oxide dismutation
254 with NOD and to understand a potential impact of pH on them. Learning how to engineer
255 systems that rely on NO dismutation could potentially decrease N_2O emissions from water
256 treatment systems.

257 In summary, a wide range of growth pH optima have been reported for different
258 microorganisms involved in the N-network (Fig. 3). Based on these optima, it is not
259 feasible to deduce favorable pH set-points for wastewater treatment processes, as pH
260 alone is likely insufficient to engineer the microbial community and select for or against
261 functional microbial guilds to an extent that would reduce net- N_2O production in these
262 systems. Instead, pH may be a suitable tool to manage fluxes through different enzymatic
263 conversion routes, when a pH control at the enzymatic level proves successful.

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

265 Outside the pH optimum of an enzyme, a reduction in catalytic activity is mainly caused by
266 changes in the enzyme's structural integrity (Illanes *et al.*, 2008). Changes of structure are
267 caused by the disruption of covalent and non-covalent bonds in the polymers.

268 Furthermore, changes of the ionization state of amino acid residues in the active site of an
269 enzyme can affect catalytic activity (Illanes *et al.*, 2008). Information about the ionization
270 state of amino acid residues is relevant to understand how the activity of an enzyme may
271 be affected by pH (Nelson and Cox, 2005). The isoelectric point (pI) defines the pH at
272 which the occurrence of an amino acid in its protonated and de-protonated form is
273 balanced; the pI is used in this review to inform the reader about the ionization states of
274 amino acids. Amino acid residues stabilize metal ions and solvents, cause the secondary,
275 tertiary and quaternary structure of an enzyme and form electron and proton transport
276 routes within enzymatic complexes (Nelson and Cox, 2005).

277 The molecular structure has been solved for a reference of most of the enzymes
278 discussed in this article (Table 1). The high-resolution structures reveal catalytically active
279 sites, intramolecular electron/proton transfer pathways and electron acceptor/donor
280 binding sites. All enzymes involved in the discussed N-conversion processes possess at
281 least one of the following inorganic co-factors: iron-sulfur clusters (Fe-S), Molybdenum (as
282 part of a molybdopterin cofactor) or Cu or Fe (often in the form of heme groups), all of
283 which play relevant functional roles in the enzymes. The effect of pH on each of these
284 structures appears to be consistent across the enzymes.

285 **Table 1 – Enzymes involved in N-transformation reactions.**

Enzyme	Full name	Reaction	pH optimum	Cellular location	Structure available	Catalytic atom
AMO	ammonia monooxygenase	$\text{NH}_3 + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O}$	Not reported (7.5 for related particulate methane monooxygenase (pMMO)) (Prior and Dalton, 1985; Zahn and DiSpirito, 1996)	Cytoplasmic membrane (Fiencke and Bock, 2006; Simon and Klotz, 2013)	Not available, refer to the structure of pMMO (Lieberman and Rosenzweig, 2005)	Likely Cu
HAO	hydroxylamine dehydrogenase	$\text{NH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{HNO}_2 + 4\text{H}^+ + 4\text{e}^-$ or $2\text{NH}_2\text{OH} + 1.5\text{O}_2 \rightarrow 2\text{NO} + 3\text{H}_2\text{O}$	8.5 (Hooper <i>et al.</i> , 1984)	Periplasm (Igarashi <i>et al.</i> , 1997)	Yes (Igarashi <i>et al.</i> , 1997)	Fe (heme)
NXR <i>Nitrobacter</i>	nitrite oxidoreductase	$\text{NO}_2^- + 0.5\text{O}_2 \rightarrow \text{NO}_3^-$	Not reported	Cytoplasmic membrane (Cobley, 1976)	No	Mo
NXR <i>Nitrospira</i>	nitrite oxidoreductase	$\text{NO}_2^- + 0.5\text{O}_2 \rightarrow \text{NO}_3^-$	Not reported	Periplasm (Koch <i>et al.</i> , 2015)	No	Mo
NAR	nitrate reductase	$\text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$	7 ± 0.5 (Carlson <i>et al.</i> , 1982)	Cytoplasm (Berks <i>et al.</i> , 1995; Bertero <i>et al.</i> , 2003)	Yes (Moura <i>et al.</i> , 2004)	Mo
NAP	nitrate reductase	$\text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$	8 (Radcliffe and Nicholas, 1970); 6.5-7.5 (Carlson <i>et al.</i> , 1982)	Periplasm (Tavares <i>et al.</i> , 2006)	Yes (Jepson <i>et al.</i> , 2007)	Mo
Cu-NIR	nitrite reductase	$\text{NO}_2^- + 2\text{H}^+ + \text{e}^- \rightarrow \text{NO} + \text{H}_2\text{O}$	<6.5 (Kim <i>et al.</i> , 2009); 5.6-6 (Wijma <i>et al.</i> , 2006)	Periplasm (Silvestrini <i>et al.</i> , 1994)	Yes (Fukuda <i>et al.</i> , 2016)	Cu
cd-NIR	nitrite reductase	$\text{NO}_2^- + 2\text{H}^+ + \text{e}^- \rightarrow \text{NO} + \text{H}_2\text{O}$	5.8 (Richter <i>et al.</i> , 2002); 6.7 (Lam and Nicholas, 1969)	Periplasm (Silvestrini <i>et al.</i> , 1994)	Yes (Farver <i>et al.</i> , 2009)	Fe (heme)
cNOR	nitric oxide reductase	$2\text{NO} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$	6 (Field <i>et al.</i> , 2002); 5 (Flock <i>et al.</i> , 2005)	Cytoplasmic membrane (Hino <i>et al.</i> , 2010)	Yes (Hino <i>et al.</i> , 2010)	Fe
NOS	nitrous oxide reductase	$\text{N}_2\text{O} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{N}_2 + \text{H}_2\text{O}$	7 (Fujita and Dooley, 2007); 8 (Johnston <i>et al.</i> , 2017)	Periplasm (Pauleta <i>et al.</i> , 2013)	Yes (Pauleta <i>et al.</i> , 2013)	Cu
HZS	hydrazine synthase	$\text{NH}_4^+ + \text{NO} + 2\text{H}^+ + 3\text{e}^- \rightarrow \text{N}_2\text{H}_4 + \text{H}_2\text{O}$	Not reported	Anammoxosome (Kartal <i>et al.</i> , 2011)	Yes (Dietl <i>et al.</i> , 2015)	Fe (heme)
HDH	hydrazine dehydrogenase	$\text{N}_2\text{H}_4 \rightarrow \text{N}_2 + 4\text{H}^+ + 4\text{e}^-$	8 (Schalk <i>et al.</i> , 2000); 8-8.5 (Shimamura <i>et al.</i> , 2008)	Anammoxosome (Kartal <i>et al.</i> , 2011)	Yes (Maalcke <i>et al.</i> , 2016)	Fe (heme)

NOD	nitric oxide dismutase	$2\text{NO} \rightarrow \text{N}_2 + \text{O}_2$	Not reported	Periplasm (Wu <i>et al.</i> , 2011)	Partly (in comparison to qNOR) (Ettwig <i>et al.</i> , 2012)	Fe (heme)
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286 3.1. The effect of pH on catalytic metal atoms

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

291 **Copper**

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

296 Cu-NIR contains two Cu-sites per homotrimer, type-1 and type-2, each of which contains
297 one Cu-atom (Jacobson *et al.*, 2007). The type-1 copper site is located close to the
298 surface of Cu-NIR and accepts an electron from electron donors like pseudoazurin
299 (Kukimoto *et al.*, 1996). The electron from the type-1 copper site is transferred to the type-
300 2 copper in the active site to reduce a NO_2^- molecule (Suzuki, 1994). The type-2 copper
301 site is located between two monomers of Cu-NIR and is directly involved in catalysis –
302 NO_2^- binds to the copper ion (Godden *et al.*, 1991). High pH (pH 8.4) leads to the
303 deprotonation of amino acid residues in the proximity of the type-2 Cu-ion and also results
304 in a loss of protons of the solvent ligand in proximity of the type 2 Cu-ion (Jacobson *et al.*,
305 2007). The solvent ligand has been suggested, depending on the pH, to be either a water

306 molecule or hydroxide ion that contributes with the presence or absence of its proton to the
307 active site geometry. With the absence of protons from amino acid residues and the
308 solvent ligand at pH 8.4 structural transformations of the type-2 copper site geometry were
309 reported, which change the site geometry from a nearly regular tetrahedron into a more
310 distorted tetrahedral geometry (Godden *et al.*, 1991; Jacobson *et al.*, 2007). It was
311 hypothesized that the type-2 copper site is unable to accept an electron from the type-1
312 copper site in the distorted geometry, which may explain the rapid decrease of enzyme
313 activity with increasing pH (measured in the range pH 6.0 to 9.0)(Jacobson *et al.*, 2007).

314 NOS also contains two Cu-centers, CuA and CuZ (Pauleta *et al.*, 2013; Carreira *et al.*,
315 2017). CuA is located close to the surface of the enzyme and accepts electrons from the
316 physiological electron donors cytochrome c_{550} and pseudoazurin, before passing them on
317 to CuZ (Mattila and Haltia, 2005; Gorelsky *et al.*, 2006). CuZ constitutes the catalytic site
318 and contains four Cu-atoms, which are bridged by two sulfur atoms (Pauleta *et al.*, 2013).
319 The intramolecular electron transfer between CuA and CuZ appears to be rate limiting for
320 N_2O reduction in the range of $4 < \text{pH} < 8$, when the electron transfer rate to CuZ is hampered
321 due to conformational changes of CuA (Gorelsky *et al.*, 2006). However, at more alkaline
322 pH ($\text{pH} > 8.9$) a loss of catalytic activity was also observed, likely due to the deprotonation
323 of a lysine residue ($\text{pI} = 9.7$) in CuZ, which in its deprotonated form cannot stabilize the
324 Cu_Z° intermediate anymore (Johnston *et al.*, 2017). Therefore, the protonation of the
325 lys397 residue appears to be essential for a successful reduction of N_2O via Cu_Z° . The
326 balance between both effects of pH, either the effect of pH on the electron transfer from
327 CuA to CuZ or the protonation state of lys397, may contribute to an observed pH optimum
328 of pH 7-8 (Fujita and Dooley, 2007; Johnston *et al.*, 2017).

329 Iron

330 Iron atoms play a central role in almost all enzymes involved in the N-network and act
331 either as catalysts in the active sites or mediate electron transfer within an enzymatic
332 complex. The iron atoms in the active sites are often (e.g. HAO, cd-NIR, HZS, HDH,
333 cNOR), but not always (e.g. cNOR; cNOR contains one heme and one non-heme Fe) part
334 of heme groups. The Fe-atoms in heme groups commonly interact with proximal histidine
335 (his) residues (pI=7.6) (Igarashi *et al.*, 1997; Maalcke *et al.*, 2016). Protonated his-residues
336 move the Fe-atom out of the heme plane and cause the Fe-atom to change its spin state
337 (Perutz *et al.*, 1998). The switch of spin state R to T results in a change of free energy that
338 eventually results in a decrease of substrate affinity and catalytic rates (details in (Perutz
339 *et al.*, 1998)). In order to successfully stabilize heme Fe-atoms, his-residues apparently
340 need to be present in their deprotonated form. However, the role of his-residues is not
341 limited to the stabilization of Fe-atoms. For instance, in cd-NIR his-residues are directly
342 involved in the binding of substrate, when a his-residue binds one of the oxygen atoms of
343 the substrate NO₂⁻ by hydrogen bonds (Tavares *et al.*, 2006). cd-NIR is highly reactive *in*
344 *vitro* at pH<7, but poorly active at alkaline pH, when his-residues are deprotonated
345 (Tavares *et al.*, 2006).

346 Molybdenum

347 The molybdenum containing molybdo-bis (pyranopterin guanine dinucleotide) co-factor is
348 characteristic for NXR, NAR and NAP and catalyzes either oxidative hydroxylation or
349 reductive dehydroxylation, in which the hydroxyl group is derived from water (Rajagopalan
350 and Johnson, 1992). In principle, one Mo-atom is coordinated with two pyranopterins and
351 interacts with cys- (NAP) or ser- or asp-residues (NAR)(pI=5.1, 5.7 and 2.8,
352 respectively)(Moura *et al.*, 2004). Two conserved his-residues in the proximity of the

353 pyranopterin rings appear to be of significance for the catalytic activity of NAR (Bertero *et*
354 *al.*, 2003; Wu *et al.*, 2015). In their protonated form the his-residues stabilize the
355 pyranopterin rings and the Mo(V) intermediate during catalysis. Upon loss of an overall
356 positive charge, the midterm potential of the molybdenum ion decreases, which is directly
357 correlated with enzyme activity (Wu *et al.*, 2015). Therefore, an increase of pH appears to
358 directly transfer into a loss of catalytic turnover of NAR.

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

363 **3.2. The effect of pH on intramolecular proton and electron transport**

364 The effect of pH on enzymes is not limited to amino acid residues in proximity of catalytic
365 metal ions. The transfer of protons and electrons towards and away from the catalytic sites
366 is also affected by pH. Proton transport usually occurs through channels in the three-
367 dimensional structure and is mediated by initially deprotonated amino acid residues. For
368 example, in cNOR the transfer of protons from the periplasm to the active site is crucial for
369 NO reduction activity (Flock *et al.*, 2008). Protons are channeled by a series of glu-
370 residues ($pI=3.2$) from the surface of the enzyme to the catalytic heme centers. The exact
371 mechanism of proton transport is unknown, but the transfer is strongly affected by pH and
372 decreases with increasing pH from $k_{max} = 250 \text{ s}^{-1}$ at pH 5 to $k_{obs}=10 \text{ s}^{-1}$ at pH 8 (Flock *et*
373 *al.*, 2005). The transfer of protons likely becomes the rate limiting step of cNOR at neutral
374 and alkaline pH (Flock *et al.*, 2005).

375 In HZS it has been proposed that a cluster of polar amino acid residues (γ Asp112, $pI=2.8$;
376 γ Arg143 and γ Arg167, $pI=10.8$) between γ Asp168 and the surface mediates proton
377 transfer to the active site (Dietl *et al.*, 2015). However, the exact mechanism is unknown.
378 The effect of changing pH on the proton transfer of HZS has not been studied and may in
379 fact be of low relevance, as the pH inside the anammoxosome has been reported to be
380 stable (approx. pH 6.3)(van der Star *et al.*, 2010).

381 Electron transfer is usually mediated by series of heme or Fe-S clusters (HAO, NXR, NAR,
382 NAP, cd-NIR, cNOR) that accept electrons from electron donors, like cytochromes, at the
383 surface of the enzymatic complexes and transfer them sequentially to the active sites. The
384 edge-to-edge distance between heme or Fe-S clusters has to be short enough for direct
385 electron transfer, e.g. 15 Å in HZS or 7 Å and 11 Å in NAR (Bertero *et al.*, 2003; Dietl *et*
386 *al.*, 2015). Larger distances can be bridged by amino acid residues (Bertero *et al.*, 2003;
387 Dietl *et al.*, 2015). For instance, in HZS it has been proposed that three electrons from the
388 redox partner kuste2854 enter the enzymatic complex via a haem (γ II) close to the surface
389 of HZS (Dietl *et al.*, 2015). The electrons may then be transferred to another haem (γ I) that
390 is situated in the active site. A his-residue (γ His144) that is located between γ I and γ II may
391 play an important role in the electron transfer between both haems and its protonation
392 state may affect electron transfer rates. However, in general, it is not well understood how
393 pH affects these electron transport paths in detail, although effects of pH have been
394 described in some cases: In NAR for example the electron transfer to the Mo-center
395 occurs via [Fe-S] clusters and is affected by pH (Bertero *et al.*, 2003; Rothery *et al.*, 2004).
396 The protonation state of Arg-residues ($pI=10.8$) may play a role in altering the midterm
397 potential of the [Fe-S] clusters, but the exact mechanism is unknown (Bertero *et al.*, 2003).

398 In summary, molecular structures with their detailed information about the position of
399 amino acid residues, ligands and metal atoms give valuable insights in how variations in
400 pH cause changes in catalytic sites, ligand affinity or electron and proton transport routes.
401 The insights on the molecular level may also facilitate the interpretation of changes of
402 overall enzyme kinetics with pH. However, findings on the molecular level have to be
403 augmented with kinetic data in order to quantify the effect of pH on enzymatic conversion
404 rates.

405 **3.3. pH optima of enzyme activity**

406 **3.3.1. The discrepancy between *in vitro* and *in vivo* enzyme kinetics.**

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

420 activity from *in vitro*-assays may serve as a starting point for considerations about
421 favorable pH set-points.

422 3.3.2. pH optima of NIR, NOR and NOS in respect to N₂O net-production

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

427 **Figure 4**

428 NOR and NIR play an important role in N₂O production, as they either produce N₂O
429 directly (NOR) or produce NO (NIR), a precursor of N₂O. NO is a free radical with
430 cytostatic or –toxic properties. In order to keep intracellular NO concentrations low,
431 microorganisms have developed rapid detoxification mechanisms that involve reduction of
432 NO to N₂O, e.g. by NOR or cytP₄₆₀ (Stein, 2011; Caranto *et al.*, 2016). Oxidation rate
433 constants for NOR are pH dependent and decrease with increasing pH (oxidation rate
434 constants of NOR at pH 6.0 = 100 s⁻¹ and at pH 7.5 = 12 s⁻¹ (Lachmann *et al.*, 2010)).
435 Therefore, N₂O production by NOR decreases at alkaline pH.

436 NIR catalyzes the reduction of NO₂⁻ to NO, a reaction that is reversible. In fact, it has been
437 reported that the directionality of Cu-NIR of *Alcaligenes faecalis* is strongly dependent on
438 pH (Wijma *et al.*, 2004). K_{cat} for nitrite reduction was twelve times higher at pH 6.0, than at
439 pH 8.0. The catalytic bias of NIR towards oxidation of NO to NO₂⁻ at pH 8, rather than
440 reduction of NO₂⁻, is caused by an altered reduction potential of the electron donor
441 pseudoazurin and hydroxyl inhibition above pH 7 (Wijma *et al.*, 2004). However, as the

442 reduction of NO_2^- to NO clearly occurs during denitrification at pH 7-8 in natural systems,
443 the effect of pH on the enzyme is unlikely the only factor that determines the catalytic
444 directionality of NIR. The findings are particularly interesting in the context of the recently
445 proposed role of Cu-NIR during nitritation (Caranto and Lancaster, 2017). Further research
446 will inform if the bidirectionality of Cu-NIR determines the fate of NO during nitrification and
447 denitrification and thus acts as a source or sink of NO as a precursor of N_2O .

448 In contrast to NIR and NOR, the activity of NOS determines, whether N_2O or N_2 is the final
449 product of denitrification (as comprehensively reviewed in (Carreira *et al.*, 2017)). High
450 activity of NOS increases N_2O consumption and has the potential to decrease net N_2O
451 production. The $\text{N}_2\text{O}:\text{N}_2$ product ratio of denitrification increases with decreasing pH and
452 conversely decreases with increasing pH (Šimek and Cooper, 2002; Bergaust *et al.*, 2010)
453 . However, an increase of $\text{N}_2\text{O}:\text{N}_2$ at acidic pH is likely not caused by lower gene copy
454 numbers of *nosZ*, nor lower transcription rates of *nosZ* vs. *nirS* that may explain an
455 imbalance between N_2O production and consumption rates; on the contrary, the ratio of
456 *nosZ/nirS* transcripts is larger at lower pH (pH = 6.1), than at higher pH (pH = 8.0)(Liu *et*
457 *al.*, 2010). pH seems to affect the $\text{N}_2\text{O}:\text{N}_2$ ratio mainly on the post-transcriptional level,
458 when higher pH determines the successful assembly of functional NOS at pH >7, whereas
459 at pH ≤ 6.1 the assembly of functional NOS seems to be disturbed (Liu *et al.*, 2014).

460 Does the effect of pH on NIR, NOR and NOS mean that for lower N_2O net-production, N-
461 removal processes should simply be operated at more alkaline pH to promote N_2O
462 consumption over production? A direct correlation between pH optima of individual
463 enzymes and the N_2O net-production of entire N-removal processes is unlikely, as
464 enzymes cannot be treated as independent entities. They depend on electrons and

465 substrates from other enzymatic conversion steps. Therefore, the comparison of individual
466 enzyme activities as a function of pH alone does not necessarily hold sufficient information
467 to evaluate the effect of pH on transformation rates of entire pathways.

468 **4. The effect of pH on pathway conversion rates and net-N₂O** 469 **production**

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

473 **Figure 5**

474 **4.1. Nitritation**

475 *Nitritation (AOB)*

476 Nitritation has been understood as the sequential oxidation of NH₃ to NO₂⁻ via NH₂OH, in
477 which the oxidation of NH₃ to NO₂⁻ is catalyzed by AMO and HAO (Kostera *et al.*, 2010).
478 Recently, *in vitro* experiments showed that HAO reduces NH₂OH in a three electron
479 oxidation to NO, instead of a four electron oxidation to NO₂⁻ (Caranto and Lancaster,
480 2017). The occurrence of NO₂⁻, as observed in previous NH₂OH oxidation experiments,
481 would then either be attributed to a subsequent non-enzymatic oxidation of NO to NO₂⁻ by
482 O₂ or an enzymatic oxidation step (potentially via Cu-NIR). An enzymatic oxidation of NO
483 to NO₂⁻ would preserve the fourth electron from NH₂OH oxidation in the electron pool to
484 contribute to the generation of the proton motive force (Caranto and Lancaster, 2017).
485 From a thermodynamic perspective, nitritation is favored at alkaline pH, when the Gibbs
486 free energy for NH₃ oxidation is larger at neutral and alkaline pH, than at acidic pH

487 ($\Delta G^{\circ}_{25^{\circ}\text{C},\text{pH } 7} = -274.7 \text{ kJ/kmol N}$ vs. $\Delta G^{\circ}_{25^{\circ}\text{C},\text{pH } 4} = -240.5 \text{ kJ/kmol N}$)(Wrage *et al.*, 2001).

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

494 The current model of electron flow in AOB during nitrification describes that the four
495 electrons released by HAO are transferred to electron acceptors, either cytochrome 554
496 (c_{554}) or directly to membrane bound c_{m552} . The electrons then enter the ubiquinone pool
497 possibly via a designated "Hydroxylamine Ubiquinone Redox Module" (HURM) (Hooper *et al.*
498 *et al.*, 1997; Simon and Klotz, 2013; Perez-Garcia *et al.*, 2014). Two electrons are channeled
499 back to AMO for the reduction of O_2 , while the remaining electrons are used to establish
500 the proton motive force. However, the exact pathways of electron transport in AOB during
501 nitrification remain to be resolved and may involve other redox mediators, such as c_{552} .
502 Additionally, direct interactions between the cytochromes that alter the electron flow
503 cannot be excluded, e.g. c_{554} with c_{m552} (Simon and Klotz, 2013; Stein *et al.*, 2013;
504 Kozłowski *et al.*, 2016). The model needs to be refined in respect to the findings of NH_2OH
505 oxidation to NO (Caranto and Lancaster, 2017). However, the proposed three electron
506 oxidation of NH_2OH to NO by HAO may be compatible with the current model. The flow of
507 one electron to NirK for the reduction of NO_2^- to NO during denitrification, which is
508 commonly mediated by cytochromes $c_{550-553}$ or (pseudo) azurins or cupredoxins (Simon
509 and Klotz, 2013), may be reversed and that way contribute an electron to the generation of
510 the proton motive force. It has been demonstrated that the reduction of NO_2^- to NO is

511 reversible and that the catalytic equilibrium is dependent on pH: at acidic pH (pH 6.2) NO_2^-
512 reduction occurred at faster rates, than NO oxidation, while at pH 8.0 NO oxidation to NO_2^-
513 was faster (Wijma *et al.*, 2004). Therefore, the pH may be relevant for the accumulation of
514 NO during nitritation.

515 In the proposed nitritation pathway by Caranto and Lancaster, i.e. $\text{NH}_3 \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO} \rightarrow$
516 NO_2^- , the balance between HAO and NO oxidation rates, potentially by NirK, are relevant:
517 a low oxidation rate of NirK would result in the accumulation of NO and potentially to
518 increased N_2O production due to reduction of accumulated NO to N_2O . Both HAO and
519 NirK are located in the periplasm and are affected by ambient pH. The pH optima of both
520 enzymes are substantially different (pH 8.5 for HAO vs. 6.0 for NirK). Lower pH would
521 result in relatively higher NO oxidation rates by NirK, compared to NH_2OH oxidation rates
522 of HAO, which would prevent accumulation of NO. In contrast, at alkaline pH NO oxidation
523 rates of NirK may be lower, than NH_2OH oxidation rates by HAO, which may lead to
524 accumulation of NO and potentially increased N_2O production rates.

525 Observations from nitrifying AOB enrichment cultures and cultures of *N. europaea* support
526 the stimulating effect of pH on nitritation and N_2O production rates (Shammas, 1986). pH
527 optima and minima for N_2O production rates were identified at pH 8.0-8.5 and below pH
528 7.0, respectively (Hynes and Knowles, 1984; Law *et al.*, 2011; Rathnayake *et al.*, 2015). In
529 summary, alkaline pH appears to favor higher nitritation rates and higher N_2O production
530 rates by AOB. The role of the different enzymes involved in nitritation and potential
531 imbalances of metabolic fluxes between them will be elucidated in future studies.

532 *Nitritation (AOA)*

533 N₂O production during ammonia oxidation has been detected in pure and enrichment
534 cultures of AOA (Jung *et al.*, 2014; Stieglmeier *et al.*, 2014). Compared to the better
535 elucidated N₂O production pathways of AOB, the mechanisms of N₂O production by AOA
536 are not well understood, due to the lack of physiological and genomic evidence for
537 enzymatic processes for N₂O production (Walker *et al.*, 2010; Tourna *et al.*, 2011; Spang
538 *et al.*, 2012). Recently, NO and NH₂OH have been identified as essential intermediates
539 during archaeal ammonia oxidation, however both intermediates are probably reduced to
540 N₂O non-enzymatically (Vajrala *et al.*, 2013; Zhu-Barker *et al.*, 2015; Kozlowski *et al.*,
541 2016). This contrasts to the enzymatic N₂O production in bacteria (Walker *et al.*, 2010;
542 Tourna *et al.*, 2011; Spang *et al.*, 2012; Stieglmeier *et al.*, 2014). Hence, the production of
543 N₂O during ammonia oxidation in soil was suggested to be lower for AOA, than AOB (Hink
544 *et al.*, 2017). pH selects for different bacterial and archaeal communities and therefore
545 determines the relative contribution of AOA and AOB to overall N₂O production (Nicol *et*
546 *al.*, 2008). In fact, AOA become the dominant contributors to nitrification and respective N₂O
547 production rates in low pH environments, e.g. soils (Li *et al.*, 2018). The production of N₂O
548 by AOA in wastewater treatment systems has, to our knowledge, not been documented as
549 relevant.

550 **4.2. Nitrifier denitrification**

551 Nitrifier denitrification (ND) describes the reduction of NO₂⁻ to N₂O by NIR and NOR in
552 AOB (Wrage *et al.*, 2001). Rates of ND increase at low oxygen tension. In the current
553 model a bottleneck of electron flow occurs at low oxygen tension between HAO and the
554 terminal oxidase, likely between HAO and c_M552 or in the c552 pool between the
555 cytochrome complexes C-III and C-IV, as a result of insufficient availability of oxygen as an
556 electron acceptor (Simon and Klotz, 2013; Kozlowski *et al.*, 2016). In order to relieve the

557 bottleneck NIR and NOR may be activated and accept electrons from c_{552} to reduce NO_2^-
558 and NO to N_2O , thus constituting the nitrifier denitrification pathway (Kozłowski *et al.*,
559 2016). However, the regulation of electron flow in AOB does not follow a single principle
560 and different phylotypes appear to apply different regulatory features, which likely depend
561 on the genomic history and environment (Stein *et al.*, 2013).

562 A relevant role of regulation may be assigned to the small red-copper protein
563 nitrosocyanin. Nitrosocyanin may regulate whether electrons from HAO are transferred to
564 c_{554} or c_{M552} , thus determining their fate in the electron flow cascade of AOB (Stein *et al.*
565 *et al.*, 2013). Electrons loaded to c_{M552} would directly enter the quinone pool to be
566 distributed to AMO, whereas electrons loaded to c_{554} would be available for further
567 distribution to other electron carriers, e.g. c_{552} , and to NIR and NOR. Therefore,
568 nitrosocyanin may act as a regulator of electron flow in order to obtain redox balance in
569 environments with sufficiently high substrate concentrations (Stein *et al.*, 2013).

570 It is challenging to study the rate of nitrifier denitrification isolated. Accordingly, a pH
571 optimum is difficult to obtain. Based on the pH optima of NIR and NOR, it may be
572 hypothesized that elevated rates of ND occur at slightly acidic pH (Table 1). However, as
573 mentioned above, ND occurs in a complex interaction with the cellular electron pool and
574 different electron carriers and a deduction of a pH optimum only from NIR and NOR is
575 likely too simple. It may be speculated that, similar to c_{554} (4.1., (McTavish *et al.*, 1995)), an
576 effect of pH also on c_{552} or nitrosocyanin may play a role for the conversion rates of NIR
577 and NOR. However, it is currently unknown, if the pH affects the concentration of c_{552} or
578 nitrosocyanin directly or indirectly or how pH affects nitrifier denitrification rates in general.

579 4.3. Anaerobic ammonia oxidation

580 The anaerobic ammonia oxidation (anammox) pathway comprises three sequential steps
581 in which the substrates NH_4^+ and NO_2^- are converted into N_2 (Strous *et al.*, 2006; Kartal *et*
582 *al.*, 2011, 2013). Initially, NO_2^- is reduced to NO by NIR. Then, NH_4^+ is oxidized with NO to
583 hydrazine (N_2H_4) by HZS. Finally, N_2H_4 is oxidized to N_2 by HDH. All enzymes involved are
584 located in the anammoxosome. The transfer of electrons and protons between the three
585 enzymatic reactions has been proposed to be mediated by the quinol::cytochrome c
586 oxidoreductase system (complex III, bc1 complex) in the membrane of the
587 anammoxosome (Kartal *et al.*, 2011). The anammox pathway does not include N_2O as an
588 intermediate (Strous *et al.*, 2006; Kartal *et al.*, 2013) and is generally not considered to
589 yield N_2O . Although the enzymes involved in anammox are located in the
590 anammoxosome, and therefore a change of ambient pH is unlikely to affect the enzymes
591 directly, anammox rates increase with pH and are highest between pH 7.5-8.3. An
592 explanation for this paradox may be found in an inhibitory effect of pH on other cellular
593 processes, than the enzymatic conversion reactions themselves, which results in changes
594 of anammox rates with pH. Substrate transporters have been proposed to play a
595 significant role in substrate trafficking of AnAOB cells and an effect of pH on these proteins
596 is likely, yet largely unknown (Kartal *et al.*, 2013).

597 4.4. Heterotrophic denitrification

598 Heterotrophic denitrification (HD) refers to the reduction of NO_3^- or NO_2^- to gaseous
599 products (NO, N_2O and N_2), in which NO_3^- or NO_2^- serve as terminal electron acceptors.
600 HD involves the enzymes NAR, NIR, NOR and NOS. HD comprises both N_2O production
601 and consumption reactions. The ratio of complete (N_2) and incomplete (N_2O) denitrification

602 as a function of pH is relevant in respect to net-N₂O production. Various studies, especially
603 from agricultural soils, report an increased N₂O/(N₂+N₂O) ratio with decreasing soils
604 (Šimek and Cooper, 2002; Bergaust *et al.*, 2010; Bakken *et al.*, 2012), which was assigned
605 to an impaired post-transcriptional assembly of NOS at pH < 7.0 (Liu *et al.*, 2014). The
606 effect of a relatively higher N₂O/(N₂+N₂O) ratio at low pH may however be compensated
607 by a decline in overall denitrification rates with decreasing pH (NO₃⁻ reduction rates
608 increase with pH with an optimum between pH 7.0 and 8.0 (Van Cleemput and Patrick,
609 1974; Knowles, 1982)). Absolute N₂O production rates from denitrification can therefore be
610 expected to be highest in the range pH 7.0-7.5, before N₂O reduction by NOS becomes
611 effective at pH > 7.5 (Liu *et al.*, 2014).

612 **4.5. Oxygenic denitrification of nitrite**

613 The oxygenic denitrification of nitrite is a two steps process, in which nitrite is first reduced
614 by cd-NIR to NO. NO is then further dismutated to N₂ and O₂ by NOD. The process
615 bypasses N₂O as an intermediate towards N₂. An effect of pH on process rates of
616 oxygenic denitrification of nitrite has, to our knowledge, not been reported yet. NIR and
617 NOD are usually located in the periplasm and are likely exposed to changes of ambient
618 pH. It may therefore be hypothesized that denitrification rates decrease, when pH diverges
619 from the pH optimum of NIR (pH 5.8-6.7) and NOD (pH optimum not reported). The pH
620 optimum of pMMO (pH 7.5; (Zahn and DiSpirito, 1996)) may also play a role, as reduced
621 activity of pMMO may lead to accumulation of oxygen that in turn inhibits the denitrification
622 process. However, with the current knowledge no reasonable pH optimum for oxygenic
623 denitrification of nitrite via NOD can be identified.

624 In summary, slightly alkaline pH results in higher rates of nitritation, anammox and
625 complete denitrification. Although changes of nitrifier denitrification and nitratation rates
626 with pH have not been assessed in detail, rates of ND and nitratation are expected to
627 decrease with increasing pH based on information available for the enzymes involved in
628 these pathways (Fig. 5). The link between enzymatic pH optima and pathway conversion
629 rates may enable predictions of net-N₂O production rates.

630 **5. Exploring favorable pH set-points for lower net-N₂O production in** 631 **wastewater bio-reactors.**

632 Based on the findings of the previous sections, this last section hypothesizes favorable pH
633 set-points (in respect to lower net-N₂O production rates) for nitrogen removing bio-reactors
634 in wastewater treatment applications. The microbial communities in these systems
635 commonly comprise AOB, AnAOB, NOB and HD. In respect to N₂O production AOB
636 appear to be of most significance, whereas HD play a dominant role for N₂O consumption.
637 As both functional guilds, i.e. AOB and HD, are present in essentially all nitrogen removing
638 wastewater systems like nitrification-denitrification, partial nitrification or partial nitritation-
639 anammox, considerations may be transferable between applications. The effect of pH on
640 net N₂O production rates in these systems is hypothesized in three scenarios: pH 6.0-6.5,
641 pH 7.0-7.5 and pH 8.0-9.0.

642 ***Slightly acidic pH 6.0-6.5***

643 At slightly acidic pH, e.g. pH 6.5, nitritation rates are not at maximum (see 4.1), as the pH
644 optimum of HAO lies at approx. 8.0-8.5. The proximity to the pH optimum of Cu-NIR
645 (approx. pH 6, see Fig. 4) would result in an efficient removal of NO. Both N₂O production

646 and consumption rates are expected to be low at slightly acidic pH, which results in low net
647 N_2O production rates. Therefore, lower net N_2O production rates can potentially be
648 achieved in N-removing bioreactors by operation at slightly acidic pH, yet at the cost of
649 reduced nitrification rates. This mode of operation may be interesting for nitrifying and
650 partially nitrifying applications.

651 ***Neutral pH 7.0-7.5***

652 At neutral pH, e.g. pH 7.0-7.5, nitrification rates increase, as reaction conditions are closer
653 to the pH optimum of HAO. NH_2OH oxidation rates by HAO would be larger than NO
654 oxidation rates by Cu-NIR and accumulated NO would be reduced to N_2O by either NOR
655 or other detoxification mechanisms. N_2O production rates are therefore expected to be
656 higher at pH 7.0-7.5, than at pH 6.5. Net N_2O production rates are also expected to be
657 high, as the pH is not high enough to allow an efficient reduction of N_2O to N_2 by NOS. Out
658 of all three pH scenarios net- N_2O production rates are expected to be highest in the range
659 pH 7.0-7.5. This pH range appears as unfavorable for nitrogen removing applications, e.g.
660 nitrification-denitrification, partial nitrification and partial nitrification-anammox, in respect to
661 net N_2O production.

662 ***Alkaline pH 8.0-9.0***

663 NH_2OH oxidation rates would be maximal at pH 8.0-9.0 and NO oxidation rates by Cu-NIR
664 would be elevated. N_2O production rates are expected to peak at alkaline pH. However,
665 other than at pH 7.0-7.5, high N_2O consumption rates may counterbalance production
666 rates at alkaline pH, thus causing low net- N_2O production rates (see Fig 5). Therefore,
667 alkaline pH of approx. 8.0 appears to be favorable for wastewater treatment applications,
668 as net N_2O production rates may be low, while ammonia removal rates can be maintained.

669 In summary, the net N₂O production curve with pH would be “bell-shaped” with low net
670 N₂O production rates at pH ≤ 6.5, high rates at pH 7.0-7.5 and low rates at pH ≥ 8.0.

671 **6. Conclusion**

672 When N₂O production exceeds N₂O consumption microbial communities constitute
673 sources of N₂O. The microbial guilds that drive nitrogen conversion in wastewater
674 treatment processes (ammonia oxidizing bacteria or archaea, complete or anaerobic
675 ammonia oxidizing bacteria, nitrite oxidizing bacteria and heterotrophic denitrifying
676 bacteria) all have physiological growth optima in the range pH 7.0-8.0. Selecting specific
677 functional microbial guilds based on pH does, therefore, not appear feasible for the
678 mitigation of net N₂O production in complex microbial communities.

679 All enzymes that are of particular relevance for N₂O production and consumption are either
680 located in (i.e. HAO, NIR and NOS) or in contact with the periplasm, when embedded in
681 the cytoplasmic membrane (i.e. NOR). Accordingly, they are exposed to changes in
682 ambient pH. In general, pH alters the catalytic activities of enzymes involved in nitrogen
683 conversion, mainly by affecting their structural integrity. However, its effects vary: in Cu-
684 NIR and NOR, protonation or deprotonation of amino acid residues alter the steric
685 organization of catalytic sites (Cu-NIR, cNOR) or the substrate affinity of catalytic metal
686 ions (Cu-NIR, cd-NIR), which leads to sub-optimal conversion rates at pH > 7.0. Electron
687 and proton transfer within enzymatic complexes is also affected by pH. In HZS and NAR
688 electron transport becomes rate limiting with increasing pH, and hampered proton
689 transport reduces the turnover of cNOR 25-fold from pH 5 to 8. Finally, pH also exerts a
690 post-translational effect and prevents the assembly of NOS to a functional enzyme at pH <
691 7.0. The result is a decrease of N₂O consumption rates at acidic pH.

692 The different effects of pH on the molecular level are consistent with rate measurements of
693 different bacterial N-conversion reactions, e.g. nitritation and denitrification, or the N_2O/N_2
694 product ratio of denitrification. Nitritation and N_2O production rates by AOB increase with
695 pH in the range pH 6.0-8.0, while N_2O consumption by heterotrophic denitrifiers increases
696 with pH > 7.0. Overall, net N_2O production in bacterial communities of wastewater
697 engineering applications at low pH < 7 appears to be dominated by N_2O production,
698 whereas at pH > 7.5 N_2O consumption counteracts production and leads to reduced net
699 N_2O production rates. Therefore, lower net N_2O production rates can be achieved at acidic
700 pH < 7.0, however at the cost of lower NH_4^+ removal rates. Low net N_2O production at
701 simultaneously high NH_4^+ removal rates is possible at alkaline pH > 7.5 due to N_2O
702 consumption in microbial communities that host denitrifying bacteria. Highest net N_2O
703 production rates would occur at pH 7.0-7.5, when N_2O consumption does not outweigh
704 increased production.

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710 **Originality-Significance Statement**

711 The presented work contributes to research in environmental microbiology by identifying
712 the effects of pH on the enzymatic level of nitrogen conversion reactions, by identifying
713 trends of conversion rates and relative N_2O production of relevant pathways upon changes

714 of pH, by connecting the effects of pH on the enzyme level with resulting changes on the
715 pathway level and by hypothesizing favorable pH set-points for wastewater treatment
716 applications with the scope of lower net-N₂O production.

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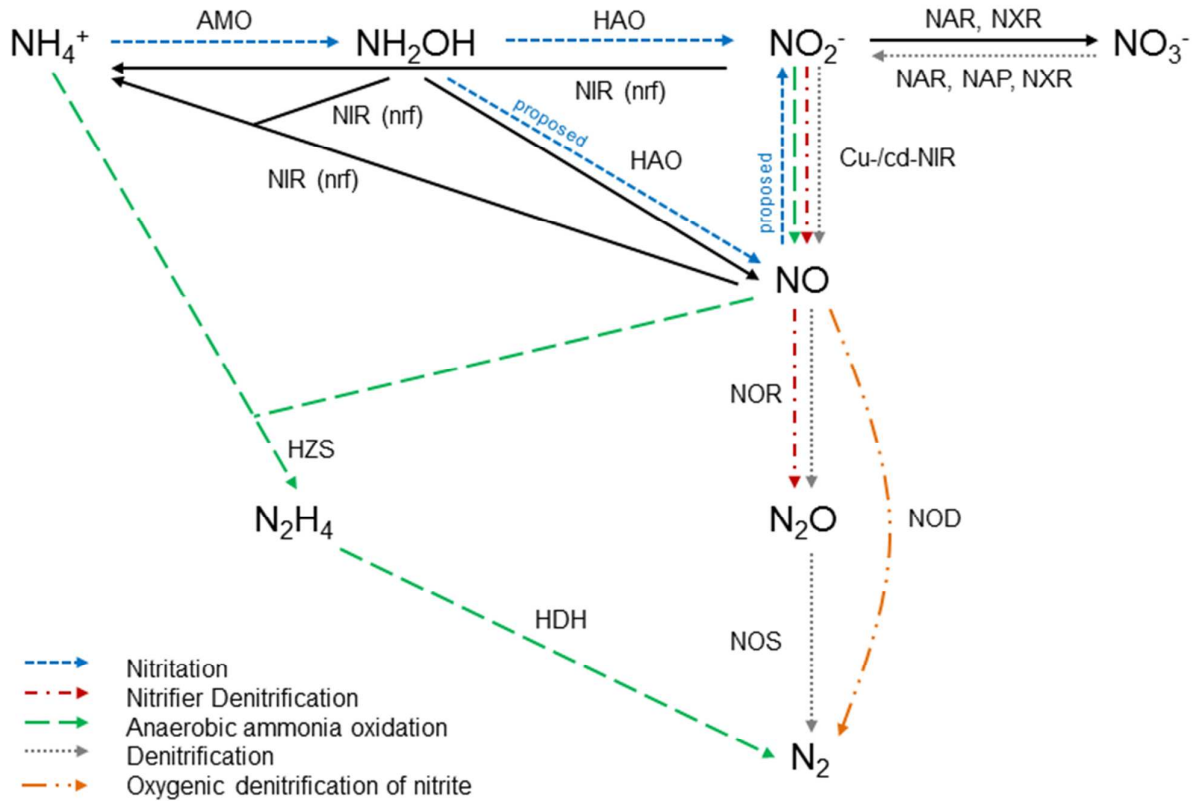


Figure 1 - Web of nitrogen conversion reactions. Enzymes involved in catalysis are ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite oxidoreductase (NXR), nitrite reductase (NIR), nitric oxide reductase (NOR), nitrous oxide reductase (NOS), hydrazine synthase (HZS), hydrazine dehydrogenase (HDH) and nitric oxide dismutase (NOD).

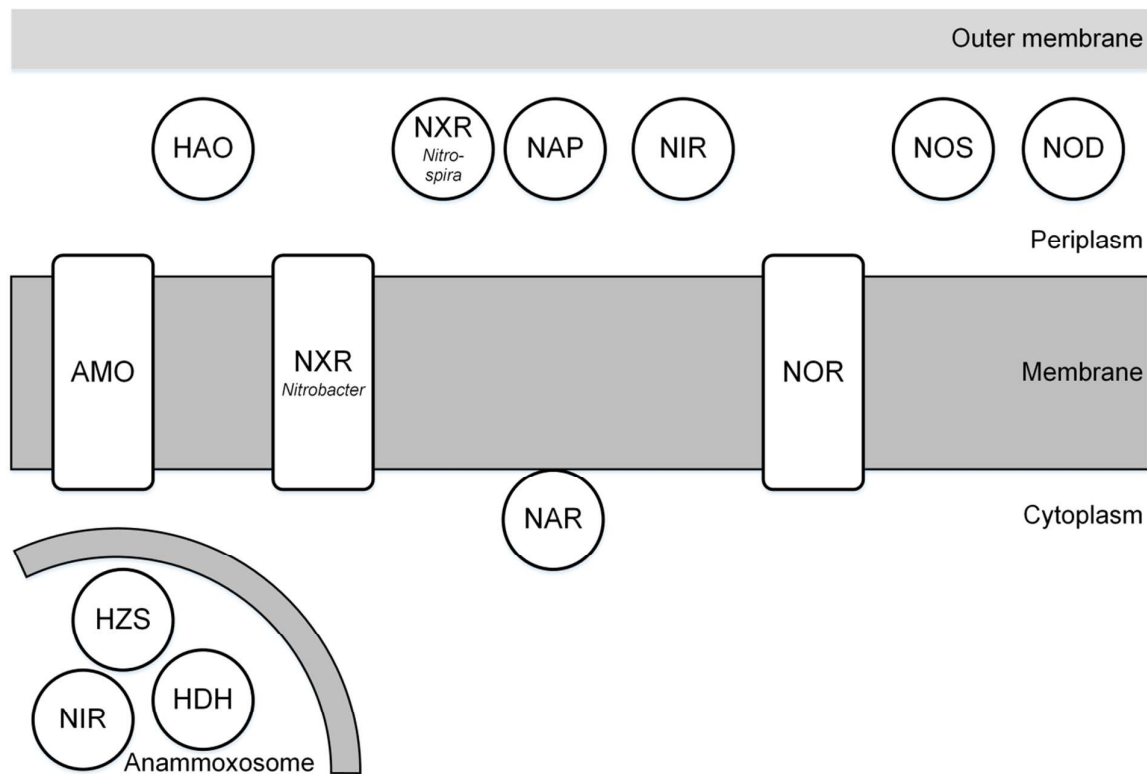


Figure 2 - Location of functional enzymes. The catalytic site of ammonia monooxygenase (AMO) and nitrite oxidoreductase (NXR) *Nitrobacter*, and nitrate reductase (NAR) are located in the cytoplasm. Hydrazine synthase (HZS), hydrazine dehydrogenase (HDH) and nitrite reductase (NIR) of anammox are located 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.

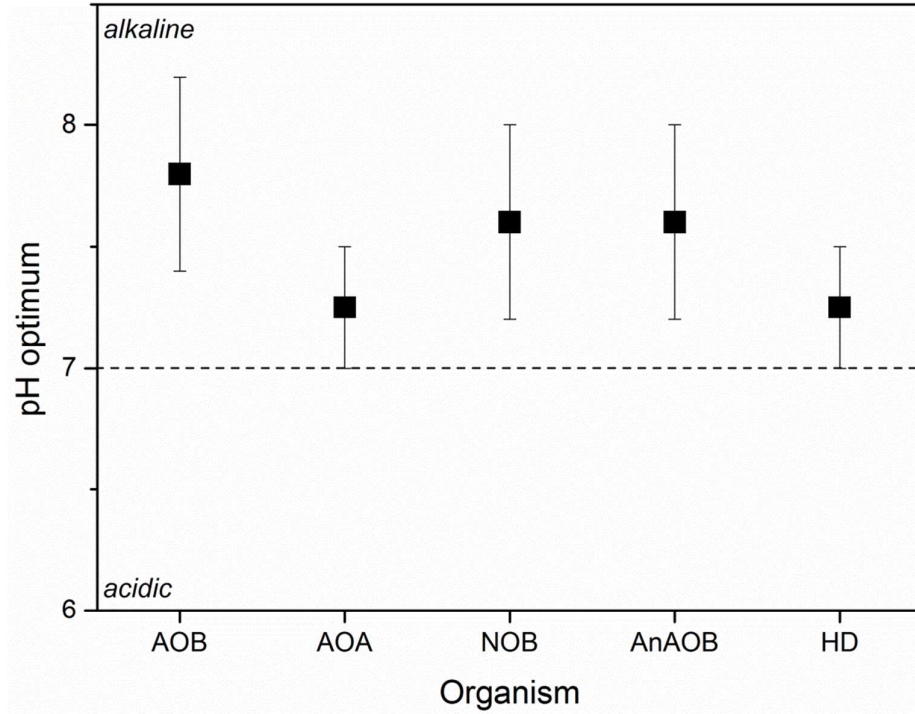


Figure 3 - Physiological pH optima of organisms reported in literature. Organisms: AOB, aerobic ammonia oxidizing bacteria; AOA, ammonia oxidizing archaea; NOB, nitrite oxidizing bacteria; AnAOB, anaerobic ammonia oxidizing bacteria; HD, heterotrophic denitrifiers. References are given in Table S1.

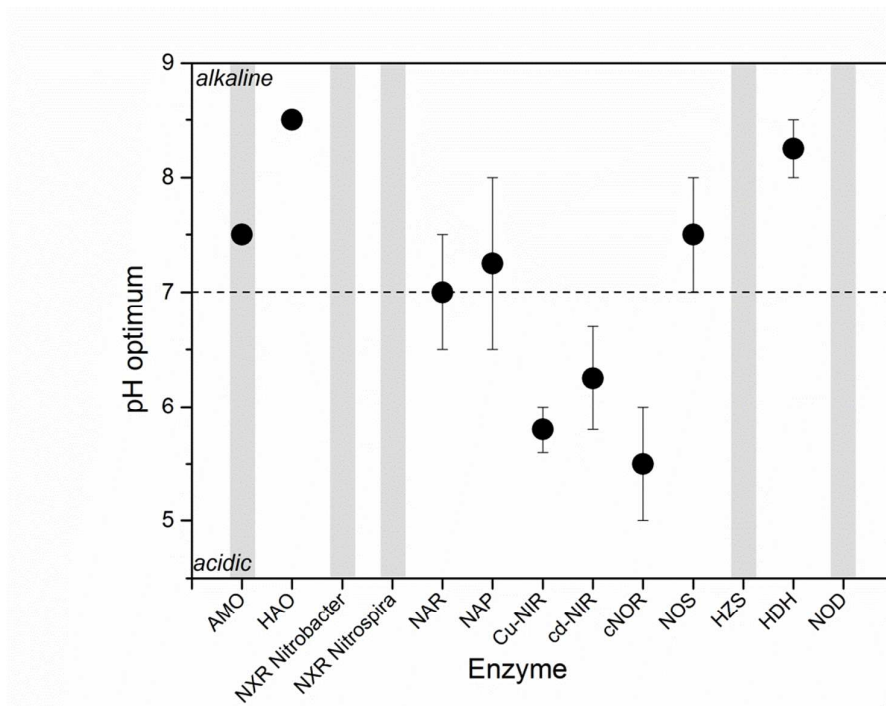


Figure 4 - pH optima of enzymes involved in N-conversion. Enzymes: ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), nitrite oxidoreductase (NXR), nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR), nitrous oxide reductase (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.

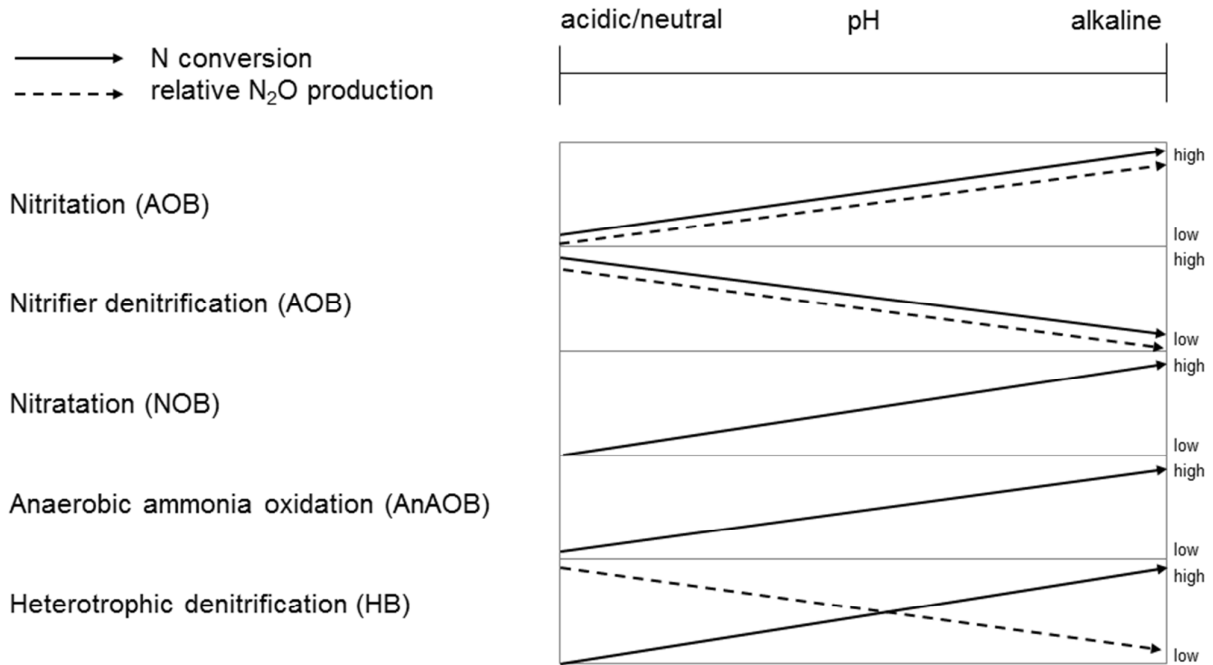


Figure 5 - Qualitative changes of N-conversion and N₂O production rates of different pathways with varying pH.