

# 1 **The microbial nitrogen-cycling network**

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9 **Abstract** | Nitrogen is an essential component of all living organisms and the main nutrient  
10 limiting life on our planet. By far the largest inventory of freely accessible nitrogen is  
11 atmospheric dinitrogen, but most organisms rely on more bioavailable forms of nitrogen, such  
12 as ammonium and nitrate, for growth. The availability of these substrates depends on diverse  
13 nitrogen transforming reactions that are carried out by complex networks of metabolically  
14 versatile microorganisms. In this Review, we summarize our current understanding of the  
15 microbial nitrogen-cycling network including novel processes, their underlying biochemical  
16 pathways, the involved microorganisms, their environmental importance and industrial  
17 applications.

## 18 19 **Introduction**

20 Nitrogen is an essential element for all living organisms and is required for the biosynthesis of  
21 key cellular components such as proteins and nucleic acids. Atmospheric dinitrogen gas is the  
22 largest inventory of freely accessible nitrogen and it is biologically available to highly diverse  
23 but rare nitrogen-fixing bacteria and archaea. Other organisms must rely for growth on more  
24 reactive forms of nitrogen, such as ammonium and nitrate. This bioavailable nitrogen is rare in  
25 many environments, and the availability of this growth-limiting nutrient is controlled primarily  
26 by microbial reactions that alter the oxidation state of nitrogen.

27 Human activity has had a profound effect on the amount of bioavailable nitrogen, mainly due  
28 to the high input of industrial nitrogen-based fertilizers<sup>1</sup>. Food production for about 50% of the  
29 human population currently relies on industrial fertilizers<sup>2</sup>. This fertilizer use and legume  
30 cultivation has nearly doubled the nitrogen input to terrestrial and marine ecosystems<sup>1</sup>. To  
31 predict the consequences of this input, there is a pressing need to understand the basic  
32 mechanisms that underlie microbial nitrogen transformations.

33 Microorganisms can transform nitrogen compounds as reactive and toxic as nitric oxide or as  
34 inert as dinitrogen gas. Microbial transformations of nitrogen are often depicted as a cycle

35 consisting of six distinct processes that proceed in an orderly fashion. This view of the nitrogen  
36 cycle implies that a molecule of dinitrogen gas is first ‘fixed’ to ammonia, which is ‘assimilated’  
37 into organic nitrogen (that is, biomass). The degradation of organic nitrogen, ‘ammonification’,  
38 releases a molecule of ammonia, which is subsequently oxidized to nitrate through  
39 ‘nitrification’ ( $\text{NH}_4^+ \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$ ) and eventually converted back to a molecule of dinitrogen  
40 gas through ‘denitrification’ ( $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$ ) or ‘anaerobic ammonium  
41 oxidation’ (anammox;  $\text{NO}_2^- + \text{NH}_4^+ \rightarrow \text{N}_2$ ). In reality, there is not one balanced nitrogen cycle.  
42 Instead, the six distinct processes are associated with nitrogen fluxes of vastly different  
43 magnitude (Box 1).

44 Nitrogen-transforming microorganisms are generally classified according to one of the six  
45 processes they are involved in: ‘nitrifiers’ carry out nitrification, ‘denitrifiers’ denitrification,  
46 ‘N<sub>2</sub>-fixers’ nitrogen fixation, etc. However, genomic data collected during the last decade  
47 challenges this classification, as it has revealed tremendous metabolic versatility within  
48 nitrogen-transforming microorganisms. We now know that diverse microorganisms can fix  
49 dinitrogen gas and denitrify simultaneously<sup>3,4</sup>, and organisms classified as nitrite oxidizers can  
50 also grow on formate, hydrogen and sulfide<sup>5,6</sup>. Thus, due to their metabolic versatility, it has  
51 become nearly impossible to objectively classify nitrogen-transforming microorganisms  
52 according to the six classical processes (Box 1). We will use process names, such as  
53 denitrification and nitrification, but refrain from classifying organisms accordingly. This  
54 Review will focus on the redox reactions that convert nitrogen compounds, biochemical  
55 pathways, and the responsible enzymes (Fig. 1) and microorganisms.

56 Based on our current understanding, microorganisms can convert nitrogen compounds spanning  
57 redox states [G] from -3 to +5 using fourteen discrete redox reactions (Fig. 1). There is no  
58 change in redox state in the interconversion of organic nitrogen to ammonia. Nitrogen-  
59 converting enzymes are often found in very diverse microorganisms (see below). Many of these  
60 enzymes have only recently been identified. Four new reactions were discovered in the last  
61 decade: hydroxylamine oxidation to nitric oxide<sup>7,8</sup> (Fig. 1; reaction 7), nitric oxide dismutation  
62 to dinitrogen gas and oxygen<sup>9</sup> (9), hydrazine synthesis<sup>10</sup> (12), and hydrazine oxidation to  
63 dinitrogen gas<sup>10</sup> (13). In addition, many new metabolic capabilities were discovered, such as  
64 phototrophic nitrite oxidation<sup>11</sup> and complete ammonia oxidation to nitrate (comammox)<sup>12,13</sup>,  
65 and novel microorganisms such as ammonia-oxidizing archaea<sup>14</sup>, denitrifying eukaryotic  
66 foraminifera<sup>15</sup> and symbiotic heterotrophic nitrogen-fixing cyanobacteria<sup>16</sup> were identified.

67 In this Review, we present these new findings in the context of our current understanding of  
68 microbial transformations of nitrogen. We describe microbial nitrogen-transforming reactions,

69 microorganisms and their physiological and environmental function, and present reactions that  
70 are likely to exist, but have not yet been discovered. Furthermore, we will discuss the complex  
71 network of interactions between nitrogen-transforming microorganisms and its impact on  
72 global biogeochemical nitrogen cycling.

73

## 74 **Nitrogen-transforming reactions**

75

### 76 *Nitrogen fixation.*

77 Atmospheric dinitrogen gas is the largest reservoir of freely accessible nitrogen, but it is  
78 biologically available only to microorganisms that carry the nitrogenase metalloenzyme and  
79 thus can fix dinitrogen into ammonia. Nitrogenase is widespread in bacteria and archaea  
80 and provides them with a competitive advantage in environments that are depleted in  
81 bioavailable nitrogen. There are three different types of nitrogenase — iron-iron (FeFe),  
82 vanadium-iron (VFe) and molybdenum-iron (MoFe) nitrogenases<sup>17</sup>. They have similar  
83 sequence, structural and functional properties, but vary in their metal cofactor. All  
84 nitrogenases are composed of two components (Fig 2a). *anfDGK*, *vnfDGK* or *nifDK*  
85 encode the catalytic component of nitrogenases that have iron, vanadium or molybdenum  
86 in the active center, respectively<sup>17,18</sup>. In addition, *anfH*, *vnfH* or *nifH* encode iron-  
87 containing electron transfer proteins (known as nitrogenase reductase or iron protein.  
88 *NifH* is used as a gene marker for the detection of nitrogen-fixing microorganisms in  
89 the environment<sup>18</sup>. The soil bacterium *Azotobacter vinelandii* encodes all three types of  
90 nitrogenases, whereas other microorganisms such as the marine nitrogen-fixers  
91 *Trichodesmium* spp. only have MoFe nitrogenase<sup>18</sup>. Whereas vanadium is seldom limiting,  
92 molybdenum and iron are rare in the terrestrial and marine environment, respectively, and  
93 can therefore limit nitrogen fixation in these ecosystems<sup>19</sup>.  
94 During nitrogen fixation, an electron carrier such as ferredoxin first reduces the iron protein,  
95 which subsequently reduces the catalytic component. This requires the iron and catalytic  
96 proteins to dissociate and reassociate<sup>20</sup>. Per molecule of N<sub>2</sub> fixed 16 molecules of adenosine  
97 triphosphate are consumed<sup>20</sup>. Additional bioenergetic costs arise from the production of  
98 powerful reductants [G] such as ferredoxin, and the protection of the oxygen-labile  
99 nitrogenase<sup>21</sup>. Because oxygen exposure deactivates nitrogenases, oxygenic phototrophic  
100 [G] cyanobacteria, such as *Trichodesmium* spp., *Crocospaera watsonii*, and *Nodularia* spp.,  
101 often separate N<sub>2</sub> fixation from photosynthesis, either spatially (for example in heterocysts,  
102 which are specialized N<sub>2</sub>-fixing cells) or temporally<sup>22</sup>. Even non-photosynthetic organisms  
living in oxic environments require mechanisms, such as enhanced oxygen respiration, 3  
detoxification via

103 superoxide dismutase and conformational changes of nitrogenase, to protect their nitrogenase  
104 from oxygen<sup>23</sup>. The existence of a completely different, oxygen-insensitive pathway of N<sub>2</sub>  
105 fixation using an ‘unusual nitrogenase’ was recently refuted<sup>24</sup>.

106 Although no N<sub>2</sub>-fixing eukaryotes have been found, many nitrogen-fixing microorganisms live  
107 in symbioses with eukaryotes. The unicellular cyanobacterium *Candidatus*  
108 *Atelocyanobacterium thalassa* (UCYN-A), which lives in symbiosis with small unicellular  
109 haptophyte algae such as *Braarudosphaera bigelowii*, is one of the most widespread nitrogen-  
110 fixing microorganisms and has a key role in marine nitrogen fixation<sup>16,25</sup>. Symbiotic nitrogen-  
111 fixing microorganisms are also part of the gut microbiota of animals such as termites and can  
112 be found in special bacteriocytes [G] in bivalves<sup>26,27</sup>. Moreover, nitrogen-fixing members of  
113 the Rhizobiales order live in special root nodules of crop legumes, such as alfalfa, beans, peas  
114 and soy, which provide 20% of food protein worldwide (Fig. 3c.)<sup>28</sup>.

115

#### 116 ***Ammonia oxidation to hydroxylamine.***

117 All known aerobic ammonia-oxidizing bacteria and archaea activate ammonia by oxidizing it  
118 to hydroxylamine using ammonia monooxygenase (AMO)<sup>29</sup>. Most ammonia-oxidizing bacteria  
119 belong to the Betaproteobacteria and Gammaproteobacteria classes and are  
120 chemolithoautotrophs that oxidize ammonia to nitrite<sup>30</sup>. They can be found in nearly all  
121 environments, including fertilized soils<sup>31</sup> and wastewater treatment plants. Archaea belonging  
122 to the Thaumarchaeota [G] phylum such as *Nitrosopumilus maritimus* can also grow  
123 chemolithoautotrophically by oxidizing ammonia to nitrite<sup>14</sup>. Their discovery resolved the long-  
124 standing mystery of the apparently rare ammonia oxidizers in the oceans<sup>32,33</sup>. Thaumarchaeota  
125 are more abundant than bacteria in some sandy and silty clay soils<sup>31,34</sup>. Furthermore, the  
126 isolation of the acidophilic [G] ammonia-oxidizing archaeon *Candidatus Nitrosotalea*  
127 *devanattera* overturned the common assumption that chemolithoautotrophic ammonia  
128 oxidation could not occur at low pH because of low ammonia availability<sup>35</sup>. Many ammonia  
129 oxidizers, such as *Nitrospira* sp. and *Nitrososphaera viennensis*, can also degrade organic  
130 nitrogen compounds, for example by hydrolyzing urea with ureases, to produce additional  
131 ammonia<sup>36,37</sup>. The archaeon *Nitrososphaera gargensis* can also produce ammonia by  
132 hydrolyzing cyanate with a cyanase<sup>38</sup>.

133 Recently, the ability to oxidize ammonia has also been found in members of the genus  
134 *Nitrospira*, which were previously assumed to only be capable of nitrite oxidation<sup>12,13</sup>. The  
135 discovery of these bacteria that oxidize ammonia to nitrate (complete ammonia oxidation  
136 (comammox)), refuted the dogma that the oxidation of ammonia and nitrite requires two distinct

137 groups of microorganisms. The bacteria that perform the comammox process such as *Nitrospira*  
138 *inopinata* appear well adapted to ammonia-limited environments and can outcompete most  
139 cultured ammonia oxidizing microorganisms for ammonia<sup>39</sup>. The transient accumulation of  
140 nitrite in comammox cultures grown on ammonia indicates that they more efficiently oxidize  
141 ammonia than nitrite<sup>12, 13, 39</sup>. We hypothesize that bacteria that perform the comammox process  
142 would oxidize ammonia to nitrate under ammonia-limited conditions and perform partial  
143 ammonia oxidation to nitrite under oxygen-limited conditions.

144 AMO is closely related to methane monooxygenase (MMO), which is found in methanotrophs  
145 [G] such as gammaproteobacteria<sup>40</sup> and NC-10<sup>9</sup> [G]. MMO can also oxidize ammonia to  
146 hydroxylamine, although very inefficiently (Fig. 1)<sup>41</sup>. Similarly, AMO can also oxidize  
147 methane, but less efficiently than MMO<sup>30</sup>. Intriguingly, *amo* sequences of bacteria that perform  
148 the comammox process were detected in the environment (for example in groundwater) already  
149 before their discovery, but were wrongly assigned as particulate methane monooxygenase  
150 (*pmo*) genes of the filamentous methane-oxidizing *Crenothrix polyspora*<sup>42</sup>. Recent  
151 resequencing of *C. polyspora* and other *Crenothrix* species revealed that they actually contain  
152 typical gammaproteobacterial *pmo* and not *amo*<sup>43</sup>.

153

#### 154 ***Hydroxylamine oxidation to nitric oxide and further to nitrite.***

155 Aerobic oxidation of ammonia to hydroxylamine is an endergonic [G] reaction. Therefore, all  
156 aerobic ammonia oxidizers conserve energy by further oxidizing hydroxylamine. It was  
157 believed that aerobic ammonia-oxidizing bacteria oxidize hydroxylamine to nitrite using  
158 octaheme hydroxylamine oxidoreductase (HAO). Recently, it was shown that the product of  
159 HAO is not nitrite but nitric oxide, which is further oxidized to nitrite by an unknown enzyme<sup>7</sup>.  
160 Although the enzyme catalyzing the latter reaction has not been conclusively identified, copper-  
161 containing nitrite reductase (Cu-NIR) working in reverse has been suggested to catalyze it<sup>7</sup>. All  
162 ammonia-oxidizing bacteria, including the newly discovered *Nitrospira* spp., which can oxidize  
163 ammonia all the way to nitrate, contain AMO and HAO<sup>12, 13</sup>. By contrast, known ammonia-  
164 oxidizing archaea do not encode HAO and the archaeal enzyme responsible for hydroxylamine  
165 oxidation remains unknown<sup>44, 45</sup>.

166 HAO belongs to a family of octaheme proteins (Fig. 2b) found in diverse microorganisms<sup>44, 46</sup>.  
167 The genomes of anaerobic ammonium-oxidizing bacteria encode ~10 HAO-like proteins<sup>46</sup>, and  
168 one of these also oxidizes hydroxylamine to nitric oxide<sup>8</sup>. In anaerobic ammonium-oxidizing  
169 bacteria this hydroxylamine oxidase (HOX) recycles hydroxylamine, which leaks from  
170 hydrazine synthase (see below).

171 Methane-oxidizing bacteria also produce hydroxylamine as a result of their unspecific ammonia  
172 oxidation activity<sup>30</sup> (see above) and diverse methanotrophs in the Proteobacteria,  
173 Verrucomicrobia [G], and NC10 phyla (for example, *Candidatus Methylophilum oxyfera*),  
174 encode HAO-like proteins that likely oxidize hydroxylamine to nitric oxide, which is further  
175 oxidized to nitrite or reduced to nitrous oxide<sup>8, 47, 48</sup>. Currently, it is unknown whether this  
176 reaction directly contributes to energy conservation in methane-oxidizing bacteria.

177

### 178 ***Nitrite oxidation to nitrate.***

179 Nitrite oxidation is the main biochemical pathway that produces nitrate, and is catalyzed by  
180 nitrite oxidoreductase (NXR). NXR is encoded by aerobic nitrite-oxidizing bacteria (members  
181 of the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Chloroflexi,  
182 Nitrospinae and Nitrospirae phyla)<sup>6</sup>, anoxygenic phototrophs [G] (for example, *Thiocapsa sp.*  
183 strain KS1 and *Rhodopseudomonas sp.* strain LQ17)<sup>11, 49</sup> and anaerobic ammonium-oxidizing  
184 bacteria<sup>50</sup>. Whereas aerobic nitrite oxidizing bacteria directly couple nitrite oxidation by NXR  
185 to energy conservation, anaerobic nitrite-oxidizing bacteria do not. *Thiocapsa sp.* Strain KS1  
186 and *Rhodopseudomonas sp.* strain LQ17 can oxidize nitrite anaerobically by coupling it directly  
187 to phototrophy<sup>11, 49</sup>. Further, anaerobic ammonium-oxidizing bacteria might couple anaerobic  
188 nitrite oxidation to carbon fixation<sup>51</sup>.

189 Nitrite-oxidizing bacteria are metabolically versatile and can grow on other substrates than  
190 nitrite<sup>6</sup>. Indeed, the comammox *Nitrospira* species oxidize ammonia to nitrate<sup>12, 13</sup>. *Nitrospira*  
191 *moscoviensis* grows aerobically on hydrogen and anaerobically on organic acids while respiring  
192 nitrate<sup>52, 53</sup>. Nitrate reduction in these nitrite-oxidizing bacteria is also catalyzed by NXR, which  
193 is related to bacterial and archaeal nitrate reductases<sup>54</sup>.

194 The concerted activity of nitrite and ammonia oxidizing microorganisms in agricultural soils  
195 converts N-based fertilizers to nitrate and has a key role in the loss of fertilizers to river and  
196 ground waters leading to the eutrophication [G] of rivers, lakes and coastal waters. The same  
197 two processes are also used in wastewater treatment plants as the first step of conventional  
198 nitrogen removal (Box 2). In marine environments, nitrite-oxidizing bacteria generate nitrate,  
199 the dominant form of biologically available nitrogen in the ocean, and contribute to carbon  
200 fixation<sup>55</sup>.

201

### 202 **[H3] Nitrate reduction to nitrite.**

203 Nitrate reduction to nitrite is used for respiration, known as dissimilatory nitrate reduction, and  
204 for nitrogen assimilation into biomass. Dissimilatory nitrate reduction to nitrite can be carried

205 out by microorganisms from all three domains of life. These microorganisms occur in all anoxic  
206 environments in which nitrate is present, including soils<sup>56</sup>, oxygen minimum zones<sup>57</sup>, marine  
207 sediments<sup>58</sup> and the human gastrointestinal system<sup>59</sup>. The reaction is catalyzed by either a  
208 membrane-bound (NAR) or a periplasmic (NAP) nitrate reductase<sup>60</sup>. Many organisms,  
209 including the model organism *Paracoccus denitrificans*, contain both NAP and NAR<sup>60</sup>. Nitrate  
210 reduction by NAR occurs in the cytoplasm and releases protons to the periplasm (Fig 2c), and  
211 thereby directly contributes to energy conservation through the proton motive force [G]. By  
212 contrast, NAP reduces nitrate to nitrite in the periplasm, and thus does not translocate protons  
213 required to create proton motive force<sup>60</sup>.

214 Dissimilatory nitrate reduction to nitrite is not merely the first step in denitrification. Some  
215 microorganisms such as the giant sulfur oxidizing *Beggiatoa* sp.<sup>61</sup>, which is widespread in  
216 freshwater and marine sediments, reduce nitrate via nitrite to ammonium and many  
217 microorganisms such as some members of the ubiquitous marine clade SAR11<sup>62</sup> only reduce  
218 nitrate to nitrite (Fig. 1). Nitrate reduction is a major source of nitrite for other nitrogen-cycling  
219 processes including aerobic nitrite oxidation and anammox<sup>62-64</sup>. Dissimilatory nitrate reduction  
220 can be coupled to the oxidation of electron donors such as organic matter<sup>65</sup>, methane<sup>66, 67</sup> (for  
221 example, in *Candidatus Methanoperedens* spp.), sulfur compounds (for example, in  
222 *Thiobacillus denitrificans*<sup>68</sup>); H<sub>2</sub> (for example, in *Alcaligenes eutrophus*) or iron (for example,  
223 *Ferroglobus placidus*<sup>69</sup>).

224 Nitrate is a major nitrogen source for eukaryotes, bacteria and archaea that contain assimilatory  
225 nitrate reductases (NAS)<sup>60</sup>. Considering that nitrate supports at least 20% of marine algal  
226 growth<sup>70</sup>, nitrate assimilation likely exceeds the magnitude of most other redox driven nitrogen-  
227 cycle process in the ocean (Box 1). NAS, together with assimilatory nitrite reductases (see  
228 below), produces ammonia, which is incorporated into biomass<sup>60</sup>. Because NAS is located in  
229 the cytoplasm, nitrate assimilation requires nitrate transport into the cell by ATP-dependent  
230 transporters<sup>60</sup>. Due to this energy requirement, NAS expression is repressed in ammonia-replete  
231 environments, such as fertilized soils<sup>60</sup>.

232 Bacterial and archaeal NAS together with NAP, NAR and NXR belong to the dimethylsulfoxide  
233 reductase family, whereas eukaryotic assimilatory nitrate reductases belong to the sulfite  
234 oxidase family<sup>71</sup>. This suggests multiple origins of nitrate reductases. The distinction between  
235 assimilatory and dissimilatory nitrate reduction pathways is not absolute. In principle, nitrite  
236 produced by assimilatory nitrate reduction could be reduced further in the respiratory chain.  
237 Conversely, *Mycobacterium tuberculosis* has been shown to use the NAR complex for nitrate  
238 assimilation<sup>72</sup>.

239

240 ***Nitrite reduction to ammonium.***

241 Nitrite reduction to ammonium is used for both dissimilatory and assimilatory purposes.  
242 Dissimilatory nitrite reduction to ammonium is carried out by most bacterial lineages, the  
243 thermophilic Crenarcheota *Pyrolobus fumarii*<sup>73</sup>, methane-oxidizing archaea<sup>67</sup>, diatoms<sup>74</sup> and  
244 fungi<sup>75</sup>. This reaction is catalyzed by the periplasmic cytochrome c nitrite reductase (ccNIR)  
245 encoded by *nrfA*, the octaheme nitrite reductase (ONR)<sup>76</sup> or the octaheme tetrathionate  
246 reductase (OTR)<sup>77</sup>. It is unclear whether the latter two enzymes are used for respiration or  
247 detoxification of nitrite or hydroxylamine. Reduction of nitrite to ammonium involves the  
248 formation of hydroxylamine as intermediate, which remains bound to the enzyme until it is  
249 reduced to ammonium<sup>78</sup>.

250 Interestingly, the anaerobic ammonium-oxidizing bacterium *K. stuttgartiensis* can reduce nitrite  
251 to ammonium, but lacks known ammonium-producing nitrite reductases. It is hypothesized that  
252 nitrite reduction to ammonium instead might be accomplished by an HAO-like protein<sup>46</sup>.  
253 Recently an HAO encoded by Epsilonproteobacteria ( $\epsilon$ HAO), such as *Campylobacter fetus* and  
254 *Nautilia profundicola*, was shown to reduce nitrite and hydroxylamine to ammonium, although  
255 with poor efficiency<sup>79</sup>.

256 Dissimilatory nitrite reduction to ammonium is the key reaction in the so-called dissimilatory  
257 nitrate reduction to ammonium (DNRA) process<sup>80</sup>. Microorganisms can grow using DNRA by  
258 coupling it to the oxidation of electron donors, such as organic matter, Fe<sup>2+</sup>, H<sub>2</sub>, sulfide and  
259 methane<sup>67, 81-83</sup>. Little is known about the environmental importance of DNRA<sup>84, 85</sup>; however, in  
260 marine and lake sediments, DNRA appears to be favored over denitrification when there is an  
261 excess of electron donor relative to nitrate<sup>58</sup>.

262 Assimilatory nitrite reductases produce ammonium and are as widespread as NAS, and both  
263 types of enzymes are often encoded on the same *nas* operon<sup>54</sup>. The formation of primary nitrite  
264 maxima [G] in the ocean has been attributed to the release of nitrite due to an uncoupling of  
265 assimilatory nitrate and nitrite reduction in phytoplankton<sup>86</sup>. The physiological reasons for this  
266 uncoupling are still unclear.

267

268 ***Nitrite reduction to nitric oxide.***

269 Many microorganisms have the ability to reduce nitrite to nitric oxide, for example,  
270 Proteobacteria, anaerobic ammonium-oxidizing bacteria, and Bacteroidetes<sup>54</sup>. These  
271 microorganisms are found in many environments, in which nitrate is available and oxygen  
272 concentrations are low, such as soils<sup>56</sup>, oxygen minimum zones<sup>57</sup> and marine sediments<sup>58</sup>. This



273 reaction can be catalyzed by two unrelated enzymes: a heme-containing cd<sub>1</sub> nitrite reductase  
274 (cd<sub>1</sub>NIR encoded by *nirS*) or a Cu-containing nitrite reductase (CuNIR encoded by *nirK*), which  
275 are widespread among bacteria and archaea<sup>87</sup>. Both enzymes are located in the periplasm and  
276 do not contribute directly to energy conservation<sup>54, 65</sup>. These two enzymes also occur together  
277 in a single microorganism, for example in *Rhodothermus marinus*<sup>87</sup>.  
278 Commonly, *nirS* and *nirK* are used in environmental studies as gene markers for ‘denitrifiers’,  
279 however, these genes are present in many other microorganisms, including anaerobic  
280 ammonium-oxidizing bacteria, nitrite and methane-oxidizing bacteria and ammonia-oxidizing  
281 bacteria and archaea<sup>88</sup>. Apart from CuNIR and cd<sub>1</sub>NIR, other nitrite-reducing enzymes might  
282 exist; for example, some anaerobic ammonium-oxidizing bacteria contain neither of them, but  
283 can reduce nitrite to nitric oxide<sup>89</sup>. To carry out this reaction, these bacteria might use an HAO-  
284 like octaheme oxidoreductase<sup>46</sup>.

285

#### 286 ***Nitric oxide reduction to nitrous oxide or dinitrogen gas.***

287 Nitric oxide is a signaling molecule, a toxin<sup>90</sup> and an intermediate of the denitrification,  
288 nitrification and anammox processes. Additionally, bacteria that perform oxygenic  
289 denitrification dismutate two molecules of nitric oxide to one molecule of dinitrogen gas and  
290 one molecule of oxygen<sup>9</sup>. Therefore, microorganisms capable of nitric oxide reduction can be  
291 found in a wide range of environments, including wastewater treatment plants<sup>46</sup>, agricultural  
292 soils<sup>56, 91</sup>, marine sediments<sup>58</sup> and marine oxygen minimum zones<sup>57</sup>. Microbial nitric oxide  
293 reduction (Fig. 1) is the main source of nitrous oxide, a powerful greenhouse gas (310 times  
294 more potent than CO<sub>2</sub>) and the dominant ozone-depleting agent<sup>92</sup>. Nitrous oxide-producing  
295 nitric oxide reductases (NOR) are used for detoxification or respiration of nitric oxide, and  
296 belong to a diverse group of enzymes ranging from flavoproteins to heme copper oxidases,  
297 which are widespread throughout the tree of life. Flavo-diiron proteins, such as flavorubredoxin  
298 nitric oxide reductase (NORvw), are used to detoxify nitric oxide, for example by the sulfate-  
299 reducing bacterium *Desulfovibrio gigas*<sup>93, 94</sup>. Other NOR-type enzymes are the NADH-  
300 dependent cytochrome P<sub>450</sub>-NOR found in the mitochondria of fungi, such as *Fusarium*  
301 *oxysporum*<sup>95</sup>, and the hybrid cluster protein HCP recently discovered in *Escherichia coli*<sup>96</sup>.

302 The heme copper oxidase family contains terminal oxidases, the cytochrome c-dependent  
303 cNOR, quinol-dependent qNOR and the copper-containing Cu<sub>A</sub>NOR, which all have a role in  
304 nitric oxide respiration<sup>97-99</sup>. Nitrous oxide is an intermediate of denitrification and NOR is  
305 present in microorganisms, such as *P. denitrificans* and *Pseudomonas stutzeri*<sup>65</sup>. Nitrous oxide  
306 can also be the end-product of denitrification in some microorganisms, such as *Pseudomonas*

307 *chlororaphis*<sup>65</sup>. Ammonia-oxidizing bacteria can produce nitrous oxide in a process termed  
308 nitrifier-denitrification, in which NOR is used to reduce nitric oxide formed upon nitrite  
309 reduction<sup>30</sup>. In cultures of ammonia-oxidizing bacteria and bacteria capable of carrying out the  
310 comammox process, nitrous oxide can also be formed through abiotic reactions of the  
311 extracellular intermediates hydroxylamine and nitric oxide<sup>100</sup>. Additionally, ammonia-oxidizing  
312 bacteria can produce nitrous oxide through the NOR-catalyzed reduction of nitric oxide, which  
313 is produced during hydroxylamine oxidation<sup>7, 30</sup>. Similar to ammonia-oxidizing bacteria,  
314 methanotrophic bacteria produce nitrous oxide through the NOR-catalyzed reduction of nitric  
315 oxide formed upon hydroxylamine-oxidation (see above) and nitrite reduction<sup>47,48</sup>. By contrast,  
316 nitrous oxide production in ammonia-oxidizing archaea might exclusively involve the abiotic  
317 reactions of the intermediates nitric oxide and hydroxylamine<sup>45</sup>.

318 The use of nitrogen-based fertilizers has drastically increased nitrous oxide emissions<sup>101</sup>. Due  
319 to the concerted activity of nitrogen-transforming microorganisms, 3 to 5% of the nitrogen used  
320 as agricultural fertilizer is converted into nitrous oxide<sup>102, 103</sup>. Nitrogen-based fertilizers are  
321 increasingly used to grow crops for biofuel production, which represents a potential  
322 replacement for fossil fuels. Herein lies a dilemma — the more fertilizer is used to produce  
323 biofuels, the more nitrous oxide emissions increase. Therefore, the fertilizer use for biofuel  
324 production counteracts the reduction in greenhouse gas emissions that is achieved by reducing  
325 the use of fossil fuels<sup>103</sup>.

326 Nitric oxide dismutation [G] to dinitrogen and oxygen gas (Fig. 1) is a recently discovered  
327 nitrogen transforming reaction<sup>104</sup>. Microorganisms such as *Ca. Methyloirabilis oxyfera* found  
328 in anoxic systems rich in methane and nitrate (for example in eutrophied lakes and wetlands)  
329 use this reaction to produce their own molecular oxygen from nitrite<sup>9</sup>. This enables *Ca.*  
330 *Methyloirabilis oxyfera* to live in anoxic environments and to use the aerobic methane  
331 oxidation pathway<sup>9</sup>. The dismutation reaction might involve an unusual qNOR, tentatively  
332 called nitric oxide dismutase (NO-D)<sup>9</sup>. Nitric oxide dismutation might be more widespread than  
333 previously thought, as similar unusual qNOR sequences are present in other phyla, such as  
334 Gammaproteobacteria (for example, HdN1 strain) and Bacteroidetes (for example, *Muricauda*  
335 *ruestringensis*)<sup>104</sup>.

336

### 337 ***Nitrous oxide reduction to nitrogen gas.***

338 Microbial nitrous oxide reduction to nitrogen gas is the main sink of this powerful greenhouse  
339 gas. The only known enzyme that catalyzes this reaction is nitrous oxide reductase (NOS),  
340 which, due to its location in the periplasm, does not directly contribute to energy conservation

341 through proton motive force<sup>105</sup>. Diverse bacteria, including members of the Proteobacteria,  
342 Bacteroidetes and Chlorobi phyla, and archaea from the Crenarchaeota and Halobacteria<sup>106</sup>  
343 utilize NOS. The discovery of a slightly different NOS-encoding gene in *Wolinella*  
344 *succinogenes*<sup>107</sup> revealed an overlooked diversity of NOS sequences in soils<sup>108</sup>. Intriguingly,  
345 organisms encoding this NOS variant often have no other nitrogen-oxide reductases<sup>87, 91, 109</sup>.  
346 Some eukaryotes, the Foraminifera and Gromiida, also reduce nitrous oxide, but their  
347 enzymatic machinery is unknown<sup>15, 110</sup>.

348 For a long time, it was believed that NOS was more sensitive to oxygen, pH and sulfide than  
349 other nitrogen-oxide reductases<sup>105</sup>. Based on that apparent sensitivity, environmental emissions  
350 of nitrous oxide were fully attributed to inhibition of NOS in organisms that reduce nitrate all  
351 the way to N<sub>2</sub>, the so-called ‘complete denitrifiers’. Additionally, interactions of so-called  
352 ‘incomplete denitrifiers’, which are microorganisms that only perform, for example, nitrite  
353 reduction to nitrous oxide or nitrous oxide reduction to dinitrogen gas, and their niche  
354 differentiation might cause imbalances between nitrous oxide production and consumption in  
355 many environments, such as soils and marine environments<sup>91, 109, 111</sup>.

356

### 357 ***Hydrazine synthesis and hydrazine oxidation to dinitrogen gas.***

358 Until recently, it was generally believed that ammonium could only be activated with molecular  
359 oxygen and that bioavailable nitrogen could only be lost as dinitrogen gas through  
360 denitrification<sup>112</sup>. The discovery of anaerobic ammonium oxidation (anammox) to dinitrogen  
361 gas with nitrite as the terminal electron acceptor overturned both of these dogmas<sup>51, 113, 114</sup>.  
362 Hydrazine synthase (HZS) is the only known enzyme that can activate ammonium  
363 anaerobically<sup>89</sup>, and it is exclusively found in anaerobic ammonium-oxidizing bacteria that  
364 belong to five genera in the phylum Planctomycetes<sup>89, 115, 116</sup>. HZS is also the only enzyme known  
365 to form an N-N bond from two discrete N-compounds, producing hydrazine as a free  
366 intermediate in a two-step reaction<sup>10, 115</sup>. The hypothetical mechanism of hydrazine synthesis  
367 starts with nitric oxide reduction to hydroxylamine (Fig 2d), which is subsequently  
368 comproportionated [G] together with ammonium into hydrazine, one of the most potent  
369 reductants in nature<sup>10, 115</sup>. During this reaction, hydroxylamine is transferred from one active site  
370 to the next (Fig. 2d), which might result in hydroxylamine-loss from HZS. Two of the genes  
371 encoding HZS, *hzsA* and *hzsB*, are used as genetic markers for anaerobic ammonium-oxidizing  
372 bacteria in the environment<sup>117, 118</sup>.

373 Hydrazine is oxidized to dinitrogen by hydrazine dehydrogenase (HDH)<sup>10, 119</sup>. Based on amino  
374 acid sequences, this enzyme is related to HOX and HAO; however, it is inhibited by

375 hydroxylamine and can only oxidize hydrazine<sup>119</sup>. Hydrazine oxidation occurs in a unique  
376 membrane-bound structure called the anammoxosome [G] and is most likely directly associated  
377 with energy conservation<sup>46, 120, 121</sup>. Intriguingly, all catabolic enzymes of anaerobic ammonium-  
378 oxidizing bacteria (HDH, HZS, NIR, HOX and NXR) are located exclusively in the  
379 anammoxosome<sup>122</sup>.  
380 HDH is responsible for the release of a substantial amount of dinitrogen to the <sup>119</sup> In the last  
381 decade, it became clear that the anammox process is a major nitrogen sink in the ocean<sup>123-125</sup>  
382 and it could also have an important role in terrestrial ecosystems<sup>126</sup>.

383

384

### 385 **Networks of nitrogen-transforming microorganisms**

386

387 There is an astonishing diversity of microorganisms that transform nitrogen and each of these  
388 microorganisms has discrete physiological requirements for optimal growth. As growth  
389 conditions in nature are highly variable and seldom optimal, nitrogen turnover by individual  
390 microorganisms is bound to be inefficient. However, nitrogen transformations in the  
391 environment are carried out by microbial communities that recycle nitrogen more efficiently  
392 than single microorganisms. Consequently, very little bioavailable nitrogen escapes to the  
393 atmosphere, and the small amount lost as dinitrogen gas is balanced by nitrogen fixation (Box  
394 1). This apparent nitrogen homeostasis not only characterizes the global biosphere, but also  
395 many ecosystems, such as forest soils and ocean gyres [G]. The microbial communities required  
396 to efficiently recycle nitrogen in these ecosystems are robust with respect to environmental  
397 changes and retain nitrogen-transforming reactions even when the species composition  
398 changes. The nitrogen-transforming reactions are linked by microorganisms that form complex  
399 networks in both natural and man-made ecosystems (Fig. 3).

400 The ocean gyres, the world's largest ecosystems, are nearly nitrogen-balanced due to extensive  
401 nitrogen recycling (Fig. 3a). Here, the main nitrogen-transforming processes are nitrogen  
402 assimilation by cyanobacteria, such as *Prochlorococcus marinus*<sup>70</sup>, ammonification by  
403 mesozooplankton<sup>127</sup> and heterotrophic bacteria, such as *Pelagibacter ubique*<sup>128</sup> and nitrification  
404 by *Nitrosopumilus* spp. and *Nitrospina* spp. (Fig. 3a; see also Box 1). Nitrogen fixation by  
405 microorganisms, such as *Trichodesmium* spp. and UCYN-A (*Atelocyanobacterium*), is a rather  
406 minor nitrogen-transforming process in the gyres<sup>70</sup>. Yet, due to the sheer extent of the area in  
407 which nitrogen fixation occurs, it is the main supply of new bioavailable nitrogen to the ocean.

408 In contrast to the ocean gyres, oxygen minimum zone waters cover less than 1% of the open  
409 ocean area, but might account for 30-50% of oceanic nitrogen loss<sup>57, 70, 125</sup> (Box 1). Here,  
410 anaerobic microorganisms such *Scalindua* spp. co-occur with aerobic organisms such as  
411 *Nitrosopumilus* spp. and *Nitrospina* spp.<sup>57</sup>. The microbial nitrogen-transforming network in  
412 open ocean oxygen minimum zones is complex<sup>57</sup> with all known nitrogen-converting processes  
413 occurring alongside each other (Fig. 3b).

414 Similar to oxygen minimum zone waters, nitrogen-removing wastewater treatment plants are  
415 characterized by imbalanced nitrogen-transformations. These man-made systems are designed  
416 to convert ammonium to dinitrogen gas, which is lost to the atmosphere (Box 2).

417 Agricultural fields are among the largest man-made ecosystems and their microbial nitrogen-  
418 transforming networks have been strongly affected by the anthropogenic input of nitrogen. The  
419 cultivation of legumes that form symbioses with nitrogen-fixing microorganisms have  
420 substantially increased the nitrogen input to the environment<sup>2, 129</sup>. Nitrogen-fixing  
421 microorganisms, such as *Bradyrhizobium* spp., often live in specialized root nodules and  
422 provide ammonium to the legumes (Fig. 3c). Ammonium that leaks out into the surrounding  
423 soil fuels other microbial nitrogen transformations, such as aerobic ammonia oxidation. In rice  
424 paddy fields, the use of industrial fertilizers has resulted in intense nitrification and enhanced  
425 nitrogen loss<sup>126</sup>. Recent studies reveal that these systems have highly complex nitrogen-  
426 transforming networks, which include nitrite-reducing (*Ca. Methylomirabilis* spp.) and nitrate-  
427 reducing (*Ca. Methanoperedens* spp.) methanotrophs<sup>130</sup> (Fig. 3d).

428 In these ecosystems, some nitrogen-transforming microorganisms, such as anaerobic  
429 ammonium-oxidizing bacteria, can perform multiple redox reactions (reactions 1, 2, 5, 7, 10,  
430 12, 13; Fig. 1). Still, processes such as nitrification and denitrification are performed by a  
431 complex network of specialists in a modular fashion (Fig. 3). Such modularity, which is a  
432 general feature of nitrogen-transforming microbial networks, results in cooperative and  
433 competitive interactions (examples in Fig. 3). A cooperative interaction exists between  
434 *Nitrosopumilus* spp. and *Nitrospina* spp. that together oxidize ammonia to nitrate (Figs. 3a, b).

435 In most environments, nitrification is carried out by diverse assemblages of ammonia- and  
436 nitrite-oxidizing microorganisms, which also compete for ammonia and nitrite, respectively.  
437 Substrate competition also exists between microorganisms with very different metabolisms,  
438 such as *Nitrospira* spp., *Ca. Methylomirabilis* spp., *Candidatus Brocadia* spp., *Ca.*  
439 *Methanoperedens* spp. and *Pseudomonas* spp., which all compete for nitrite (Fig. 3d). Microbial  
440 interactions can also be simultaneously cooperative and competitive: *Nitrosopumilus* spp.  
441 produces nitrite for *Scalindua* spp., but both also compete for ammonia (Fig. 3b).

442 The factors that control these interactions are poorly understood. Sometimes, a single  
443 physiological characteristic is used to explain the dominance of certain nitrogen-transforming  
444 microorganisms in the environment. For example, the abundance of ammonia oxidizing archaea  
445 relative to bacteria in ammonia-depleted environments was attributed to the superior ammonia  
446 affinity of the archaea<sup>31, 131, 132</sup>. Recently, however, it was shown that the terrestrial bacterium  
447 *Nitrospira inopinata*, which performs the comammox process, has a higher ammonia affinity  
448 than all cultured terrestrial ammonia-oxidizing archaea<sup>39</sup>. Yet, the microorganisms that perform  
449 the comammox process do not dominate all ammonia-depleted terrestrial environments<sup>133</sup>. The  
450 success of nitrogen-transforming microorganisms also depends on other factors, such as the use  
451 of alternative substrates and cellular energy requirements. Such variables might have general  
452 roles in shaping nitrogen-transforming microbial networks.

453

#### 454 **Concluding remarks**

455

456 Identifying the factors that shape nitrogen-transforming networks will require greater insight  
457 into the physiology of the involved microorganisms and a deeper understanding of their ecology  
458 and evolution. Only a fraction of all microorganisms has been cultivated, and the uncultivated  
459 majority likely contains undiscovered metabolic pathways (Box 3). Cultivation, followed by  
460 painstaking biochemical, physiological and genomic characterisation has already changed our  
461 perspective of key nitrogen-cycle processes. Aerobic nitrite-oxidizing bacteria and anaerobic  
462 ammonium-oxidizing bacteria have a hitherto unexpected metabolic versatility that renders  
463 their classification as mere ‘aerobic nitrite oxidizers’ or ‘anaerobic ammonia oxidizers’  
464 inadequate. Many aerobic nitrite oxidizers might grow as hydrogen-oxidizers, ammonia-  
465 oxidizers or nitrate reducers in the environment<sup>6</sup>. Anaerobic ammonium-oxidizing bacteria can  
466 also use short-chain fatty acids, methylamines and FeII as electron donors<sup>46, 134</sup> and they can use  
467 nitrate, MnIV and FeIII as electron acceptors<sup>46, 135, 136</sup>.

468 On the other hand, there is a growing realization that complete denitrification by single  
469 microorganisms is the exception rather than the rule, with many microorganisms being  
470 specialists that perform only one or a few nitrogen-oxide reduction reactions<sup>3, 91, 137</sup>. Specialized  
471 nitrogen-oxide reducers often lack known genes enabling them to reduce nitrate all the way to  
472 N<sub>2</sub><sup>87, 138</sup>. These specialist nitrogen-oxide reducers are often described as ‘incomplete  
473 denitrifiers’, which is comparable to describing ammonia oxidizers such as *Nitrosomonas* spp.  
474 as ‘incomplete nitrifiers’.

475 Undoubtedly, it will become increasingly difficult to classify organisms according to the  
476 classical six nitrogen-cycling processes, leaving it up to the eye of the beholder to define the  
477 function of an organism. If we can learn one thing from the last few decades of research, it is  
478 that microorganisms do not conform to boundaries. They will do whatever necessary in the  
479 perpetual struggle to survive.

480

481

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885

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### **Author contributions**

889 M.M.M.K., H.K.M. and B.K. researched data for the article, made substantial contributions to  
890 discussions of the content, wrote the article and reviewed and edited the manuscript before  
891 submission.

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902

### **Box 1. Biogeochemical nitrogen cycling: global inventories, processes and fluxes.**

903  
904 The largest global nitrogen inventory, with  $1.8 \times 10^{10}$  Tg nitrogen, is ammonia bound in rocks  
905 and sediments<sup>139</sup>. Although this bound ammonia becomes available upon erosion, it has a minor  
906 role in annual biogeochemical nitrogen cycling. Whereas the terrestrial inventory of freely  
907 accessible ammonia is unknown<sup>140</sup>, the marine inventory<sup>70, 139</sup> is estimated to be between 340

908 and 3600 Tg nitrogen (see grey numbers in the figure). The largest freely accessible global  
909 nitrogen inventory is dinitrogen gas with  $3.9 \times 10^9$  Tg nitrogen followed by organic nitrogen,  
910 nitrate, and nitrous oxide inventories<sup>70, 139</sup>. Global nitrite and nitric oxide inventories are  
911 negligible.

912 Biogeochemical nitrogen cycling between these inventories is often attributed to the following  
913 six distinct nitrogen-transforming processes: assimilation, ammonification, nitrification,  
914 denitrification, anaerobic ammonium oxidation (anammox) and nitrogen-fixation (see the  
915 figure). We estimated the annual nitrogen fluxes for a number of these processes from the  
916 available literature<sup>129, 141-143</sup> and based on simple assumptions (see below). In the figure, the  
917 fluxes between major nitrogen species are shown in Tg nitrogen per year, with green, blue and  
918 red numbers referring to terrestrial, marine and anthropogenic nitrogen fluxes, respectively. The  
919 best-defined fluxes involve nitrogen loss and fixation, because they have been the focus of  
920 many studies<sup>129, 141, 143</sup>. These fluxes are comparatively small (see the figure) but regulate the  
921 availability of bioavailable nitrogen, which largely controls the removal of atmospheric CO<sub>2</sub>  
922 through the biological carbon pump<sup>129</sup>. Current estimates suggest that biological N<sub>2</sub>-fixation  
923 ( $\sim 300$  Tg nitrogen y<sup>-1</sup>) combined with industrial nitrogen-fixation and fossil fuel burning ( $\sim 125$   
924 Tg nitrogen y<sup>-1</sup>)<sup>129, 143</sup> exceeds the production of dinitrogen gas by anammox and denitrification  
925 ( $\sim 350$  Tg nitrogen y<sup>-1</sup>)<sup>129, 141</sup>. Not all nitrous oxide produced from nitric oxide reduction is  
926 further reduced to dinitrogen gas. The resulting nitrous oxide release from the marine and  
927 terrestrial environment is 4 and 12 Tg nitrogen y<sup>-1</sup>, respectively<sup>129</sup>. Although the nitrous oxide  
928 flux is small compared to the other nitrogen fluxes, it has a profound effect on the environment  
929 because nitrous oxide is the main ozone depleting agent and a powerful greenhouse gas<sup>92</sup>.

930 As shown in the figure, the nitrogen-transforming processes have vastly different fluxes and do  
931 not form one balanced nitrogen cycle as often depicted in papers and textbooks. The largest  
932 nitrogen fluxes are associated with the interconversion of ammonia and organic nitrogen. In the  
933 marine environment alone, the fluxes associated with ammonification and ammonium  
934 assimilation are an order of magnitude larger ( $\sim 8800$  Tg nitrogen y<sup>-1</sup>)<sup>142</sup> than marine nitrogen  
935 loss and gain combined ( $\sim 300$  Tg nitrogen y<sup>-1</sup>)<sup>141</sup>. Another substantial nitrogen flux is associated  
936 with the oxidation of ammonia to nitrate via nitric oxide and nitrite (that is, nitrification).  
937 Marine nitrification is associated with a flux of  $\sim 2000$  Tg nitrogen per year, which explains  
938 why marine ammonia-oxidizing archaea are among the most abundant microorganisms even  
939 though ammonia concentrations are low in the ocean. Nitrate-assimilation related fluxes are in  
940 the same order of magnitude. Marine phytoplankton accounts for 2000 Tg nitrate reduced per  
941 year<sup>142</sup>. Compared to this, the fluxes associated with dissimilatory nitrate reduction to



942 ammonium are most likely smaller. Although there are no available estimates for the terrestrial  
943 environment, assimilation related fluxes are likely six times smaller due to the lower nitrogen  
944 requirement of land plants, which require about 1 molecule of nitrogen for every 40 carbon  
945 molecules fixed<sup>144</sup>, compared to 1 molecule nitrogen per 6.6 molecules of carbon fixed by  
946 marine algae. Assuming steady state conditions (when gain of a nitrogen compound equals its  
947 loss), we estimated the terrestrial nitrification and ammonification fluxes by dividing the marine  
948 fluxes by six.

949

## 950 **Box 2. Nitrogen removal by microorganisms in wastewater treatment**

951 Since the industrial revolution, agriculture, burning of fossil fuel and domestic and industrial  
952 wastewater production have been the major drivers of nitrogen pollution, which severely affects  
953 life on earth<sup>141, 142</sup>. Nitrogen has been recognized as an important pollutant in wastewater only  
954 in the last 40 years, when it became clear that excess nitrogen leads to eutrophication and fish  
955 mortality due to ammonia toxicity. Consequently, nitrogen-removing systems were added to  
956 many wastewater treatment plants, which were originally used to remove organic carbon.  
957 Nevertheless, most conventional wastewater treatment plants do not remove nitrogen.

958 In contrast to most natural ecosystems, in which precious nitrogen is recycled and retained,  
959 nitrogen-removing treatment plants are designed to convert ammonium to dinitrogen gas, which  
960 is lost to the atmosphere. In these treatment plants, organic carbon is removed first. This results  
961 in organic carbon-poor and ammonium-rich wastewater, which is fed into a nitrogen-removal  
962 system. Conventional systems rely on nitrification ( $\text{NH}_4^+ \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$ ) to oxidize ammonium  
963 to nitrate, which is subsequently reduced to dinitrogen gas by denitrification ( $\text{NO}_3^- \rightarrow \text{NO}_2^-$   
964  $\rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$ ). Nitrification requires extensive aeration to create conditions that are  
965 suitable for ammonium oxidation to nitrate (2 molecules of  $\text{O}_2$  is needed per molecule of  
966 ammonium). Subsequently, external organic carbon (often methanol) is added to induce  
967 heterotrophic denitrification, which reduces nitrate to  $\text{N}_2$ . Hence, conventional nitrogen removal  
968 is costly, energy- and resource-intensive, and also produces nitrous oxide, which contributes to  
969 global warming. To alleviate these problems, different reactor configurations have been  
970 implemented to minimize external carbon addition and aeration. For example, in some systems,  
971 part of the raw wastewater, which is rich in organic carbon, is fed directly to the denitrification  
972 step or in others, intermittent aeration is used to promote nitrification and denitrification in a  
973 single tank<sup>145</sup>.

974 In the last decade, anaerobic ammonium oxidation (anammox) emerged as an alternative  
975 process for nitrogen removal. In compact bioreactors, aerobic ammonia-oxidizing bacteria,

976 such as *Nitrosomonas europaea*, convert half of the available ammonia to nitrite under oxygen  
977 limitation, which is termed ‘partial-nitrification’. This is followed by the conversion of nitrite  
978 with the remaining ammonium to dinitrogen gas by bacteria performing the anammox process,  
979 such as *Kuenenia stuttgartiensis* (solid arrows, see the figure)<sup>146</sup>. In these partial nitrification-  
980 anammox systems, nitrate production by aerobic nitrite oxidizers such as *Nitrospira* spp. or  
981 *Nitrobacter* spp. is undesired as it decreases the efficiency of nitrogen removal. Oxygen-limited  
982 partial-nitrification-anammox reactors have lower aeration requirements than conventional  
983 nitrogen-removal systems, do not require organic carbon addition, and produce less nitrous  
984 oxide. Currently, partial-nitrification-anammox systems are increasingly applied to ammonium-  
985 rich wastewaters<sup>146, 147</sup>, such as effluents from anaerobic sludge digesters [G]. Implementation  
986 of these systems in full-scale municipal wastewater treatment, which have much lower  
987 ammonium concentrations, could pave the way to more sustainable sewage treatment<sup>146</sup>.

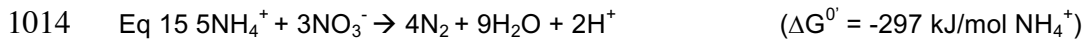
988 Some of the recently discovered nitrogen-cycling microorganisms could also be applied in  
989 wastewater treatment. Archaea that oxidize ammonia to nitrite and bacteria that oxidize  
990 ammonia to nitrate (in the comammox process) have been detected in nitrogen-removing  
991 wastewater treatment plants<sup>133, 148</sup> but their role in these systems is unclear. In oxygen-limited  
992 nitrogen-removal systems, such as partial-nitrification-anammox bioreactors, bacteria performing  
993 comammox<sup>12, 13, 39</sup> will most likely act as conventional ammonia oxidizers that produce nitrite.  
994 Exciting new possibilities for wastewater treatment are offered by the newly discovered nitrite-  
995 and nitrate-dependent anaerobic methane-oxidizing microorganisms<sup>66, 149</sup>. A bioreactor that  
996 combines anaerobic methanotrophs, such as *Candidatus Methylothermobacter* spp. and *Candidatus*  
997 *Methanoperedens* spp. with microorganisms that perform the anammox process could  
998 simultaneously remove ammonium, nitrate and methane (dashed arrows; see the figure). Such  
999 co-cultures have already been established under laboratory conditions; however, a full-scale  
1000 wastewater treatment system has not been implemented<sup>66, 149</sup>. In these systems, aerobic methane  
1001 oxidizers such as *Methylobacter* spp. would also contribute to methane removal.

1002 Fundamental physiological and biochemical research into nitrogen-cycling microorganisms  
1003 and their application have always progressed hand in hand — newly discovered  
1004 microorganisms led to more efficient and sustainable treatment systems, and vice versa. It is  
1005 apparent that this trend will continue to help safeguard the environment for future generations.

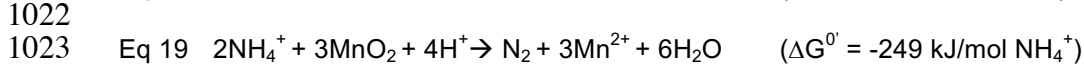
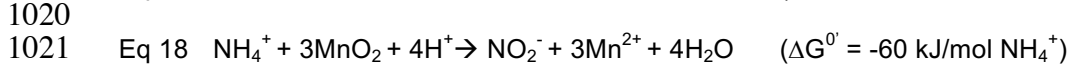
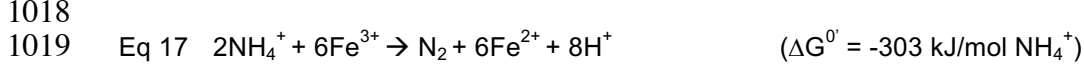
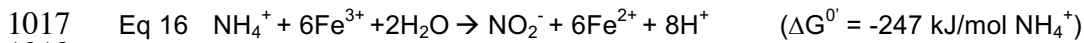
1006 **Box 3: Undiscovered biochemical reactions**

1007 Numerous new microbial nitrogen-transforming reactions and pathways have been discovered  
1008 in the last decade. Based on thermodynamic considerations further exergonic [G] reactions exist  
1009 that could be exploited by microorganisms (Equations 15-26). Whereas some reactions could

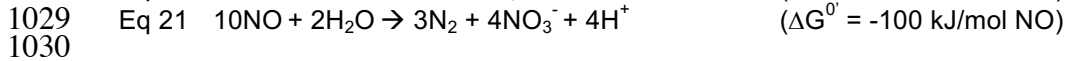
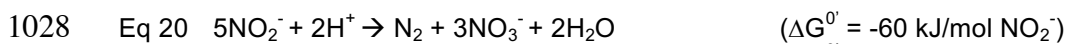
1010 be catalyzed by known enzymes, others would require hitherto unknown biochemistry (Eq. 15-  
1011 19, 25 and 26). For example, nitrate-dependent ammonium oxidation (Eq. 15) cannot proceed  
1012 through the known anaerobic ammonium oxidation pathway because ammonia first needs to be  
1013 oxidized to the intermediate hydroxylamine or a similar oxygen containing species<sup>46</sup>.



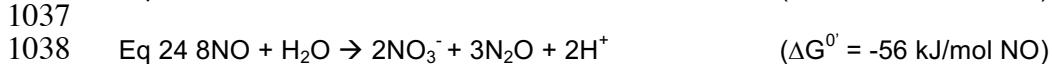
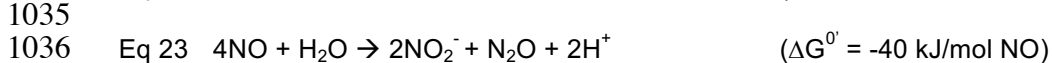
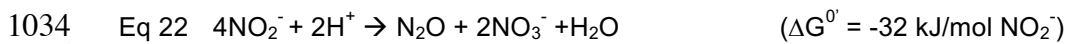
1015 Similarly, novel biochemical pathways for ammonia activation would be necessary for iron-  
1016 and manganese-dependent ammonium oxidation (Eq. 16-19).



1021 On the other hand, several disproportionation [G] reactions (Eq. 20-24) could be carried out by  
1022 known microorganisms using the existing biochemical machinery. Anaerobic ammonium-  
1023 oxidizing bacteria could perform nitrite (Eq. 20) and nitric oxide (Eq. 21) disproportionation<sup>46</sup>.



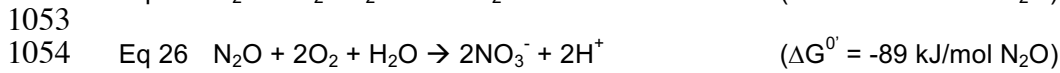
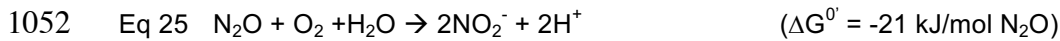
1026 Similarly, disproportionation of nitrite into nitrous oxide and nitrate (Eq. 22), nitric oxide into  
1027 nitrite and nitrous oxide (Eq. 23) or nitrate and nitrous oxide (Eq. 24) could theoretically be  
1028 carried out by aerobic nitrite-oxidizing bacteria.



1032 These microorganisms could use nitrite oxidoreductase to oxidize nitrite to nitrate and nitrite  
1033 reductases present in *Nitrobacter* spp., *Nitrococcus marinus*, *Nitrospira* spp. and *Nitrospina*  
1034 spp. could reduce nitrite to nitric oxide<sup>6</sup>. Nitric oxide oxidation has been observed in *Nitrobacter*  
1035 spp.,<sup>150, 151</sup> but it is unclear whether this reaction is biotic or abiotic and the responsible enzyme  
1036 remains unknown. Nitric oxide oxidation to nitrite could also be catalyzed by Cu-containing  
1037 nitrite reductases (nirK), which are known to be bidirectional<sup>152</sup>. The remaining reaction,  
1038 reduction of nitric oxide to nitrous oxide, can be carried out by terminal oxidases, which are  
1039 evolutionarily related to nitric oxide reductases<sup>153</sup>.

1040 Nitrous oxide, a potent greenhouse gas, is reduced to dinitrogen gas in the absence of oxygen,  
1041 whereas it is assumed to be biologically stable under oxic conditions. Intriguingly, aerobic

1050 nitrous oxide oxidation to either nitrite or nitrate is thermodynamically feasible (Eq. 25, 26),  
1051 but this reaction would also require a new biochemical pathway.



1055  
1056 The only way to identify microorganisms that catalyze these undiscovered reactions is to grow  
1057 them under controlled laboratory conditions. It is clear that the physiology and biochemistry of  
1058 nitrogen-transforming microorganisms will remain fertile fields of research for years to come.

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**Figure 1. Microbial transformations of nitrogen compounds.** Microorganisms carry  
1065 enzymes that perform fourteen redox reactions involving eight key inorganic nitrogen species  
1066 of different oxidation states (enzyme-bound intermediates and their redox-states are not shown).  
1067 The interconversion of ammonia and organic nitrogen does not involve a change in the redox  
1068 state of the nitrogen atom. The reactions involve reduction (red), oxidation (blue) and  
1069 disproportionation and comproportionation (green). The following enzymes perform the  
1070 nitrogen transformations: assimilatory nitrate reductase (NAS, *nasA* and *nirA*); membrane-  
1071 bound (NAR, *narGH*) and periplasmic (NAP, *napA*) dissimilatory nitrate reductases; nitrite  
1072 oxidoreductase (NXR, *nxrAB*); nitric oxide oxidase (NOD, *hmp*); heme-containing (cd1-NIR,  
1073 *nirS*) and copper-containing (Cu-NIR, *nirK*) nitrite reductases; cytochrome c-dependent  
1074 (cNOR, *cnorB*), quinol-dependent (qNOR, *norZ*) and copper-containing quinol-dependent  
1075 nitric oxide reductases (Cu<sub>A</sub>NOR); NADH-dependent cytochrome P<sub>450</sub> nitric oxide reductase  
1076 (P<sub>450</sub>NOR, *p450nor*); flavodiiron nitric oxide reductase (NOR<sub>VW</sub>, *norVW*); hybrid cluster protein  
1077 (HCP, *hcp*); hydroxylamine oxidoreductase (HAO, *hao*); hydroxylamine oxidase (HOX, *hox*);  
1078 nitrous oxide reductase (NOS, *nosZ*); nitric oxide dismutase (NO-D, *norZ*); assimilatory nitrite  
1079 reductase (cNIR); *nasB* and *nirB*); dissimilatory periplasmic cytochrome c nitrite reductase  
1080 (ccNIR, *nrfH*); epsilon hydroxylamine oxidoreductase ( $\epsilon$ HAO, *haoA*); octaheme nitrite  
1081 reductase (ONR); octaheme tetrathionate reductase (OTR); molybdenum-iron (MoFe,  
1082 *nifHDK*), iron-iron (FeFe, *anfHGDK*) and vanadium-iron (VFe, *vnfHGDK*) nitrogenases;  
1083 hydrazine hydrolase (HDH, *hdh*); hydrazine synthase (HZS, *hzsCBA*); ammonia  
1084 monooxygenase (AMO, *amoCAB*); particulate methane monooxygenase (*pMMO*, *pmoBAC*);  
1085 cyanase (CYN, *cynS*); urease (URE, *ureABC*).

1086

1087 **Figure 2. Enzymes catalyzing four key nitrogen cycling reactions. a.** The molybdenum-iron  
1088 (MoFe) nitrogenase enzyme contains the electron transfer protein (green; encoded by *nifH*) and  
1089 the alpha- (magenta; encoded by *nifD*) and beta-subunits (purple; encoded by the *nifK*) of the  
1090 catalytic enzyme. *nifH* is used to detect nitrogen fixing-microorganisms in the environment.  
1091 The iron sulfur clusters mediate electron transfer to the catalytic center. The association and  
1092 dissociation of the electron transfer and catalytic proteins requires the input of ATP. **b.** In the  
1093 anaerobic ammonium-oxidizing bacterium *Kuenenia stuttgartiensis*, electrons flow through the  
1094 hemes of the octaheme hydroxylamine oxidase (HOX) (red arrows). Hemes belonging to  
1095 different monomers are depicted in green, blue and gray. Heme 4 is the catalytic center. **c.** In  
1096 the membrane-bound bacterial nitrate reductase (NAR), the catalytic dimer is encoded by *narG*  
1097 and *narH*, whereas the membrane anchor is encoded by *narI*. *narG* is used to detect denitrifying  
1098 microorganisms in the environment. Nitrate reduction to nitrite occurs in the cytoplasm and  
1099 protons are translocated into the periplasm. Thereby, NAR contributes to the proton motive  
1100 force. **d.** In *Kuenenia stuttgartiensis*, *hzsA*, *hzsB* and *hzsC* encode a hydrazine synthase. The  
1101 former two genes are used to detect anaerobic ammonium-oxidizing bacteria in the  
1102 environment. This enzyme is proposed to perform a two-step reaction. It starts in the gamma  
1103 subunit (gray) with the reduction of nitric oxide to hydroxylamine, which is transported through  
1104 the substrate channel (brown) to the alpha subunit (green). The alpha subunit comproportionates  
1105 hydroxylamine with ammonia into hydrazine. Both reactions are catalyzed by cytochrome *c*-type  
1106 heme proteins. Figure 2a was adapted from <http://pdb101.rcsb.org/motm/26>, 2b from Ref. 8, 2c  
1107 adapted from Ref. 154, and 2d was adapted from Ref. 115.  
1108

1109 **Figure 3. Potential nitrogen-transforming microbial networks in different ecosystems. a)**  
1110 The open ocean gyres are vast nutrient-limited regions, in which nitrogen is extensively  
1111 recycled. In the sunlit surface waters, cyanobacteria mainly assimilate ammonium and/or  
1112 organic nitrogen compounds for growth. Viral lysis and grazing by mesozooplankton releases  
1113 organic nitrogen (for example, urea), which is subsequently mineralized back to ammonium by  
1114 heterotrophic bacteria. Nitrogen-fixing bacteria provide additional ammonium. In deeper  
1115 waters, ammonium is oxidized to nitrate. Some of this nitrate diffuses up into the surface waters  
1116 and is assimilated by phytoplankton. **b)** Marine oxygen minimum zones (OMZs) are found on  
1117 the eastern boundaries of oceans, where wind-driven upwelling of nutrient rich waters  
1118 stimulates primary productivity in the surface waters. The subsequent aerobic mineralization of  
1119 sinking organic matters depletes oxygen in the underlying waters. Aerobic nitrifying

1120 communities that are well adapted to low oxygen-conditions perform ammonia oxidation to  
1121 nitrite and nitrate. The OMZs are major regions of nitrogen loss due to the activity of anaerobic  
1122 ammonium-oxidizing bacteria and to a lesser extent denitrification. Complex communities of  
1123 microorganisms are involved in the denitrification process. **c)** Amongst the largest man-made  
1124 ecosystems are agricultural fields that are used for crop production. Legumes are common crops  
1125 and an important source of protein. They influence the microbial community in the surrounding  
1126 soil by releasing organic carbon and live in symbiosis with N<sub>2</sub>-fixing microorganisms, such as  
1127 *Bradyrhizobium* spp.. Ammonium that leaks out into the surrounding soil can fuel aerobic  
1128 ammonia and nitrite oxidation. Subsequent diffusion of nitrate to anoxic zones in soil fuels  
1129 nitrogen-transforming processes such as dissimilatory nitrate reduction to ammonium, nitrous  
1130 oxide and dinitrogen gas. **d)** Rice paddies are flooded agricultural fields, which are fertilized  
1131 with nitrogen-containing compounds such as urea to grow rice<sup>155</sup>. Urea hydrolysis and nitrogen  
1132 fixation generate ammonia, which is oxidized to nitrate in oxic soils surrounding the rice-plant  
1133 roots. Subsequent diffusion of nitrate to the underlying anoxic soil fuels processes, such as  
1134 denitrification, anaerobic ammonium oxidation (anammox) and the oxidation of methane  
1135 produced by methanogenesis.

1136

### 1137 **Key points**

1138

- 1139 • Nitrogen is an essential component of all living organisms and the main nutrient limiting  
1140 life on our planet. Its availability depends on diverse nitrogen transforming reactions  
1141 that are carried out by microorganisms.
- 1142 • Nitrogen-transforming microorganisms are metabolically versatile rendering their  
1143 classification as mere ‘nitrifiers’ or ‘denitrifiers’ etc. inadequate.
- 1144 • The classical nitrogen cycle consisting of distinct processes that follow each other in an  
1145 orderly fashion does not exist. In nature, microorganisms form complex networks that  
1146 link nitrogen-transforming reactions.
- 1147 • Microbial nitrogen-transforming networks both attenuate and exacerbate human-  
1148 induced global change. They produce and consume the powerful greenhouse gas nitrous  
1149 oxide; lead to eutrophication of aquatic systems and at the same time remove nitrogen  
1150 from wastewater.
- 1151 • There are still many undiscovered nitrogen-transforming reactions that are  
1152 thermodynamically feasible. The microorganisms catalyzing these reactions and the  
1153 involved biochemical pathways are waiting to be discovered.

1154

1155 **Glossary Terms**

1156

1157 **Reductant**

1158 The electron-donating compound in a redox reaction.

1159

1160 **Oxygenic phototroph**

1161 Oxygenic phototrophs obtain energy from light and use water as the electron donor, forming

1162 molecular oxygen and sugar as products.

1163

1164 **Bacteriocytes**

1165 Special cells in animals that contain endosymbiotic bacteria.

1166

1167 **Thaumarchaeota**

1168 The phylum Thaumarchaeota contains the ammonia-oxidizing archaea.

1169

1170 **Acidophile**

1171 An organism that grows in acidic environments (<pH 6).

1172

1173 **Methanotroph**

1174 An organism that oxidises methane to conserve energy.

1175

1176 **NC10**

1177 A candidate bacterial phylum, named after Nullarbor Caves in Australia, which contains

1178 *Candidatus Methylophilum oxyfera*, which is the first organism discovered that performs

1179 methane oxidation coupled to oxygenic denitrification.

1180

1181 **Endergonic**

1182 A reaction that requires energy input.

1183

1184 **Verrucomicrobia**

1185 A bacterial phylum with only a few described species, some of which appear to be important in

1186 the methane cycle.

1187

1188 **Anoxygenic phototroph**

1189 These microorganisms obtain energy from light and use compounds such as hydrogen sulfide  
1190 instead of water as electron donor and thus do not produce molecular oxygen.

1191

1192 **Eutrophication**

1193 The excessive growth of algae or cyanobacteria due to increased input of nutrients.

1194

1195 **Proton motive force**

1196 Proton dislocation creates a difference of charge and pH between two sides of a cell membrane  
1197 and thereby generates an electrochemical potential, which is used for energy conservation.

1198

1199 **Primary nitrite maxima**

1200 The peak in nitrite concentrations at the base of the euphotic zone.

1201

1202 **Nitric oxide dismutation**

1203 Two molecules of nitric oxide are disproportionated into one molecule of molecular oxygen  
1204 and one molecule of dinitrogen gas.

1205

1206 **Comproportionation**

1207 A chemical reaction in which two reactants containing the same element with a different  
1208 oxidation state react to create a product with a single oxidation state.

1209

1210 **Anammoxosome**

1211 A bacterial organelle found in anammox bacteria, which is the only known prokaryotic  
1212 membrane-bound structure that is equally divided into daughter cells upon cell division.

1213

1214 **Exergonic**

1215 A reaction that results in the release of free energy.

1216

1217 **Disproportionation**

1218 A chemical reaction in which a reactant is split into two species containing the same element  
1219 with different oxidation states, one more oxidized and the other more reduced than the reactant.

1220

1221 **Anaerobic sludge digesters**

1222



1223 Bioreactors in which excess microbial biomass (sludge) produced during wastewater treatment  
1224 is anaerobically converted to carbon dioxide, methane, ammonium and reduced sulfur  
1225 compounds.

1226

1227 **Subject categories**

1228 Biological sciences / Microbiology / Biogeochemistry / Element cycles

1229 [URI /631/326/47/4112]

1230 Biological sciences / Ecology / Microbial ecology

1231 [URI /631/158/855]

1232 Biological sciences / Microbiology / Environmental microbiology

1233 [URI /631/326/171]

1234 Biological sciences / Biochemistry / Enzymes / Oxidoreductases

1235 [URI /631/45/607/1168]

1236 Biological sciences / Ecology / Ecological networks

1237 [URI /631/158/2463]

1238

1239

1240 **ToC blurb**

1241 Nitrogen-transforming microorganisms shape global biogeochemical nutrient cycles. In this

1242 Review, Kuypers, Marchant and Kartal explore the vast diversity of these microorganisms

1243 and their enzymes, highlighting novel pathways, and discuss how nitrogen-transforming

1244 microorganisms form complex nitrogen-cycling networks in different environments.

1245

1246

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1248

Fig. 1

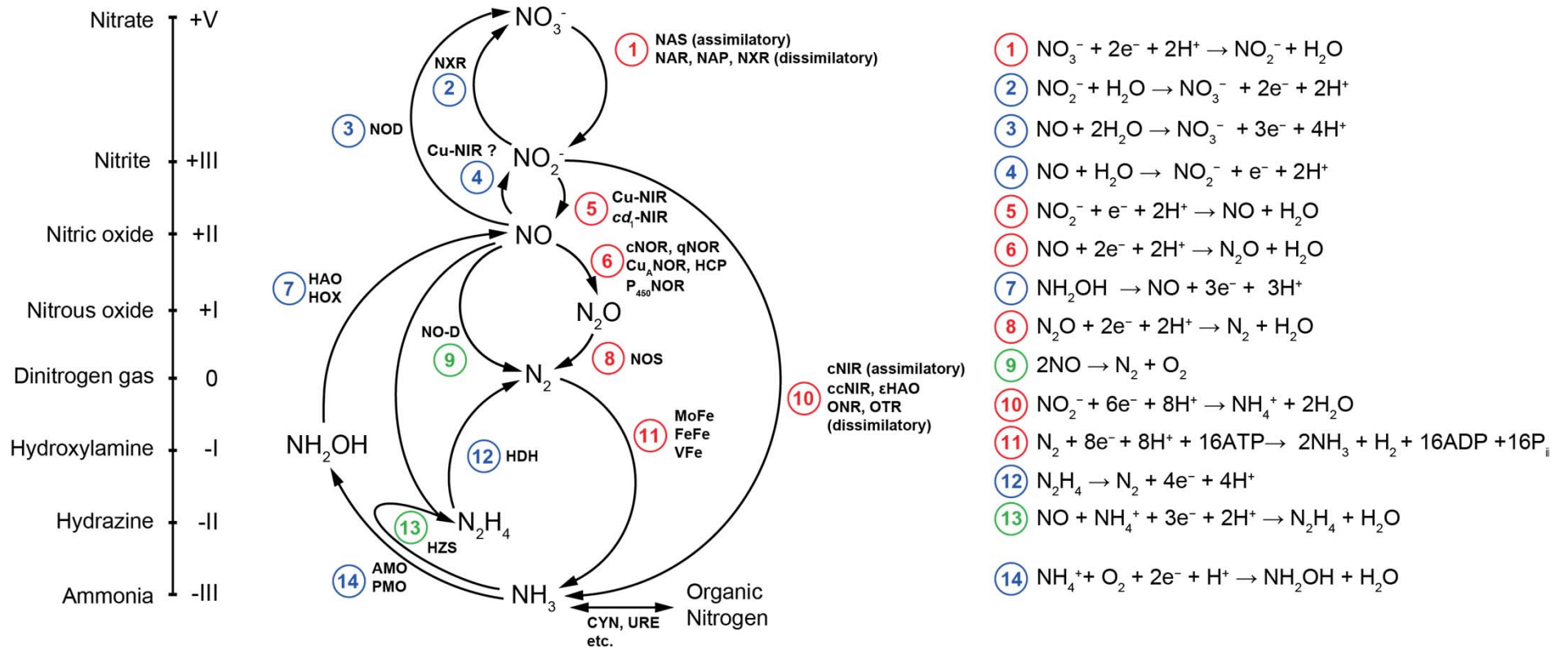


Fig. 2

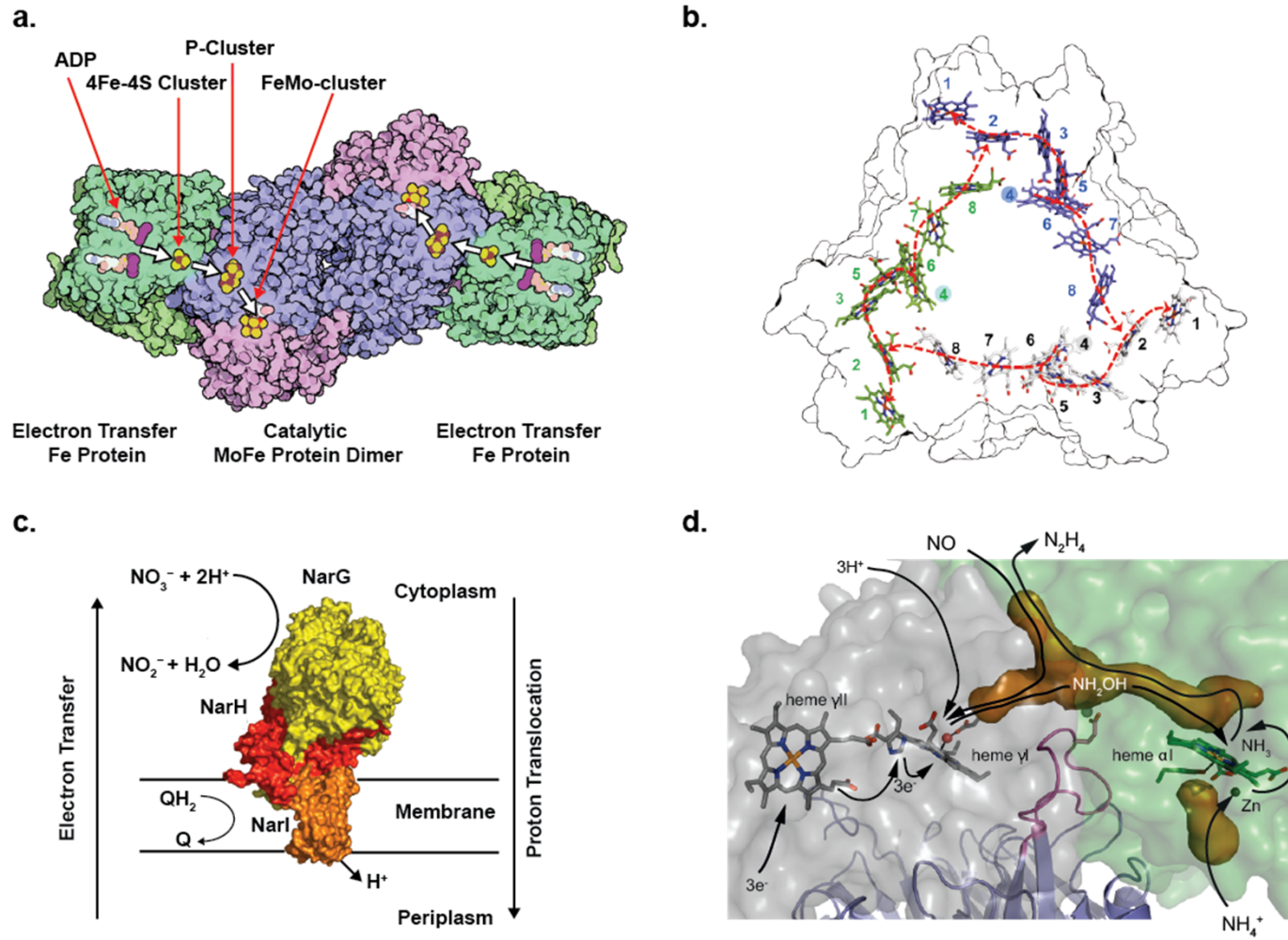
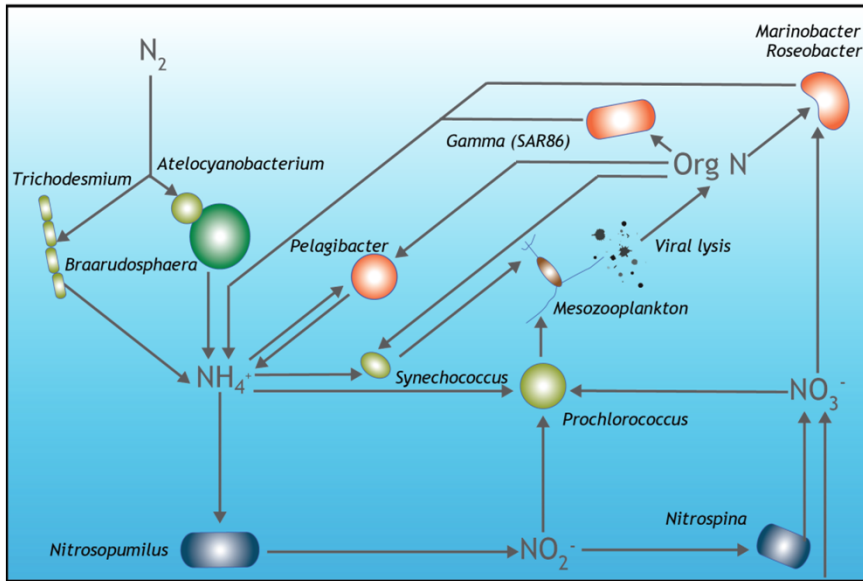
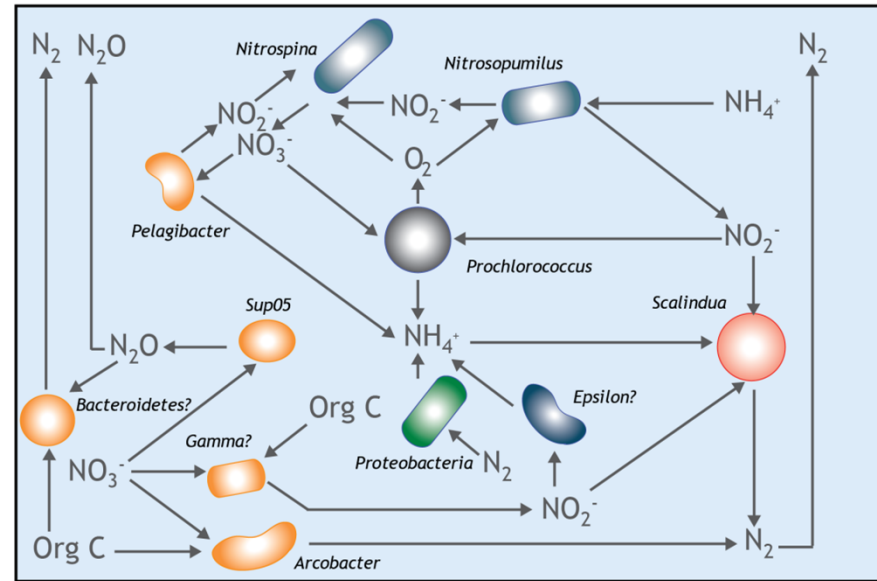


Fig. 3

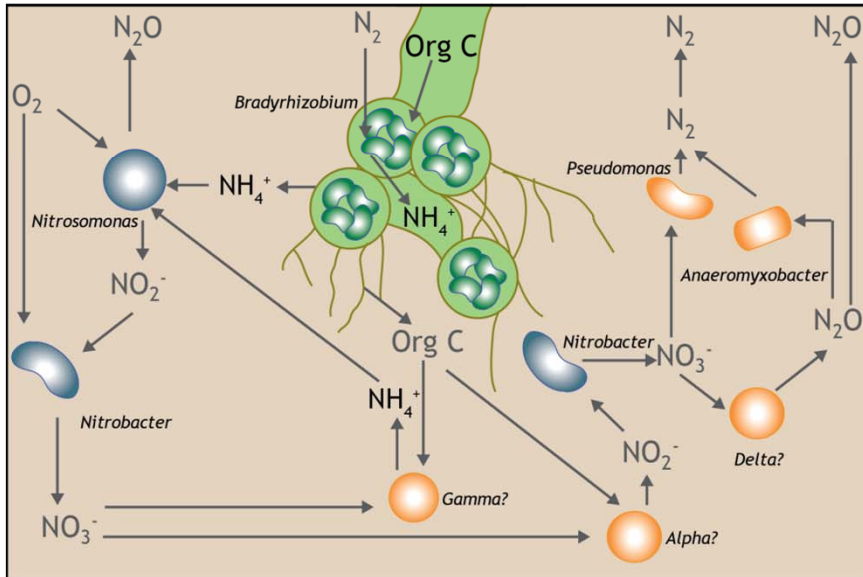
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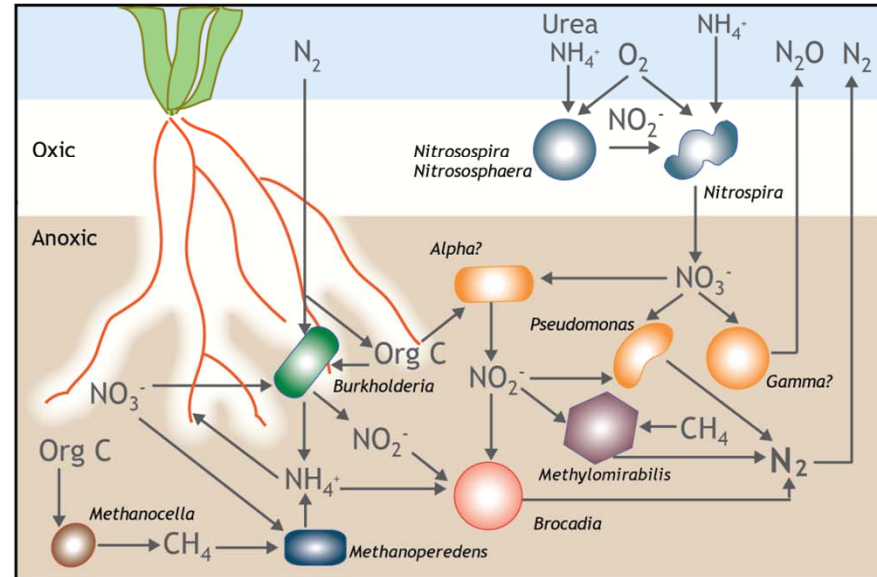
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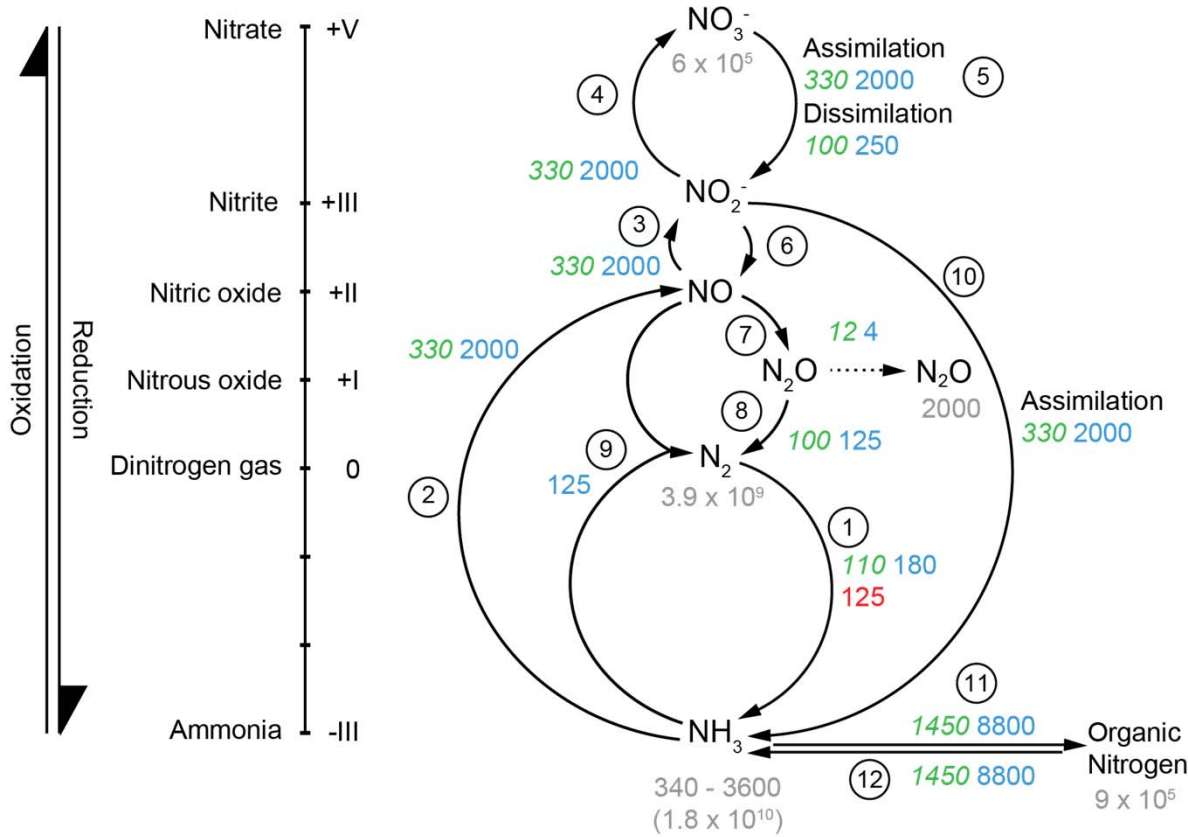
c.



d.



# Text Box 1



Text Box 2

