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1 **Nitrous Oxide Emissions from Biofilm Processes for Wastewater**  
2 **Treatment**

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42 **Abstract**

43 This paper discusses the microbial basis and the latest research on nitrous oxide (N<sub>2</sub>O) emissions  
44 from biofilms processes for wastewater treatment. Conditions that generally promote N<sub>2</sub>O  
45 formation in biofilms include (1) low DO values, or spatial DO transitions from high to low within  
46 the biofilm; (2) DO fluctuations within biofilm due to varying bulk DO concentrations or varying  
47 substrate concentrations; (3) conditions with high reaction rates, which lead to greater formation  
48 of intermediates, e.g., hydroxylamine (NH<sub>2</sub>OH) and nitrite (NO<sub>2</sub><sup>-</sup>), that promote N<sub>2</sub>O formation;  
49 and (4) electron donor limitation for denitrification. Formation of N<sub>2</sub>O directly results from the  
50 activities of ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and  
51 heterotrophic denitrifying bacteria. More research is needed on the roles of AOA, comammox, and  
52 specialized denitrifying microorganisms. In nitrifying biofilms, higher bulk ammonia (NH<sub>3</sub>)  
53 concentrations, higher nitrite (NO<sub>2</sub><sup>-</sup>) concentrations, lower dissolved oxygen (DO), and greater  
54 biofilm thicknesses result in higher N<sub>2</sub>O emissions. In denitrifying biofilms, N<sub>2</sub>O accumulates at  
55 low levels as an intermediate, and at higher levels at the oxic/anoxic transition regions of the  
56 biofilms and where COD becomes limiting. N<sub>2</sub>O formed in the outer regions can be consumed in  
57 the inner regions if COD penetrates sufficiently. In membrane-aerated biofilms, where  
58 nitrification takes place in the inner, aerobic biofilm region, the exterior anoxic biofilm can serve  
59 as a N<sub>2</sub>O sink. Reactors that include variable aeration or air scouring, such as denitrifying filters,  
60 trickling filters, or rotating biological contactors (RBCs), can form peaks of N<sub>2</sub>O emissions during  
61 or following a scouring or aeration event. N<sub>2</sub>O emissions from biofilm processes depend on the  
62 microbial composition, biofilm thickness, substrate concentrations and variability, and reactor type  
63 and operation. Given the complexity and difficulty in quantifying many of these factors, it may  
64 be difficult to accurately predict emissions for full-scale treatment plants. However, a better  
65 understanding of the mechanisms, and the impacts of process configurations, can help minimize  
66 N<sub>2</sub>O emission from biofilm processes for wastewater treatment.

67

68 **Keywords:** N<sub>2</sub>O, biofilms, hydroxylamine, MBBR, MABR, MBfR, granules

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71

72

## 73 INTRODUCTION

74

75 Wastewater treatment processes can be a significant source of nitrous oxide (N<sub>2</sub>O), a powerful  
76 greenhouse gas (GHG) with a global warming potential around 300 times that of carbon dioxide  
77 (CO<sub>2</sub>) (Montzka et al. 2011). N<sub>2</sub>O is very stable, and may persist in the atmosphere for over 120  
78 years (Kampschreur et al. 2009; Schreiber et al. 2012). The U.S. Environmental Protection  
79 Agency (EPA) estimates that U.S. wastewater treatment plants emit around 5.2 Tg N<sub>2</sub>O yr<sup>-1</sup> as  
80 CO<sub>2</sub> equivalents (Ritter 2014), and these amounts are expected to increase with time (Law et al.  
81 2012; Okabe et al. 2011).

82 Much past research has addressed N<sub>2</sub>O emissions from suspended growth processes (Ahn  
83 et al. 2010; Kampschreur et al. 2009; Law et al. 2012). However, much less is known about  
84 emissions from biofilm processes, such as the moving bed biofilm reactor (MBBR), integrated  
85 fixed-film activated sludge (IFAS), biological aerated filter (BAF), granular sludge, and  
86 membrane-aerated biofilm reactors (MABRs) (Henze et al. 2008; Martin and Nerenberg 2012;  
87 Syron and Casey 2008). Biofilm processes are becoming increasingly popular due to their higher  
88 volumetric treatment rates, reduced operational costs, minimal need for settling, and operational  
89 simplicity (Henze et al. 2008; Khan et al. 2013; Nicoletta et al. 2000; WEF 2010).

90 While the microbial basis of N<sub>2</sub>O formation, i.e., the microorganisms and metabolic  
91 pathways leading to its formation, are the same for suspended-growth and biofilm systems, the  
92 observed behavior may be very different. This results from the microbial stratification, microbial  
93 interactions, substrate gradients, and substrate interactions unique to biofilms, as well as the  
94 biofilm reactor configuration (Henze et al. 2008; Law et al. 2012; Vlaeminck et al. 2010a). Thus,  
95 the “mechanisms” leading to N<sub>2</sub>O emissions in biofilms may significantly differ from those of  
96 suspended growth systems.

97 Todt and Dorsch (2016) provided a comprehensive review of N<sub>2</sub>O emissions from biofilm  
98 systems. They explored the biochemistry of N<sub>2</sub>O production/consumption in relevant organisms,  
99 discussed current biofilm models, evaluated possible environmental factors affecting N<sub>2</sub>O  
100 emissions, and tabulated emission factors for different processes. Massara et. al (2017) briefly  
101 addressed biofilms as part of a comprehensive review of N<sub>2</sub>O emissions from wastewater  
102 processes. This review provides an update, considering new information on the N<sub>2</sub>O emissions

103 from microbial systems. It also discusses **new types of microbial metabolism and different biofilm**  
104 **reactor configurations, and their impacts on N<sub>2</sub>O emissions.**

105

## 106 **BIOFILMS VS. SUSPENDED-GROWTH SYSTEMS**

107

108 Biofilms are aggregates of microbial cells embedded in a network of self-produced extracellular  
109 polymeric substances (EPS) (Flemming et al. 2016; Stoodley et al. 2002). Biofilms are widespread  
110 in natural systems (Donlan 2002), and increasingly used in engineered treatment processes,  
111 especially for those with low substrate concentrations and high flows (Henze et al. 2008;  
112 Nicoletta et al. 2000; WEF 2010). Unlike with suspended bacteria, diffusion and reaction in  
113 biofilms lead to substrate gradients. As a result, concentrations in the biofilm may differ  
114 significantly from those in the bulk liquid (Fig. 1). In addition, bacteria stratify into layers,  
115 where different types of metabolism may predominate at different depths within the biofilm.

116

### 117 **FIGURE 1**

118

119 The dynamics of growth, decay, and detachment influence the microbial community  
120 structure of biofilms (Elenter et al. 2007). Slow growing organisms may be “pushed out” of the  
121 biofilm by faster growing organisms (Lackner et al. 2008; Xavier et al. 2005). Metabolic products  
122 may diffuse out of the biofilm or may be consumed by other populations. pH gradients may form  
123 due to proton-producing or consuming processes within the biofilm (Vroom et al. 1999). The  
124 greater complexity of biofilms, compared to suspended growth processes, makes their behavior  
125 more difficult to predict.

126

## 127 **N<sub>2</sub>O AND NITROGEN CYCLE**

128

129 **This section discusses basic microbial transformations that affect N<sub>2</sub>O formation in wastewater**  
130 **treatment processes. These processes are relevant to both suspended growth and biofilm processes.**  
131 **The relationship between these transformations and N<sub>2</sub>O formation in biofilms is discussed in**  
132 **subsequent sections.**

133 The nitrogen cycle includes a number of N species and both microbial and abiotic  
134 transformations, where N varies in redox state between -3 and +5. While most of the nitrogen  
135 cycle is well established, new biotic and abiotic transformation processes continue to be discovered  
136 (Daims et al. 2016; Kuypers et al. 2018; Schreiber et al. 2012; Stein and Klotz 2016). Figure 2  
137 schematically shows key N species and biological transformations. For wastewater treatment  
138 processes, the key transformations include nitrification and denitrification, where nitrate ( $\text{NO}_3^-$ ) is  
139 sequentially reduced to nitrogen gas ( $\text{N}_2$ ). Both processes can lead to  $\text{N}_2\text{O}$  formation.

140

141 FIGURE 2

142

### 143 **$\text{N}_2\text{O}$ from Microorganisms Related to Nitrification**

144

145 Nitrification is carried out by the sequential activity of ammonia-oxidizing bacteria (AOB)  
146 and archaea (AOA), and nitrite-oxidizing bacteria (NOB). **AOB and AOA oxidize ammonia ( $\text{NH}_3$ )  
147 to nitrite ( $\text{NO}_2^-$ ), with hydroxylamine ( $\text{NH}_2\text{OH}$ ) as an intermediate (Fig. 3) (Daims et al. 2016;  
148 Guo et al. 2017), while NOB oxidize  $\text{NO}_2^-$  to  $\text{NO}_3^-$ . AOB directly produce  $\text{N}_2\text{O}$  through two main  
149 pathways: nitrifier denitrification and  $\text{NH}_2\text{OH}$  oxidation (Fig. 3). NOB, AOA, anammox, and  
150 comammox microorganisms may play an indirect role in  $\text{N}_2\text{O}$  formation by affecting the  
151 availability of  $\text{NH}_3$  and  $\text{NO}_2^-$ .**

152

153 FIGURE 3

154

155 **In the nitrifier denitrification pathway, AOB reduce  $\text{NO}_2^-$  to nitric oxide (NO) and  $\text{N}_2\text{O}$**   
156 **(Chandran et al. 2011; Kampschreur et al. 2007; Kim et al. 2010; Tallec et al. 2006) (Fig. 3). The**  
157  **$\text{NH}_2\text{OH}$  oxidation pathway involves the oxidation of  $\text{NH}_2\text{OH}$  to NO by hydroxylamine**  
158 **oxidoreductase (HAO) and subsequent reduction to  $\text{N}_2\text{O}$  catalyzed by the enzyme NO reductase**  
159 **(Chandran et al. 2011; Law et al. 2012; Stein 2011) (Fig. 3).**

160 **Recent findings show that, in the canonical nitrifying bacteria *N. europaea*, two other**  
161 **routes for  $\text{N}_2\text{O}$  production exist under anaerobic conditions. One is the direct oxidation of  $\text{NH}_2\text{OH}$**   
162 **to  $\text{N}_2\text{O}$  by cytochrome P460 (Caranto et al. 2016) and the nitrification intermediate NO (Caranto**  
163 **and Lancaster 2017). Although not all AOB share the same route for  $\text{N}_2\text{O}$  production, these recent**

164 findings expand on previous knowledge where chemical reactions were thought to be mainly  
165 important at higher oxygen (O<sub>2</sub>) levels (Liu et al. 2017a).

166 N<sub>2</sub>O can also be produced biologically or abiotically by coupling NH<sub>2</sub>OH oxidation with  
167 the reduction of NO<sub>2</sub><sup>-</sup> (Harper et al. 2015; Terada et al. 2017), free nitrous acid (HNO<sub>2</sub>) (Soler-  
168 Jofra et al. 2016), or NO (Spott et al. 2011). These are termed N-nitrosation hybrid reactions, or  
169 simply “hybrid” reactions (Spott and Stange 2011). In addition, metals such as copper (Harper et  
170 al. 2015) and manganese (Heil et al. 2015) can catalyze abiotic N<sub>2</sub>O production from NH<sub>2</sub>OH via  
171 the hybrid reaction. Under some conditions, the hybrid reaction can become a predominant  
172 pathway for N<sub>2</sub>O production in a partial nitrifying reactor (Soler-Jofra et al. 2018; Terada et al.  
173 2017). N<sub>2</sub>O production via the hybrid reaction is enhanced in the presence of AOB (Liu et al.  
174 2017a; Terada et al. 2017).

175 Under aerobic conditions, N<sub>2</sub>O is mainly formed via the NH<sub>2</sub>OH pathway, and rates are  
176 relatively low. When DO concentrations decrease, the nitrifier denitrification pathway becomes  
177 more important, leading to higher rates of N<sub>2</sub>O formation (Chung and Chung 2000; Kampschreur  
178 et al. 2009; Ma et al. 2017a; Park et al. 2000; Tallec et al. 2008). However, under **complete** anoxic  
179 conditions N<sub>2</sub>O emissions are again low due to the lack of DO for NH<sub>3</sub> oxidation (Fig. 3). Spikes  
180 of N<sub>2</sub>O production can occur at transitions from anoxic to aerobic, or aerobic to anoxic, conditions,  
181 due to an electron imbalance (Domingo-Felez et al. 2014; Kampschreur et al. 2008; Sabba et al.  
182 2015; Yu et al. 2010). Thus, N<sub>2</sub>O emissions can be significant in processes with anoxic/aerobic  
183 stages or intermittent aeration (Chandran et al. 2011).

184 Unlike AOB, which have well elucidated N<sub>2</sub>O production pathways, the pathways for AOA  
185 are yet to be fully understood (Blum et al. 2018b). They perform NH<sub>3</sub> oxidation in a similar way  
186 to AOB (Kozlowski et al. 2016); however, they lack the ability to produce N<sub>2</sub>O enzymatically  
187 through side reactions of NH<sub>3</sub> oxidation or nitrifier denitrification, as mediated by AOB (Spang et  
188 al. 2012; Tourna et al. 2011; Walker et al. 2010). Stieglmeier et al. (2014) showed that  
189 *Nitrososphaera viennensis*, a pure culture of AOA from soil, produces N<sub>2</sub>O via a hybrid reaction.  
190 While AOA are found in WWTPs (Park et al. 2006; Sauder et al. 2012; Zhang et al. 2009), AOA  
191 are more common in marine environments (Santoro et al. 2011) and soils (Gubry-Rangin et al.  
192 2010; Li et al. 2018; Nicol et al. 2008; Zhang et al. 2012).

193 Anammox bacteria convert NH<sub>3</sub> and NO<sub>2</sub><sup>-</sup> to N<sub>2</sub> under anoxic conditions (Kuypers et al.  
194 2003). NO is a key intermediate in anammox metabolism (Kartal et al. 2011), and genomic

195 evidence suggests that anammox species have the potential to produce N<sub>2</sub>O via NO reduction  
196 (Kartal et al. 2007; Strous et al. 2006). However, research suggests that N<sub>2</sub>O production under  
197 **process**-relevant conditions is negligible (Blum et al. 2018a). Anammox may indirectly affect  
198 N<sub>2</sub>O formation by heterotrophs and AOB by reducing the concentrations of NH<sub>3</sub> and NO<sub>2</sub><sup>-</sup>.

199 Comammox bacteria are a subset of the genus *Nitrospira* capable of **complete ammonia**  
200 **oxidation** (comammox) via oxidation of NH<sub>3</sub> to NO<sub>3</sub><sup>-</sup> (Daims et al. 2015; van Kessel et al. 2015).  
201 **Comammox are thought to have a competitive advantage over conventional ammonia oxidizers**  
202 **(e.g. AOA and AOB) under ammonia-limiting conditions** (Costa et al. 2006; Daims et al. 2015;  
203 **Kits et al. 2017; van Kessel et al. 2015). While little is known about comammox in wastewater**  
204 **biofilms, van Kessel et al. (2015) and Daims et al. (2015) obtained comammox enrichments in the**  
205 **lab by operating their systems with low NH<sub>3</sub> concentrations. Thus, it is likely they play a role in**  
206 **wastewater biofilms under similar conditions.**

207 Evidence suggests that comammox *Nitrospira*, as opposed to canonical *Nitrospira*, harbor  
208 genomic NH<sub>3</sub> and NO<sub>2</sub><sup>-</sup> oxidation machinery homologous to classical AOB and NOB, respectively  
209 (e.g., gene clusters encoding *amo*, *hao*, and *nxr*) (Daims et al. 2015; van Kessel et al. 2015).  
210 However, very little is known about their capacity for N<sub>2</sub>O production. NH<sub>2</sub>OH appears to be an  
211 obligate intermediate of comammox metabolism, analogous to AOB catabolism, and it is likely  
212 that N<sub>2</sub>O can be formed by comammox via the NH<sub>2</sub>OH pathway (Fig. 3). Comammox genomes  
213 recovered to date also harbor capacity for NO<sub>2</sub><sup>-</sup> reduction to NO (NirK), similar to non-comammox  
214 *Nitrospira* (Camejo et al. 2017; Lawson and Lucker 2018). Comammox clades A and B genomes  
215 reported to date lack a known NOR **or proteins related to NO<sub>x</sub> metabolism** (Palomo et al. 2018),  
216 similarly to common *Nitrospira* taxa (Lawson and Lucker 2018) and therefore may be incapable  
217 of nitrifier denitrification. **Thus, the presence of reactive nitrogen species produced by comammox**  
218 **biomass, e.g. NO or NH<sub>2</sub>OH, could lead to abiotic reactions with the production of N<sub>2</sub>O as a**  
219 **final product.**

220 Comammox may be detrimental to PN/A systems, where NO<sub>2</sub><sup>-</sup> production is needed.  
221 However, they may also reduce N<sub>2</sub>O emissions by minimizing NO<sub>2</sub><sup>-</sup> accumulation. The presence  
222 of comammox in wastewater treatment processes, both in suspended growth and biofilm processes,  
223 and the metabolic versatility of *Nitrospira* species including the two comammox *Nitrospira* clades  
224 is currently an active area of research. Future research should also address the selecting factors



225 for partitioning between comammox and canonical *Nitrospira*, and clarify the potential role for  
226 comammox in N<sub>2</sub>O emissions.

227

## 228 **N<sub>2</sub>O from Microorganisms Related to Denitrification**

229

230 Denitrification is the sequential reduction of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> to NO, N<sub>2</sub>O, and finally N<sub>2</sub>  
231 (Ni and Yuan 2015). It involves four enzymes: the nitrate reductase (NAR), nitrite reductase  
232 (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS). A schematic of the  
233 denitrification metabolism is shown in Figure 3.

234 The formation of N<sub>2</sub>O in wastewater denitrification processes is often due to selective  
235 inhibition of the NOS enzyme (Guo et al. 2017). This can be caused by its greater sensitivity to  
236 DO (Firestone et al. 1979; Tallec et al. 2008), pH (Firestone et al. 1979; Hanaki et al. 1992), NO<sub>2</sub><sup>-</sup>  
237 (Alinsafi et al. 2008), carbon source type and concentration (Tallec et al. 2006), carbon limitation  
238 (Alinsafi et al. 2008; Tallec et al. 2006), and hydrogen sulfide (H<sub>2</sub>S) (Schonharting et al. 1998).

239 While denitrifying bacteria produce N<sub>2</sub>O during denitrification, they also can reduce N<sub>2</sub>O  
240 to N<sub>2</sub> (Read-Daily et al. 2016). Externally supplied N<sub>2</sub>O can be reduced concurrently with NO<sub>3</sub><sup>-</sup>  
241 and NO<sub>2</sub><sup>-</sup> (Conthe et al. 2018; Pan et al. 2015; Pan et al. 2013a; Read-Daily et al. 2016).

242 While many denitrifying bacteria have a complete reduction pathway and can reduce NO<sub>3</sub><sup>-</sup>  
243 and NO<sub>2</sub><sup>-</sup> all the way to N<sub>2</sub>, less is known about bacteria that can grow with N<sub>2</sub>O but not with NO<sub>3</sub><sup>-</sup>  
244 or NO<sub>2</sub><sup>-</sup>. Newly classified clade II-type *nosZ* N<sub>2</sub>O reducing bacteria were recently discovered  
245 (Jones et al. 2013; Sanford et al. 2012). These have since been detected in a granular sludge reactor  
246 (Lawson et al. 2017), a membrane-aerated biofilm reactor (MABR) (Kinh et al. 2017b) and a  
247 biofiltration system (Yoon et al. 2017). Some isolates harboring clade II type *nosZ* have higher  
248 affinity for N<sub>2</sub>O reduction than those harboring clade I type *nosZ* (Suenaga et al. 2018; Yoon et al.  
249 2016) whereas a contradictory finding was reported (Conthe et al. 2018), requiring more in-depth  
250 analysis concerning bacteria as an N<sub>2</sub>O sink at a low N<sub>2</sub>O concentration. Some clade II type *nosZ*  
251 bacteria appear to lack genes encoding for NIR and/or NOR, suggesting their potential as an N<sub>2</sub>O  
252 sink but not an N<sub>2</sub>O source (Graf et al. 2014). As reviewed elsewhere, these non-denitrifying N<sub>2</sub>O-  
253 reducing bacteria in wastewater engineering are yet to be explored in detail (Hallin et al. 2018).  
254 The ecophysiology of non-denitrifying N<sub>2</sub>O reducers in a biofilm system warrants further research.

255 There are a wide range of denitrifying microorganisms, and some with special behavior

256 with respect to N<sub>2</sub>O formation and reduction. Some can fully reduce NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> to NH<sub>3</sub> in an  
257 ecologically important process called dissimilatory nitrate or nitrite reduction to ammonium  
258 (DNRA) (Stein and Klotz 2016) (Fig. 2). In this process, NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> is reduced to NH<sub>3</sub>, with  
259 N<sub>2</sub>O produced at the NO<sub>2</sub><sup>-</sup> reduction stage as a by-product (Fig. 2) (Kelso et al. 1997; Rutting et  
260 al. 2011; Streminska et al. 2012). Unlike denitrification, this process conserves N in the ecosystem  
261 (Rutting et al. 2011; Tiedje et al. 1982). Many DNRA microorganisms can produce N<sub>2</sub>O as a by-  
262 product (Stevens and Laughlin 1998; Stevens et al. 1998). Some of these microorganisms employ  
263 DNRA as a detoxification mechanism in order to avoid high concentration of NO<sub>2</sub><sup>-</sup> (Kaspar 1982).  
264 However, the actual contribution of DNRA to N<sub>2</sub>O formation in these species remains uncertain  
265 (Butterbach-Bahl et al. 2013).

266 Behavior regarding N<sub>2</sub>O emissions may also vary based on the type of electron donor. For  
267 example, elemental-sulfur (S<sup>0</sup>) oxidizing denitrifiers (Di Capua et al. 2015; Liu et al. 2017b),  
268 methane (CH<sub>4</sub>) oxidizing denitrifiers (He et al. 2018), phosphate-accumulating (PAO) denitrifiers  
269 (Gao et al. 2017; Wang et al. 2011; Wang et al. 2014; Zhou et al. 2012), H<sub>2</sub> oxidizing denitrifiers  
270 (Li et al. 2017), and bacteria growing with an electrode as an electron donor (Jiang et al. 2018)  
271 display different behavior with respect to N<sub>2</sub>O emissions. Methane-oxidizing denitrifiers appear  
272 to reduce NO<sub>2</sub><sup>-</sup> to N<sub>2</sub> without forming N<sub>2</sub>O as an intermediate, and therefore are thought to  
273 minimize N<sub>2</sub>O emissions (He et al. 2018). While the details on each of these donors are beyond  
274 the scope of this review, the kinetics for each donor can have important impacts on N<sub>2</sub>O formation  
275 and consumption.

276

## 277 **TYPES OF BIOFILM REACTORS AND IMPACTS ON N<sub>2</sub>O EMISSIONS**

278

279 This section describes different type of biofilm reactors, and their special characteristics as relate  
280 to N<sub>2</sub>O emissions. Based on the analysis in the previous section, and also following Todt et al.  
281 (2016) and Massara et al. (2017), conditions that promote N<sub>2</sub>O emission include (1) low DO  
282 values, or DO spatially transitioning from high to low within the biofilm, as this leads to nitrifier  
283 denitrification or incomplete heterotrophic denitrification; (2) conditions where the DO fluctuates  
284 temporally from high to low values, (3) conditions with high reaction rates, which lead to greater  
285 formation of intermediates (e.g., NH<sub>2</sub>OH, NO<sub>2</sub><sup>-</sup>) that promote N<sub>2</sub>O formation; and (4) limiting  
286 electron donor for denitrification.

287           The above factors may have different impacts for different types of biofilm reactors. There  
288 is a wide range of biofilm reactors, and they can be classified based on the arrangement of their  
289 solid, liquid, and gas phases, whether the carriers are fixed or moving, their carrier specific surface  
290 area (area of carrier per unit volume of reactor), their mixing regime (completely mixed or plug  
291 flow), and the mechanisms of transfer of gases and electron donor or acceptor substrates. Typical  
292 biofilm reactor configurations are shown schematically in Figure 4.

293

294           **FIGURE 4**

295

296           Trickling filters (Fig. 4A) are commonly used for COD removal and nitrification. The  
297 media is non-submerged, and is kept aerobic by convective air currents within the bed. While  
298 considered aerobic, anoxic niches can form in the deeper biofilm (Dalsgaard and Revsbech 1992).  
299 The variations in DO and donor concentration in the biofilm between passes of the wastewater  
300 distributor arm can lead to N<sub>2</sub>O emissions. When used for nitrification, N<sub>2</sub>O is likely to form within  
301 the bed, with some stripped by the air currents and present in the effluent (Melse and Mosquera  
302 2014). There is little experimental data on N<sub>2</sub>O emissions from trickling filters, possibly due to the  
303 difficulty in capturing the off-gases, and further research is needed in this area.

304           Biofilters (Fig. 4A) are similar to trickling filters, but used to treat gaseous contaminants  
305 such as odorous compounds in air or volatile organic compounds (VOCs). Air is passed through a  
306 non-submerged packed bed with biofilms growing on the media, and the contaminants partition  
307 into the liquid phase coating the biofilm. Yoon et al. (2017) proposed using a biofilter supplied to  
308 remove N<sub>2</sub>O in off gases from an activated sludge aeration basin. Raw wastewater was used as the  
309 electron donor. In lab tests, 99.9% of N<sub>2</sub>O was removed when supplied at 100 ppmV in N<sub>2</sub>, i.e.,  
310 without any O<sub>2</sub>. However, removals decreased significantly when supplied in air. Biofilters are  
311 likely an expensive approach to mitigating N<sub>2</sub>O emissions, as they require covering aeration basin  
312 to collect off gases, treating large volumes of gas, and adding an additional process and complexity  
313 to the treatment train.

314           Packed bed reactors (Fig. 4B and 4C) are fully submerged fixed bed biofilm reactors. They  
315 can be operated in upflow or downflow mode, and either aerated (e.g., for nitrification) or  
316 unaerated with electron donor addition (denitrifying filters). Upflow packed bed reactors, such as  
317 nitrifying or denitrifying filters, typically operate in plug flow fashion. Thus, the filters experience

318 high substrate concentrations at the influent end and low concentrations at the effluent end. The  
319 concentration gradients (e.g., high  $\text{NH}_3$  at influent, low DO at effluent) can impact  $\text{N}_2\text{O}$  formation  
320 processes. When used for denitrification, air pulses are periodically performed at the bottom of the  
321 filter to release  $\text{N}_2$  bubbles accumulating in the reactor. These pulses can strip  $\text{N}_2\text{O}$  formed at the  
322 beginning of the bed, when normally it would be reduced to  $\text{N}_2$  further within the bed (Bollon et  
323 al. 2016). Whenever air is added to a denitrifying filter, there is potential for  $\text{N}_2\text{O}$  formation at  
324 some location within the biofilm due to the greater sensitivity of  $\text{N}_2\text{OR}$  to  $\text{O}_2$  inhibition.  $\text{N}_2\text{O}$  may  
325 also accumulate due to insufficient electron donor supply. For nitrifying and denitrifying packed  
326 bed reactors, backwashing is carried out regularly to remove excess biomass. Thinner biofilms  
327 may not allow full treatment, leading  $\text{N}_2\text{O}$  breakthrough from the reactor. For denitrifying biofilms,  
328 breakthrough can also be caused by donor limitation. Bollon et al. (2016) found that a full-scale  
329 denitrifying filter with a C/N of 3 or higher had up to 93%  $\text{N}_2\text{O}$  reduction. However, during a  
330 carbon supply failure removals lowered 26%. Similar results were found by Capodici et al. (2018)  
331 and Zhang et al. (2016). In the latter study, the authors found that a decrease of the C/N from 3 to  
332 0.65 led to an increase of the genes encoding for NOR that would enhance the transformation of  
333 NO to  $\text{N}_2\text{O}$  and lead to increased  $\text{N}_2\text{O}$  emissions. Zhang et al. (2017) studied the behavior of lab-  
334 scale denitrification filters and found a complex interaction of the denitrification with anammox  
335 and DNRA. Gene abundance, together with accumulation of  $\text{NO}_2^-$  at temperatures between 5 and  
336 15 °C, were found important factors for  $\text{N}_2\text{O}$  accumulation. Further research is required to  
337 investigate the impact of influent  $\text{NO}_2^-$  and possible adaptation of bacteria to variable influent  
338 loadings of both  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in denitrifying filters.

339 RBCs (Fig. 4D) use rotating wheels of media partially submerged in wastewater. When the  
340 wheels are outside the water, the biofilm can experience  $\text{O}_2$  concentrations in the biofilm exterior,  
341 while the DO concentrations can drop significantly when immersed in the wastewater (Pynaert et  
342 al. 2002). This cycling of high and low DO concentrations, as well variations in donor  
343 concentration when the biofilm is submerged vs. when it is out of the wastewater, can potentially  
344 lead to higher  $\text{N}_2\text{O}$  emissions. There does not appear to be any published findings of  $\text{N}_2\text{O}$   
345 emissions from RBCs. Note that RBCs are often covered to prevent from UV toxicity and to  
346 protect from low temperatures in winter. In these cases, it may be possible to pump air from the  
347 enclosures through an anoxic zone or into a biofilter, such as that described above, to reduce  $\text{N}_2\text{O}$   
348 to  $\text{N}_2$ .

349 Airlift, MBBRs, and IFAS (Fig. 4E and 4G) use carriers that “float” in the water, and  
350 therefore have little relative velocity between the carrier and the water. They can be operated under  
351 aerobic or anoxic conditions. In continuous systems, the biofilm carriers are kept in a single zone,  
352 experiencing consistent bulk environments. This can avoid the high N<sub>2</sub>O emissions in suspended  
353 growth systems transitioning from anoxic to aerobic zones (Chandran et al. 2011). Recent research  
354 on N<sub>2</sub>O emissions from MBBRs are consistent with the factors described at the beginning of this  
355 section, depending on the application (Mannina et al. 2018a; Mannina et al. 2017; Mannina et al.  
356 2018b; Wei et al. 2017).

357 Fluidized bed reactors (Fig. 4F) behave similarly to a BAF, but use much finer media. This  
358 provides a high specific surface area, and allows the particles to become suspended in the upward  
359 wastewater flow. These reactors also experience a somewhat higher degree of mixing, compared  
360 to packed bed reactors, but still have some plug flow behavior. Excess biofilm is continuously  
361 removed by abrasion, and biofilms typically are thinner than in BAFs. The behavior with respect  
362 to N<sub>2</sub>O emissions should be similar to the BAFs. Note that aerobic granular sludge can behave  
363 similarly to a fluidized bed reactor. However, granular sludge is typically operated in sequencing  
364 batch mode (Castro-Barros et al. 2015). Recent research on N<sub>2</sub>O emission from granular sludge  
365 also confirm the above mechanisms (Jia et al. 2018; Lu et al. 2018; Peng et al. 2017; Reino et al.  
366 2017).

367 Counter-diffusional biofilms are those where one substrate diffuses from the bulk liquid,  
368 while the other penetrates the biofilm from the attachment surface. The counter-diffusion of  
369 substrates leads to a range of different behaviors with respect to conventional, co-diffusional  
370 biofilms (Nerenberg, 2016). Examples of counter-diffusional biofilms include MABRs, where the  
371 membranes are used to supply air or O<sub>2</sub>; membrane-biofilm reactors (MBfRs) where membranes  
372 supply H<sub>2</sub> or CH<sub>4</sub> (Liu et al., 2017b); sulfur-based biofilms, where solid S<sup>0</sup> particles support a  
373 biofilm (Wang et al. 2016a); and even bioelectrochemical biofilms (Jiang et al., 2018). MABR  
374 behavior is discussed in more detail in the next section.

375  
376  
377  
378

379 **MECHANISMS OF N<sub>2</sub>O FORMATION IN BIOFILM PROCESSES FOR**  
380 **WASTEWATER TREATMENT**

381  
382 Because of their special layered structure and organization, biofilms allow unique niche formation  
383 with specific metabolic functions. In addition, intermediates formed in one biofilm location can  
384 diffuse to another with different environments, leading to transformations that would not normally  
385 occur in a suspended growth system (Dalsgaard et al. 1995; de Beer 1997; Nielsen et al. 1990;  
386 Sabba et al. 2017b; Schreiber et al. 2009). **This section discusses basic behavior of biofilms for**  
387 **some key processes, including nitrification, denitrification, combined nitrification and**  
388 **denitrification, and partial nitrification/anammox. The behavior is common for most biofilm**  
389 **reactors except for MABRs, which are described separately. The figures in this section are intended**  
390 **to illustrate typical behavior. They are only schematics, not meant to reflect an actual operating**  
391 **condition.**

392  
393 **Nitrifying biofilms**

394  
395 Nitrifying biofilms form when NH<sub>3</sub> is the dominant or sole electron donor. While AOB and NOB  
396 are primary population members in nitrifying biofilms, heterotrophic bacteria typically co-exist  
397 (Kindaichi et al. 2004), growing on the decay products from nitrifying microorganisms (Gieseke  
398 et al. 2005; Okabe et al. 2005). However, N<sub>2</sub>O production in nitrifying biofilms is likely dominated  
399 by AOB, with a minor contribution from heterotrophic bacteria. In this section, we focus on the  
400 mechanisms of N<sub>2</sub>O from the nitrifying population. In the subsequent section, we discuss the  
401 impact of heterotrophs on nitrifying biofilms, especially when organic carbon is present in the  
402 bulk.

403 Typical substrate profiles in nitrifying biofilms, and zones of N<sub>2</sub>O formation and emission,  
404 are shown schematically in Figure 5. In conventional, co-diffusional biofilms, the outer biofilm is  
405 aerobic and has the highest NH<sub>3</sub> concentrations. As a result, the NH<sub>3</sub> oxidation rates are high,  
406 leading to high NH<sub>2</sub>OH concentrations. In addition, the nitrifier denitrification pathway is  
407 inhibited by the high DO in this zone. Thus, the NH<sub>2</sub>OH **oxidation** pathway is likely to dominate,  
408 and N<sub>2</sub>O formation rates are likely to be relatively low. Nitrifier denitrification may become  
409 significant in the aerobic/anoxic transition zone (Mao et al. 2008; Schreiber et al. 2009; Schreiber

410 et al. 2008). In the anoxic zone,  $N_2O$  formation rates are low. This is because  $NH_3$  oxidation,  
411 which is the source of electrons for nitrifier denitrification, requires  $O_2$ . However, Sabba et al.  
412 (2015) proposed that  $NH_2OH$  formed in the aerobic biofilm exterior would diffuse to the interior  
413 anoxic zones. AOB in this zone could utilize  $NH_2OH$  as a rich electron source, enabling the  
414 nitrifier denitrification pathway and resulting in a spike of  $N_2O$ . **Further research is needed to**  
415 **confirm this mechanism experimentally.** In Figure 5, the  $N_2O$  concentration profile slopes towards  
416 the outer biofilm, indicating diffusive mass transfer towards the bulk. If diffused aeration is used,  
417 the  $N_2O$  is readily stripped from the liquid phase (Law et al. 2012; Rassamee et al. 2011; Wu et al.  
418 2014).

419 Membrane-aerated biofilms (MABs) are a novel biofilm process for wastewater treatment,  
420 where  $O_2$  is supplied from the membrane and  $NH_3$  from the bulk (Martin and Nerenberg 2012;  
421 Syron and Casey 2008) (Fig. 5b). Because of the unique penetration of  $NH_3$  and  $O_2$  from opposite  
422 sides of the biofilm, they are called, as mentioned above counter-diffusional biofilms (Nerenberg  
423 2016).  $N_2O$  can also occur in MABRs systems. In MABs, the highest nitrification rates usually  
424 occur in the biofilm interior, not at the outer edge. Thus,  $N_2O$  formation via the  $NH_2OH$  pathway  
425 is likely to occur in the deep biofilm. In addition, the aerobic/anoxic transition occurs in the  
426 biofilm interior, and the bulk is anoxic. Thus, while  $N_2O$  can be stripped from suspended growth  
427 systems by bulk aeration (Law et al. 2012; Rassamee et al. 2011; Wu et al. 2014),  $N_2O$  in MABRs  
428 can be consumed by denitrifying bacteria in the outer biofilm or bulk liquid. Conversely, some  
429  $N_2O$  may be stripped from MABR biofilms by air flowing through the membrane lumen, if  
430 operated with open end membranes (Kinh et al. 2017a). Stripping from the lumen is indicated in  
431 Figure 5b by the slope of the  $N_2O$  concentration profile towards the membrane in its proximity.

432

433 FIGURE 5

434

435 NOB can contribute indirectly to  $N_2O$  emissions by scavenging DO and favoring the  
436 formation of a steeper gradient for transitioning from oxic to anoxic conditions (Sabba et al. 2017a;  
437 Sabba et al. 2015). They also can play a key role in reducing the  $NO_2^-$  concentration, which reduces  
438 the rates of nitrifier denitrification (Schreiber et al., 2009). Anammox bacteria can play a similar  
439 role in decreasing  $N_2O$  emissions (Pellicer-Nacher et al. 2010). **As mentioned previously, NOB**

440 do not play a direct role for NO and N<sub>2</sub>O emissions, but may affect emission by modifying the  
441 NO<sub>2</sub><sup>-</sup> concentrations (Wang et al. 2016b).

442

#### 443 Denitrifying biofilms

444

445 Denitrifying biofilms are those where NO<sub>3</sub><sup>-</sup> is the primary electron acceptor. We also consider  
446 biofilms with an aerobic exterior and denitrifying interior, but neglect any nitrification in the  
447 aerobic zone. In denitrifying biofilms, N<sub>2</sub>O is an obligate intermediate. It is typically present at  
448 higher concentrations in the outer biofilm region, where NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> reduction activity is higher,  
449 but can diffuse and be consumed in deeper regions where NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> concentrations are lower  
450 (Fig. 6a). Thus, biofilms can have regions that can serve as an N<sub>2</sub>O sink, mitigating N<sub>2</sub>O emissions  
451 (Dalsgaard and Revsbech 1992; Nielsen et al. 1990).

452

#### 453 FIGURE 6

454

455 In the presence of high DO, denitrification is usually inhibited and therefore little N<sub>2</sub>O is  
456 formed (Fig. 6b). However, biofilms typically have DO gradients, and denitrification and N<sub>2</sub>O  
457 formation may occur deeper in the biofilm (Dalsgaard and Revsbech 1992; Nielsen et al. 1990).  
458 In the transition zone from oxic to anoxic, higher amounts of N<sub>2</sub>O will be formed due to the higher  
459 sensitivity of NOS to O<sub>2</sub> inhibition (Bonin et al. 1992; Lu and Chandran 2010; Morley et al. 2008;  
460 Otte et al. 1996). When this transition zone is near the outer biofilms, more N<sub>2</sub>O may be exported  
461 to the bulk liquid. When the transition occurs deeper in the biofilm, i.e., at higher bulk DO  
462 concentrations, and when electron donor is sufficient, N<sub>2</sub>O is more likely to be reduced in the  
463 deeper biofilm and less emissions will occur (Dalsgaard and Revsbech 1992).

464

465 If N<sub>2</sub>O is formed in the outer biofilm, and if sufficient electron donor is available in the  
466 deeper zones of the biofilm, denitrifying biofilms can serve as an N<sub>2</sub>O sink (Eldyasti et al. 2014;  
467 Sabba et al. 2017b). However, if sulfate reduction occurs in the deeper biofilm where NO<sub>3</sub><sup>-</sup> has  
468 been depleted, H<sub>2</sub>S may accumulate and inhibit N<sub>2</sub>O reduction (Pan et al. 2013b). Electron donor  
469 limitation in the denitrifying zone also may result in greater N<sub>2</sub>O formation (Dalsgaard and  
470 Revsbech 1992; Nielsen et al. 1990; Todt and Dorsch 2015) (Fig. 6c).

470



471 **Combined nitrifying/denitrifying biofilms**

472

473 Biofilms exposed to both organic carbon and  $\text{NH}_3$  usually have an outer layer dominated  
474 by fast-growing heterotrophic bacteria (Henze et al. 2008). In the presence of non-limiting organic  
475 substrates,  $\text{O}_2$  is usually consumed by heterotrophic activity with little formation nitrifying  
476 biomass. However, in presence of low or transient organic carbon concentrations, nitrifying  
477 organisms can develop in the biofilm. These biofilms are here referred as “combined  
478 nitrifying/denitrifying biofilms”.

479 In combined nitrifying/denitrifying biofilms, the mechanisms of  $\text{N}_2\text{O}$  formation can be  
480 quite complex. Both co- and counter- diffusional combined nitrifying/denitrifying biofilms are  
481 characterized by the presence of complex communities where  $\text{N}_2\text{O}$  is formed by both nitrifiers and  
482 denitrifiers, but also reduced by denitrifiers (Matsumoto et al. 2007; Nerenberg 2016). Various  
483 intermediates play roles in both pathways, as indicated in Figure 2. For example,  $\text{NO}_2^-$  and  $\text{NO}$ ,  
484 two crucial components of both nitrifier denitrification and  $\text{NH}_2\text{OH}$  oxidation pathways, also play  
485 a role as intermediates in the denitrification pathway (Todt and Dorsch 2015). Thickness is also a  
486 crucial component for both co- and counter- diffusional biofilm, if adequate thickness and COD  
487 concentrations are present, then  $\text{N}_2\text{O}$  reduction can occur (Eldyasti et al. 2014; He et al. 2017).

488 Co-diffusional combined nitrifying/denitrifying biofilms receive both electron donor and  
489 acceptor from the bulk (Fig. 7a). In this type of biofilm, heterotroph are typically more abundant  
490 in the outer biofilm, due to their faster growth rates and the greater availability of COD. This zone  
491 is typically aerobic, so little or no denitrification or  $\text{N}_2\text{O}$  reduction occurs. Nitrifiers are typically  
492 located in the aerobic zone below the heterotrophs. If enough COD is present, then  $\text{N}_2\text{O}$  reduction  
493 can occur in the deeper biofilm (Fig. 7a) (Chae et al. 2012; Eldyasti et al. 2014; He et al. 2017).  
494 When the bulk is aerated in co-diffusional combined nitrifying/denitrifying biofilms, there is  
495 greater  $\text{N}_2\text{O}$  mass transfer towards the bulk rather than towards the anoxic zone where it can be  
496 reduced. This translates in higher  $\text{N}_2\text{O}$  emissions.

497

498 **FIGURE 7**

499

500 In counter-diffusional combined nitrifying/denitrifying biofilms,  $\text{DO}$  penetrates the biofilm  
501 from the attachment surface. In this case, and assuming the bulk liquid is anoxic, the nitrifiers

502 would only be active near the membrane surface (Kinh et al. 2017a). In addition, N<sub>2</sub>O formed by  
503 the nitrifiers could potentially be reduced by the heterotrophs in outer, anoxic region of the biofilm,  
504 where the COD concentrations are highest (Cole et al. 2004; Kinh et al. 2017b; LaPara et al. 2006).  
505 As seen for nitrifying biofilms (Fig. 5b), there could also be N<sub>2</sub>O stripping by the membrane, as  
506 indicated from a negative slope of the N<sub>2</sub>O profile towards the membrane (Fig. 7b). The lack of  
507 bulk aeration reduces N<sub>2</sub>O mass transfer to the bulk. **Note that MABR membranes can also strip**  
508 **CO<sub>2</sub> from the biofilm, leading to pH shifts that can impact the microbial community and potentially**  
509 **impact N<sub>2</sub>O emissions (Ma et al. 2017b).**

510 Based on the above, the type of biofilm (co- vs. counter- diffusional) also can affect the  
511 microbial community structure and therefore the N<sub>2</sub>O emissions. For each bulk substrate condition  
512 and detachment regime, there may be a different microbial community structure, which in turn can  
513 affect the formation/reduction and emissions of N<sub>2</sub>O. Therefore, the behavior of these biofilms is  
514 complex and hard to predict (Martin and Nerenberg 2012; Nerenberg 2016).

515

#### 516 **Partial nitritation/anammox biofilms**

517

518 In combined partial nitritation/anammox (PN/A) reactors, NH<sub>3</sub> is partially oxidized to NO<sub>2</sub><sup>-</sup>  
519 by AOB. The remainder of the NH<sub>3</sub> is then oxidized to N<sub>2</sub> gas via NO<sub>2</sub><sup>-</sup> reduction by anammox  
520 bacteria. NOB are undesirable in PN/A reactors, and diverse strategies are employed to outselect  
521 these organisms. PN/A reactors typically also harbor a diverse flanking community, many of  
522 which are capable of heterotrophic denitrification (Lawson et al. 2017).

523 A distinguishing feature of PN/A systems is the presence of multiple biological sinks for  
524 NO<sub>2</sub><sup>-</sup>. Biofilm-based PN/A systems are further distinguished by strong spatial segregation of AOB  
525 (in oxic layers) and anammox and denitrifiers (in anoxic, usually deep, layers) (Hubaux et al. 2015;  
526 Laurenzi et al. 2016; Okabe et al. 2011). Crossfeeding within the biofilm and capacity of certain  
527 denitrifiers to act as internal N<sub>2</sub>O sinks, likely differentiates N<sub>2</sub>O emissions in biofilms from  
528 suspended growth PN/A processes.

529 The potential of PN/A systems to act as significant N<sub>2</sub>O sources, particularly from biofilm  
530 or hybrid PN/A reactors, is poorly understood. Results suggest that emissions depend strongly on  
531 bulk O<sub>2</sub> concentration (Harris et al. 2015), NO<sub>2</sub><sup>-</sup> concentration (Van Hulle et al. 2012), NH<sub>3</sub>  
532 oxidation activity (Blum et al. 2018a; Domingo-Felez et al. 2014), nitrogen loading (Yang et al.

533 2016), aeration regime (intermittent vs. continuous aeration) (Blum et al. 2018a; Domingo-Felez  
534 et al. 2014; Kampschreur et al. 2008; Ma 2018), presence of organic matter (Jia et al. 2018), and  
535 biofilm thickness (Vlaeminck et al. 2010b).

536 Intermittent aeration mirrors conditions recently shown to promote N<sub>2</sub>O generation  
537 (Chandran et al. 2011; Kampschreur et al. 2008; Kampschreur et al. 2009; Yu et al. 2010), but has  
538 also been suggested that appropriate intermittent aeration can facilitate control or minimization of  
539 N<sub>2</sub>O emissions from PN/A processes (Castro-Barros et al. 2015; Domingo-Felez et al. 2014; Su et  
540 al. 2017).

541 While sources of N<sub>2</sub>O in PN/A systems are still not well understood, multiple studies have  
542 indicated it may derive predominantly from AOB. Ali et al. (2016) provided evidence based that  
543 nitrifier denitrification and NH<sub>2</sub>OH pathways were equally important to N<sub>2</sub>O formation in the oxic  
544 surface region of granules from a PN/A reactor. However, ~30% of N<sub>2</sub>O emissions in this system  
545 could be attributed to the anammox dominated anoxic interior of granules due to either  
546 heterotrophic denitrification or a yet unidentified pathway. Harris et al. (2015) showed that N<sub>2</sub>O  
547 site preference data from a suspended growth PN/A reactor was inconsistent with current  
548 understanding of N<sub>2</sub>O production pathways, and further suggested that N<sub>2</sub>O emissions in this  
549 system could be due in part to an unknown inorganic or anammox-associated N<sub>2</sub>O production  
550 pathway. In general, biofilm-based PN/A processes appear to emit less N<sub>2</sub>O than suspended  
551 nitrifying processes (Gilmore et al. 2013). Further research is needed to better identify sources of  
552 N<sub>2</sub>O in biofilm-based and hybrid biofilm suspended growth PN/A systems, and to quantitatively  
553 evaluate how spatial structuring, biofilm thickness, and aggregate architecture influence N<sub>2</sub>O  
554 emissions in these emerging low energy N removal systems.

555

## 556 CONCLUSIONS

557

558 N<sub>2</sub>O formation is promoted when there are (1) low DO values, or DO spatially transitioning from  
559 high to low within the biofilm; (2) conditions where the DO fluctuates temporally from high to  
560 low values; (3) conditions with high reaction rates, which lead to greater formation of  
561 intermediates (e.g., NH<sub>2</sub>OH and NO<sub>2</sub><sup>-</sup>) that promote N<sub>2</sub>O formation; and (4) limiting electron  
562 donor for denitrification. The microbial basis of N<sub>2</sub>O formation in biofilms and suspended growth  
563 systems are similar, yet N<sub>2</sub>O emissions in biofilm systems depend greatly on microbial

564 stratification, the formation of substrate gradients, the exchange of intermediates within the  
565 biofilm, and the type of biofilm reactor. This can lead to different patterns and quantities of N<sub>2</sub>O  
566 emission for the same bulk environment, and make it more difficult to predict N<sub>2</sub>O emissions. Co-  
567 diffusional and membrane-aerated biofilms may have substantially different behavior, due to the  
568 unique microbial and stratifications and substrate profiles. In order to predict N<sub>2</sub>O emissions from  
569 biofilm processes, and develop strategies to minimize them, it is important to understand the  
570 microbiological and biochemical basis for N<sub>2</sub>O formation, the factors affecting N<sub>2</sub>O formation in  
571 biofilms, as well as the impacts of reactor configurations and operating modes. Future research  
572 should address the pathways and kinetics of N<sub>2</sub>O emissions from AOA, comammox bacteria,  
573 methane-oxidizing denitrifying bacteria, and others. It also is important to explore their abundance  
574 in biofilms. Given the complexity of biofilms and biofilm processes, empirical assessments of N<sub>2</sub>O  
575 emissions from the broad range of biofilm reactors type and operating conditions is needed, and  
576 application-specific recommendations to minimize emissions should be developed.

577

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582

#### 583 **Compliance with Ethical Standards**

584

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589

590 Conflict of Interest:

591 F. Sabba declares he has no conflict of interest.

592 A. Terada declares he has no conflict of interest.

593 G. Wells declares he has no conflict of interest.

594 B. F. Smets declares he has no conflict of interest.

595 R. Nerenberg declares he has no conflict of interest.

596

597 Ethical approval:

598 This article does not contain any studies with human participants or animals performed by any of  
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600

601

602

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## Figure Captions

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**Fig. 1** Idealized schematics of (a) a floc, and (b) a biofilm. The biofilm schematic shows the liquid diffusion layer (LDL), as well as profiles of a substrate and metabolic product. Note that real flocs are highly complex and heterogeneous in morphology, and biofilms may have rough or dendritic surfaces with internal pores.

**Fig. 2** Key processes in the N-cycle.  $\text{N}_2\text{O}$  is highlighted in gray (adapted from Daims et al. 2016 and Schreiber et al. 2012). The dashed line for comammox shows the formation of  $\text{NO}_2^-$  as intermediate but also its oxidation to  $\text{NO}_3^-$  by the same organism. Abbreviations in figure: DNRA is dissimilatory nitrite reduction to ammonia; assimil. is assimilatory; dissimil. is dissimilatory. Note that denitrification can produce  $\text{N}_2\text{O}$ , but it is also the only known process that can reduce it.

**Fig. 3** Nitrogen transformations in AOB, NOB and DNB. Abbreviations: AOB, ammonia-oxidizing bacteria; NOB, nitrite-oxidizing bacteria; DNB, denitrifying bacteria, AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase (hydroxylamine dehydrogenase in *Nitrospira*); NXR, nitrite oxidoreductase; NirK, copper-containing nitrite reductase; NirS, cytochrome cd1 type nitrite reductase; NOR, nitric oxide reductase; and NOS, nitrous oxide reductase. Purple arrows show intermediates potentially shared between nitrification and denitrification pathways. Abiotic reactions (gray) are further discussed in the text.

**Fig. 4** Types of biofilm reactors. (A) Unsubmerged filter (e.g., trickling filter or biofilter), (B) upflow fixed-bed reactor (e.g., biologically active filter (BAF)), (C) downflow fixed-bed reactor (e.g., BAF), (D) rotating biological contactor (RBC), (E) suspended or airlift biofilm reactor, (F) fluidized-bed biofilm reactor (FBBR or granular sludge), (G) moving-bed biofilm reactor (MBBR), integrated fixed film activated sludge (IFAS), and (H) membrane-supported biofilm reactor (e.g., MBfR or MABR). Note:  $i$  = influent;  $e$  = effluent;  $r$  = recycle;  $w$  = wasting flow;  $g$  = gas flow (typically air) in or out. Black dots in figures E, F, and G are biofilm carriers. Adapted from (Morgenroth 2008) and (WEF 2010)

**Fig. 5**  $\text{N}_2\text{O}$  formation in nitrifying biofilms. (a) Co-diffusional and (b) counter-diffusional. Solid black arrow indicates  $\text{N}_2\text{O}$  loss towards either bulk or membrane lumen.  $\text{NO}_2^-$  and  $\text{NO}$  are not shown for clarity.

**Fig. 6**  $\text{N}_2\text{O}$  formation in denitrifying biofilms. (a) Excess  $e^-$  donor, (b) excess  $e^-$  donor with  $\text{O}_2$ , and (c) limiting  $e^-$  donor. Solid black arrow indicates  $\text{N}_2\text{O}$  loss towards bulk and dashed black arrow indicates reduction within the biofilm depth.  $\text{NO}_2^-$  and  $\text{NO}$  are not shown for clarity.

**Fig. 7**  $\text{N}_2\text{O}$  formation in combined nitrifying/denitrifying biofilms. (a) Co-diffusional and (b) counter-diffusional. Solid black arrow indicates  $\text{N}_2\text{O}$  loss towards either bulk or membrane lumen; dashed black arrow indicates reduction within the biofilm depth.  $\text{NO}_2^-$  and  $\text{NO}$  are not shown for clarity

(a)

20 - 50  $\mu\text{m}$   
diameter

50 - 1000  $\mu\text{m}$   
thickness



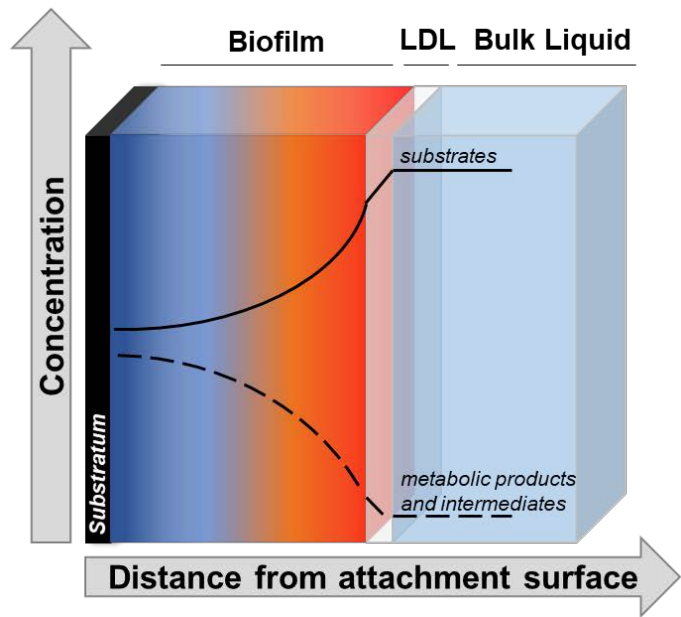
Floc



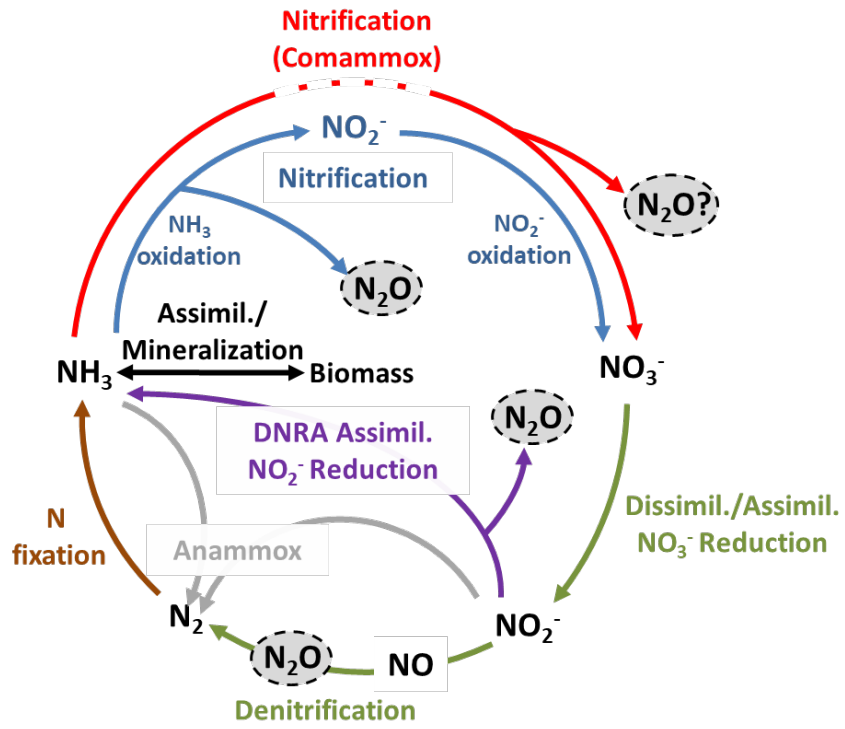
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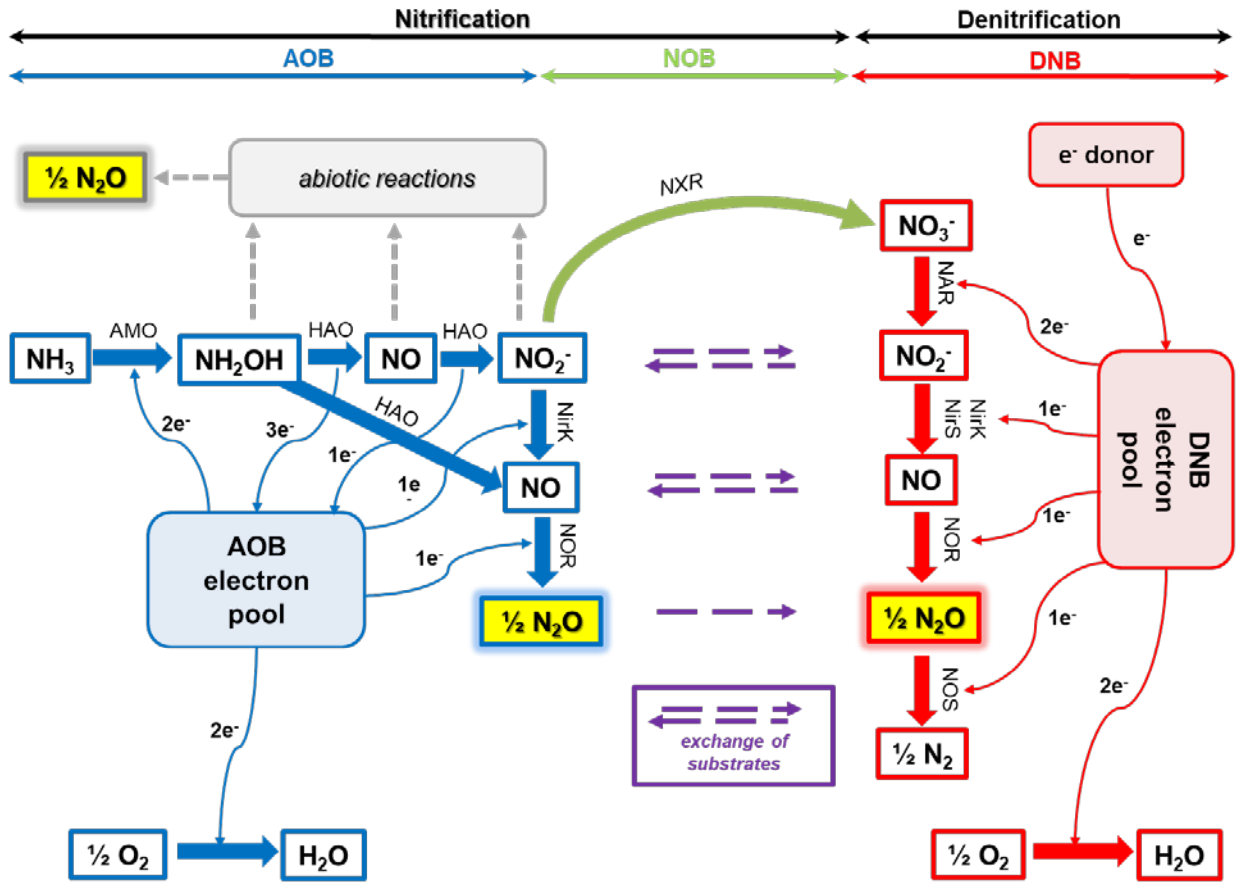
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(b)









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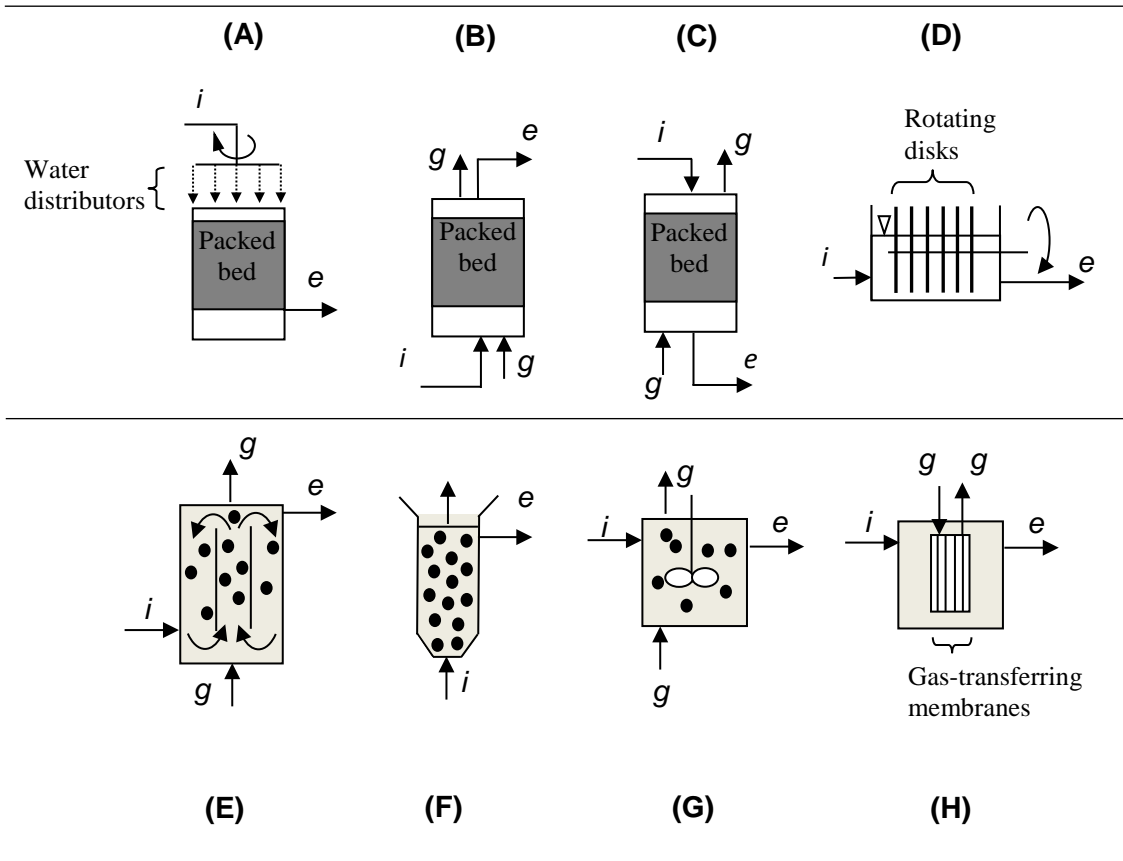
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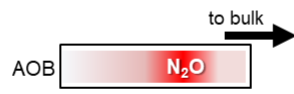
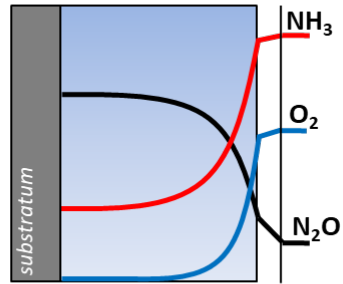
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Nitrifying biofilms

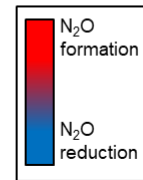
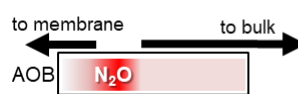
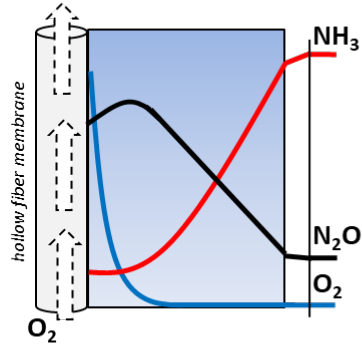
(a)

Co-diffusional



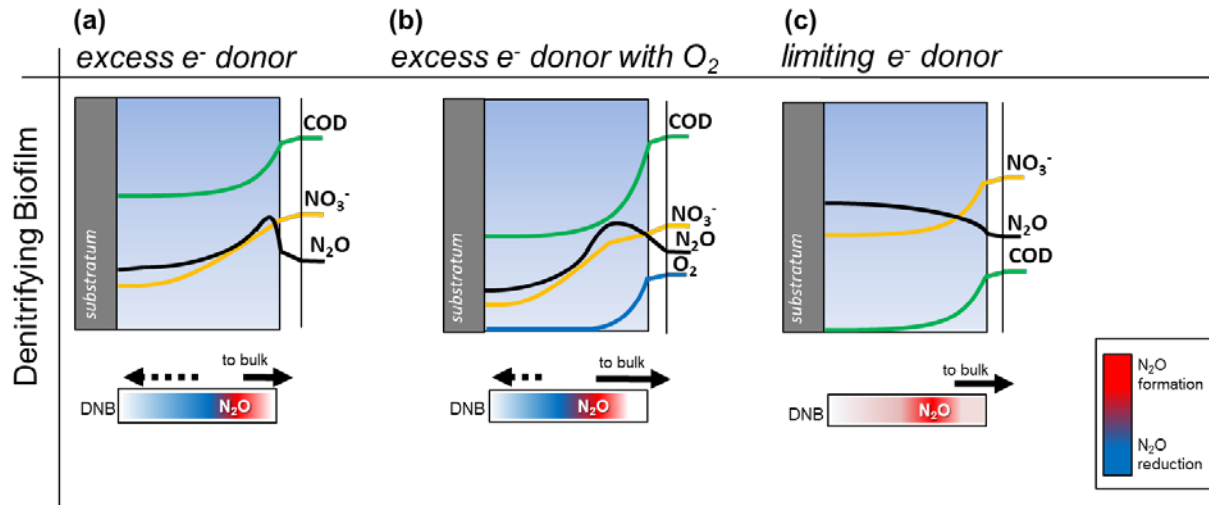
(b)

Membrane aerated  
(Counter-diffusional)



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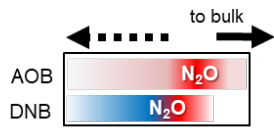
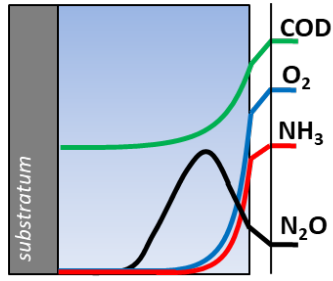
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SND Biofilms

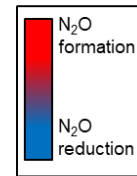
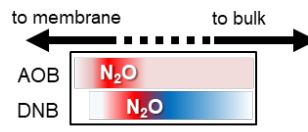
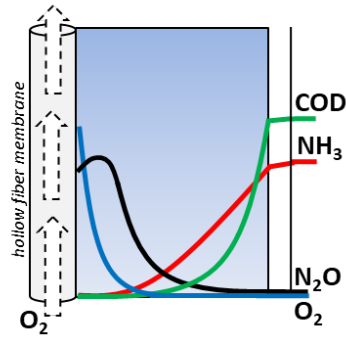
(a)

Co-diffusional



(b)

Membrane aerated  
(Counter-diffusional)



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