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Published in:
Applied Microbiology and Biotechnology

Link to article, DOI:
[10.1007/s00253-018-9332-7](https://doi.org/10.1007/s00253-018-9332-7)

Publication date:
2018

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Sabba, F., Terada, A., Wells, G., Smets, B. F., & Nerenberg, R. (2018). Nitrous oxide emissions from biofilm processes for wastewater treatment. *Applied Microbiology and Biotechnology*, 102(22), 9815–9829. <https://doi.org/10.1007/s00253-018-9332-7>

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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 (N₂O) emissions
44 from biofilms processes for wastewater treatment. Conditions that generally promote N₂O
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 (NH₂OH) and nitrite (NO₂⁻), that promote N₂O formation;
49 and (4) electron donor limitation for denitrification. Formation of N₂O 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 (NH₃)
53 concentrations, higher nitrite (NO₂⁻) concentrations, lower dissolved oxygen (DO), and greater
54 biofilm thicknesses result in higher N₂O emissions. In denitrifying biofilms, N₂O 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. N₂O 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 N₂O 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 N₂O emissions during
61 or following a scouring or aeration event. N₂O 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 N₂O emission from biofilm processes for wastewater treatment.

67

68 **Keywords:** N₂O, biofilms, hydroxylamine, MBBR, MABR, MBfR, granules

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72

73 INTRODUCTION

74

75 Wastewater treatment processes can be a significant source of nitrous oxide (N₂O), a powerful
76 greenhouse gas (GHG) with a global warming potential around 300 times that of carbon dioxide
77 (CO₂) (Montzka et al. 2011). N₂O 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 N₂O yr⁻¹ as
80 CO₂ 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 N₂O 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 N₂O 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 N₂O emissions in biofilms may significantly differ from those of
96 suspended growth systems.

97 Todt and Dorsch (2016) provided a comprehensive review of N₂O emissions from biofilm
98 systems. They explored the biochemistry of N₂O production/consumption in relevant organisms,
99 discussed current biofilm models, evaluated possible environmental factors affecting N₂O
100 emissions, and tabulated emission factors for different processes. Massara et. al (2017) briefly
101 addressed biofilms as part of a comprehensive review of N₂O emissions from wastewater
102 processes. This review provides an update, considering new information on the N₂O emissions

103 from microbial systems. It also discusses **new types of microbial metabolism and different biofilm**
104 **reactor configurations, and their impacts on N₂O 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 **N₂O AND NITROGEN CYCLE**

128

129 **This section discusses basic microbial transformations that affect N₂O formation in wastewater**
130 **treatment processes. These processes are relevant to both suspended growth and biofilm processes.**
131 **The relationship between these transformations and N₂O 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 (NO_3^-) is
139 sequentially reduced to nitrogen gas (N_2). Both processes can lead to N_2O formation.

140

141 FIGURE 2

142

143 **N_2O 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 (NH_3)**
147 **to nitrite (NO_2^-), with hydroxylamine (NH_2OH) as an intermediate (Fig. 3) (Daims et al. 2016;**
148 **Guo et al. 2017), while NOB oxidize NO_2^- to NO_3^- . AOB directly produce N_2O through two main**
149 **pathways: nitrifier denitrification and NH_2OH oxidation (Fig. 3). NOB, AOA, anammox, and**
150 **comammox microorganisms may play an indirect role in N_2O formation by affecting the**
151 **availability of NH_3 and NO_2^- .**

152

153 FIGURE 3

154

155 **In the nitrifier denitrification pathway, AOB reduce NO_2^- to nitric oxide (NO) and N_2O**
156 **(Chandran et al. 2011; Kampschreur et al. 2007; Kim et al. 2010; Tallec et al. 2006) (Fig. 3). The**
157 **NH_2OH oxidation pathway involves the oxidation of NH_2OH to NO by hydroxylamine**
158 **oxidoreductase (HAO) and subsequent reduction to N_2O 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 N_2O production exist under anaerobic conditions. One is the direct oxidation of NH_2OH**
162 **to N_2O 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 N_2O production, these recent**

164 findings expand on previous knowledge where chemical reactions were thought to be mainly
165 important at higher oxygen (O₂) levels (Liu et al. 2017a).

166 N₂O can also be produced biologically or abiotically by coupling NH₂OH oxidation with
167 the reduction of NO₂⁻ (Harper et al. 2015; Terada et al. 2017), free nitrous acid (HNO₂) (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 N₂O production from NH₂OH via
171 the hybrid reaction. Under some conditions, the hybrid reaction can become a predominant
172 pathway for N₂O production in a partial nitrifying reactor (Soler-Jofra et al. 2018; Terada et al.
173 2017). N₂O 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, N₂O is mainly formed via the NH₂OH pathway, and rates are
176 relatively low. When DO concentrations decrease, the nitrifier denitrification pathway becomes
177 more important, leading to higher rates of N₂O 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 N₂O emissions are again low due to the lack of DO for NH₃ oxidation (Fig. 3). Spikes
180 of N₂O 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, N₂O 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 N₂O production pathways, the pathways for AOA
185 are yet to be fully understood (Blum et al. 2018b). They perform NH₃ oxidation in a similar way
186 to AOB (Kozlowski et al. 2016); however, they lack the ability to produce N₂O enzymatically
187 through side reactions of NH₃ 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 N₂O 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 NH₃ and NO₂⁻ to N₂ 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 N₂O via NO reduction
196 (Kartal et al. 2007; Strous et al. 2006). However, research suggests that N₂O production under
197 **process**-relevant conditions is negligible (Blum et al. 2018a). Anammox may indirectly affect
198 N₂O formation by heterotrophs and AOB by reducing the concentrations of NH₃ and NO₂⁻.

199 Comammox bacteria are a subset of the genus *Nitrospira* capable of **complete ammonia**
200 **oxidation** (comammox) via oxidation of NH₃ to NO₃⁻ (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 NH₃ 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 NH₃ and NO₂⁻ 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 N₂O production. NH₂OH appears to be an
211 obligate intermediate of comammox metabolism, analogous to AOB catabolism, and it is likely
212 that N₂O can be formed by comammox via the NH₂OH pathway (Fig. 3). Comammox genomes
213 recovered to date also harbor capacity for NO₂⁻ 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_x 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 NH₂OH, could lead to abiotic reactions with the production of N₂O as a**
219 **final product.**

220 Comammox may be detrimental to PN/A systems, where NO₂⁻ production is needed.
221 However, they may also reduce N₂O emissions by minimizing NO₂⁻ 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 N₂O emissions.

227

228 **N₂O from Microorganisms Related to Denitrification**

229

230 Denitrification is the sequential reduction of NO₃⁻ and NO₂⁻ to NO, N₂O, and finally N₂
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 N₂O 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), NO₂⁻
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 (H₂S) (Schonharting et al. 1998).

239 While denitrifying bacteria produce N₂O during denitrification, they also can reduce N₂O
240 to N₂ (Read-Daily et al. 2016). Externally supplied N₂O can be reduced concurrently with NO₃⁻
241 and NO₂⁻ (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 NO₃⁻
243 and NO₂⁻ all the way to N₂, less is known about bacteria that can grow with N₂O but not with NO₃⁻
244 or NO₂⁻. Newly classified clade II-type *nosZ* N₂O 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 N₂O 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 N₂O sink at a low N₂O concentration. Some clade II type *nosZ*
251 bacteria appear to lack genes encoding for NIR and/or NOR, suggesting their potential as an N₂O
252 sink but not an N₂O source (Graf et al. 2014). As reviewed elsewhere, these non-denitrifying N₂O-
253 reducing bacteria in wastewater engineering are yet to be explored in detail (Hallin et al. 2018).
254 The ecophysiology of non-denitrifying N₂O 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 N₂O formation and reduction. Some can fully reduce NO₃⁻ and NO₂⁻ to NH₃ 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, NO₃⁻ or NO₂⁻ is reduced to NH₃, with
259 N₂O produced at the NO₂⁻ 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 N₂O 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 NO₂⁻ (Kaspar 1982).
264 However, the actual contribution of DNRA to N₂O formation in these species remains uncertain
265 (Butterbach-Bahl et al. 2013).

266 Behavior regarding N₂O emissions may also vary based on the type of electron donor. For
267 example, elemental-sulfur (S⁰) oxidizing denitrifiers (Di Capua et al. 2015; Liu et al. 2017b),
268 methane (CH₄) 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), H₂ 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 N₂O emissions. Methane-oxidizing denitrifiers appear
272 to reduce NO₂⁻ to N₂ without forming N₂O as an intermediate, and therefore are thought to
273 minimize N₂O 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 N₂O formation
275 and consumption.

276

277 **TYPES OF BIOFILM REACTORS AND IMPACTS ON N₂O EMISSIONS**

278

279 This section describes different type of biofilm reactors, and their special characteristics as relate
280 to N₂O 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 N₂O 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., NH₂OH, NO₂⁻) that promote N₂O 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 N₂O emissions. When used for nitrification, N₂O 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 N₂O 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 N₂O in off gases from an activated sludge aeration basin. Raw wastewater was used as the
309 electron donor. In lab tests, 99.9% of N₂O was removed when supplied at 100 ppmV in N₂, i.e.,
310 without any O₂. However, removals decreased significantly when supplied in air. Biofilters are
311 likely an expensive approach to mitigating N₂O 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 NH_3 at influent, low DO at effluent) can impact N_2O formation
320 processes. When used for denitrification, air pulses are periodically performed at the bottom of the
321 filter to release N_2 bubbles accumulating in the reactor. These pulses can strip N_2O formed at the
322 beginning of the bed, when normally it would be reduced to N_2 further within the bed (Bollon et
323 al. 2016). Whenever air is added to a denitrifying filter, there is potential for N_2O formation at
324 some location within the biofilm due to the greater sensitivity of N_2OR to O_2 inhibition. N_2O 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 N_2O 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% N_2O 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 N_2O and lead to increased N_2O 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 NO_2^- at temperatures between 5 and
336 15 °C, were found important factors for N_2O accumulation. Further research is required to
337 investigate the impact of influent NO_2^- and possible adaptation of bacteria to variable influent
338 loadings of both NO_2^- and 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 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 N_2O emissions. There does not appear to be any published findings of N_2O
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 N_2O
348 to 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 N₂O emissions in suspended
353 growth systems transitioning from anoxic to aerobic zones (Chandran et al. 2011). Recent research
354 on N₂O 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 N₂O 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 N₂O 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 O₂; membrane-biofilm reactors (MBfRs) where membranes
372 supply H₂ or CH₄ (Liu et al., 2017b); sulfur-based biofilms, where solid S⁰ 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 N₂O 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 NH₃ 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, N₂O 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 N₂O 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 N₂O 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 NH₃ concentrations. As a result, the NH₃ oxidation rates are high,
406 leading to high NH₂OH concentrations. In addition, the nitrifier denitrification pathway is
407 inhibited by the high DO in this zone. Thus, the NH₂OH **oxidation** pathway is likely to dominate,
408 and N₂O 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 N₂O emissions, but may affect emission by modifying the
441 NO₂⁻ concentrations (Wang et al. 2016b).

442

443 Denitrifying biofilms

444

445 Denitrifying biofilms are those where NO₃⁻ 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, N₂O is an obligate intermediate. It is typically present at
448 higher concentrations in the outer biofilm region, where NO₃⁻ and NO₂⁻ reduction activity is higher,
449 but can diffuse and be consumed in deeper regions where NO₃⁻ and NO₂⁻ concentrations are lower
450 (Fig. 6a). Thus, biofilms can have regions that can serve as an N₂O sink, mitigating N₂O 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 N₂O is
456 formed (Fig. 6b). However, biofilms typically have DO gradients, and denitrification and N₂O
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 N₂O will be formed due to the higher
459 sensitivity of NOS to O₂ 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 N₂O 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, N₂O is more likely to be reduced in the
463 deeper biofilm and less emissions will occur (Dalsgaard and Revsbech 1992).

464

465 If N₂O 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 N₂O sink (Eldyasti et al. 2014;
467 Sabba et al. 2017b). However, if sulfate reduction occurs in the deeper biofilm where NO₃⁻ has
468 been depleted, H₂S may accumulate and inhibit N₂O reduction (Pan et al. 2013b). Electron donor
469 limitation in the denitrifying zone also may result in greater N₂O 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 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, 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 N_2O 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 N_2O 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, NO_2^- and NO ,
484 two crucial components of both nitrifier denitrification and NH_2OH 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 N_2O 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 N_2O reduction occurs. Nitrifiers are typically
492 located in the aerobic zone below the heterotrophs. If enough COD is present, then N_2O 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 N_2O mass transfer towards the bulk rather than towards the anoxic zone where it can be
496 reduced. This translates in higher N_2O emissions.

497

498 **FIGURE 7**

499

500 In counter-diffusional combined nitrifying/denitrifying biofilms, 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, N₂O 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 N₂O stripping by the membrane, as
506 indicated from a negative slope of the N₂O profile towards the membrane (Fig. 7b). The lack of
507 bulk aeration reduces N₂O mass transfer to the bulk. **Note that MABR membranes can also strip**
508 **CO₂ from the biofilm, leading to pH shifts that can impact the microbial community and potentially**
509 **impact N₂O 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 N₂O 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 N₂O. 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, NH₃ is partially oxidized to NO₂⁻
519 by AOB. The remainder of the NH₃ is then oxidized to N₂ gas via NO₂⁻ 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 NO₂⁻. 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 N₂O sinks, likely differentiates N₂O emissions in biofilms from
528 suspended growth PN/A processes.

529 The potential of PN/A systems to act as significant N₂O sources, particularly from biofilm
530 or hybrid PN/A reactors, is poorly understood. Results suggest that emissions depend strongly on
531 bulk O₂ concentration (Harris et al. 2015), NO₂⁻ concentration (Van Hulle et al. 2012), NH₃
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 N₂O 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 N₂O emissions from PN/A processes (Castro-Barros et al. 2015; Domingo-Felez et al. 2014; Su et
540 al. 2017).

541 While sources of N₂O 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 NH₂OH pathways were equally important to N₂O formation in the oxic
544 surface region of granules from a PN/A reactor. However, ~30% of N₂O 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 N₂O
547 site preference data from a suspended growth PN/A reactor was inconsistent with current
548 understanding of N₂O production pathways, and further suggested that N₂O emissions in this
549 system could be due in part to an unknown inorganic or anammox-associated N₂O production
550 pathway. In general, biofilm-based PN/A processes appear to emit less N₂O than suspended
551 nitrifying processes (Gilmore et al. 2013). Further research is needed to better identify sources of
552 N₂O 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 N₂O
554 emissions in these emerging low energy N removal systems.

555

556 CONCLUSIONS

557

558 N₂O 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., NH₂OH and NO₂⁻) that promote N₂O formation; and (4) limiting electron
562 donor for denitrification. The microbial basis of N₂O formation in biofilms and suspended growth
563 systems are similar, yet N₂O 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 N₂O
566 emission for the same bulk environment, and make it more difficult to predict N₂O 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 N₂O emissions from
569 biofilm processes, and develop strategies to minimize them, it is important to understand the
570 microbiological and biochemical basis for N₂O formation, the factors affecting N₂O 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 N₂O 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 N₂O
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

578 **Acknowledgments**

579 F.S. and R.N. were partially supported by NSF project CBET0954918 and WERF project U2R10.
580 A. T. was partially funded by Grant-in-Aid for Scientific Research (17H01893) - Japan Society
581 for the Promotion of Science and BFSM was funded by the DFF project N2OMan.

582

583 **Compliance with Ethical Standards**

584

585 Funding: This study was funded by the Water Environment Research Foundation (grant U2R10),
586 the USA National Science Foundation (grant CBET0954918), the Japanese Society for the
587 Promotion of Science (grant 17H01893), and the Danish Council for Independent (Project
588 N2OMan, File No. 1335-00100B).

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
599 the authors.

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. N_2O is highlighted in gray (adapted from Daims et al. 2016 and Schreiber et al. 2012). The dashed line for comammox shows the formation of NO_2^- as intermediate but also its oxidation to 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 N_2O , 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 N_2O formation in nitrifying biofilms. (a) Co-diffusional and (b) counter-diffusional. Solid black arrow indicates N_2O loss towards either bulk or membrane lumen. NO_2^- and NO are not shown for clarity.

Fig. 6 N_2O formation in denitrifying biofilms. (a) Excess e^- donor, (b) excess e^- donor with O_2 , and (c) limiting e^- donor. Solid black arrow indicates N_2O loss towards bulk and dashed black arrow indicates reduction within the biofilm depth. NO_2^- and NO are not shown for clarity.

Fig. 7 N_2O formation in combined nitrifying/denitrifying biofilms. (a) Co-diffusional and (b) counter-diffusional. Solid black arrow indicates N_2O loss towards either bulk or membrane lumen; dashed black arrow indicates reduction within the biofilm depth. NO_2^- and NO are not shown for clarity

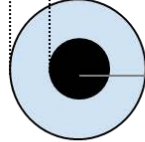
(a)

20 - 50 μm
diameter

50 - 1000 μm
thickness



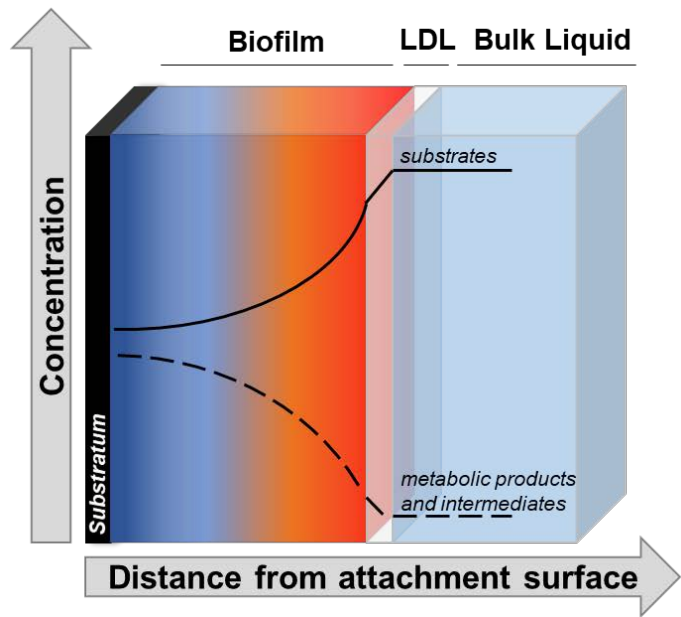
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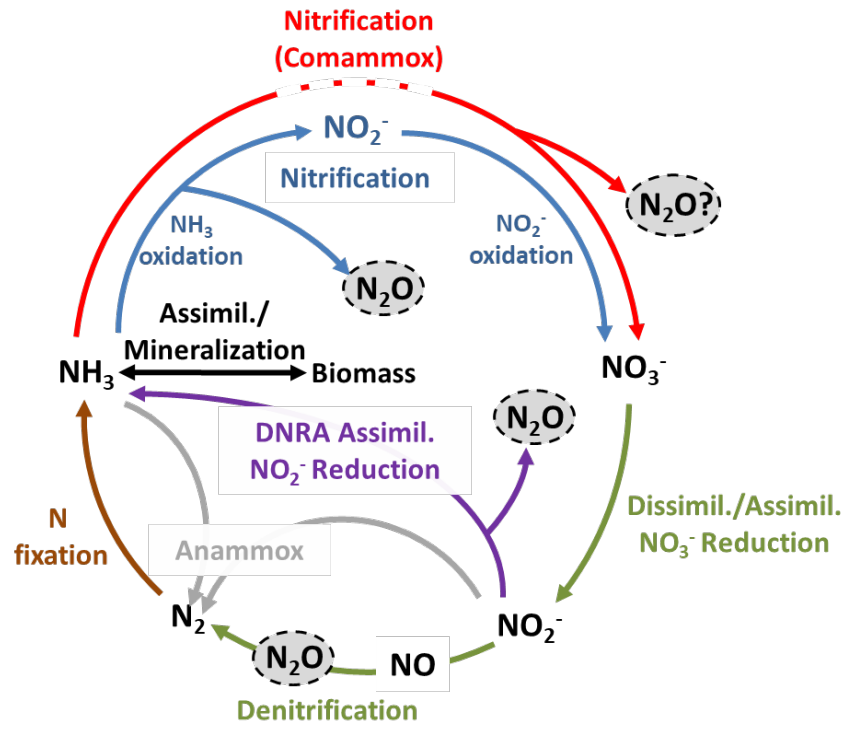


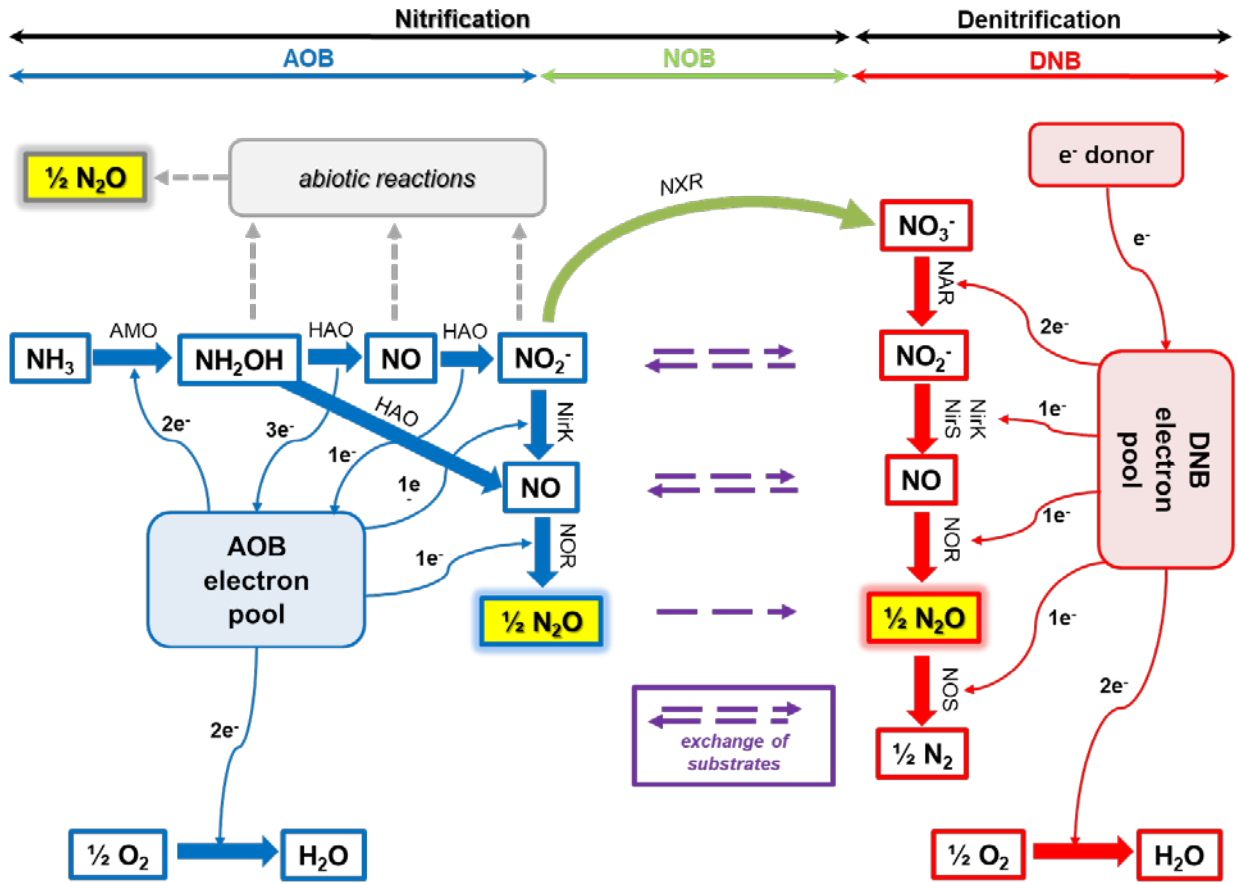
biofilm
support

Biofilm

(b)







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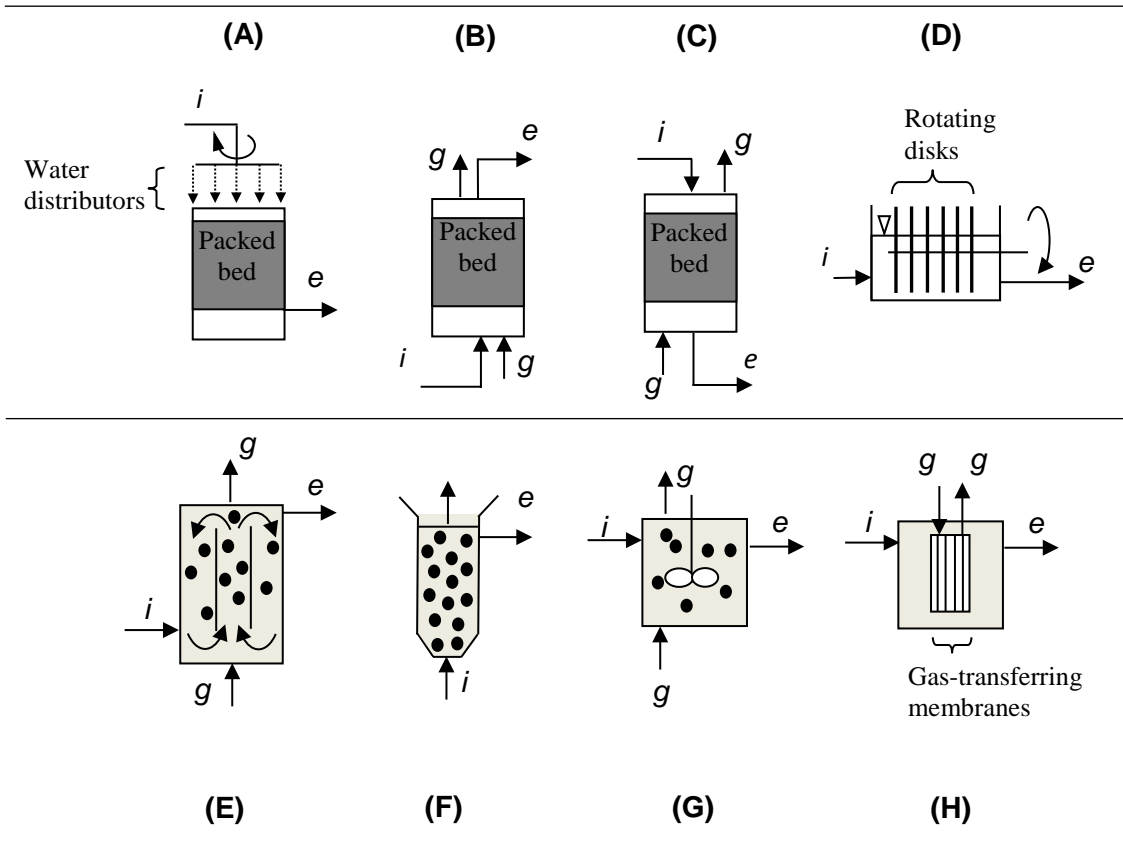
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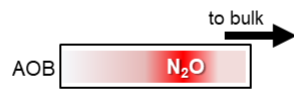
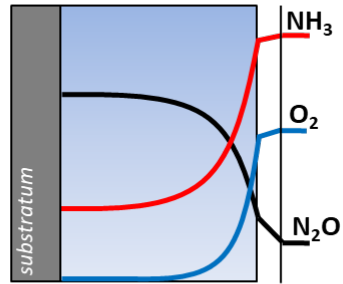
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Nitrifying biofilms

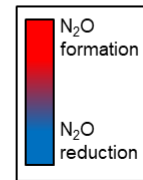
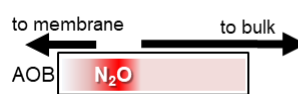
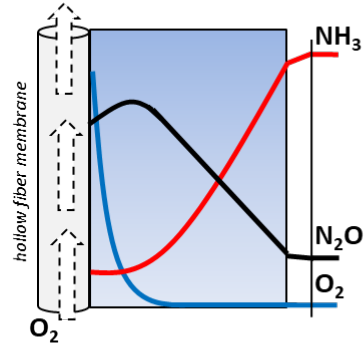
(a)

Co-diffusional



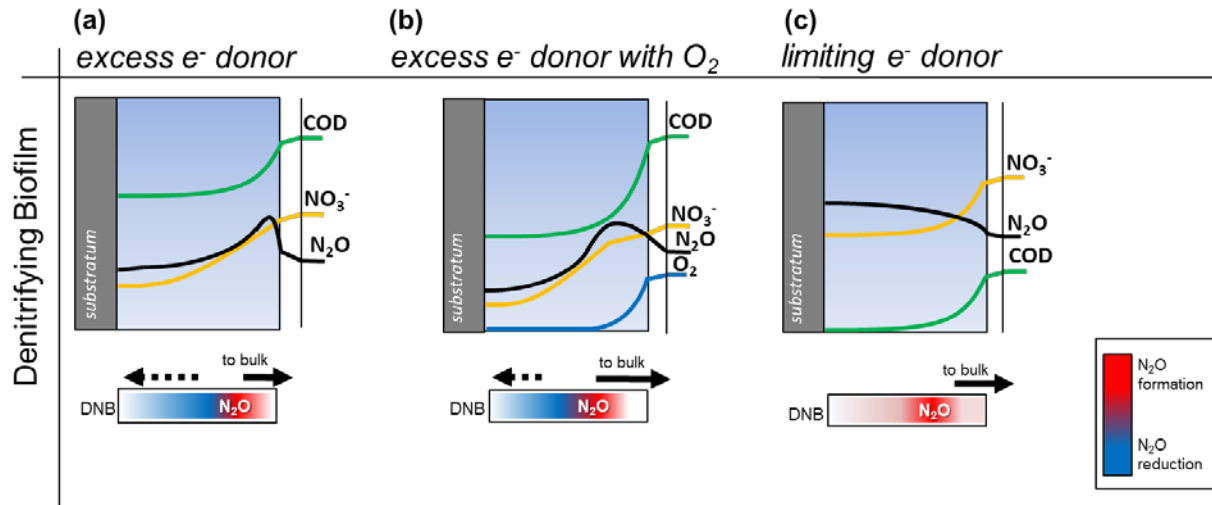
(b)

Membrane aerated
(Counter-diffusional)



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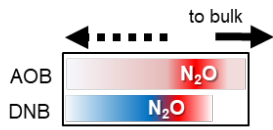
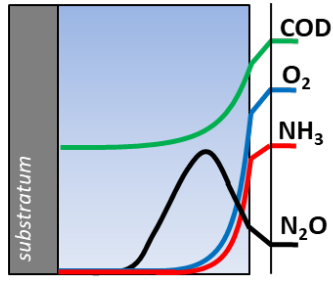
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SND Biofilms

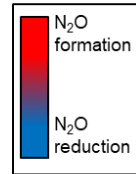
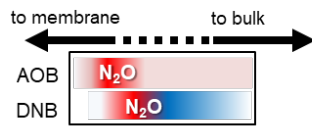
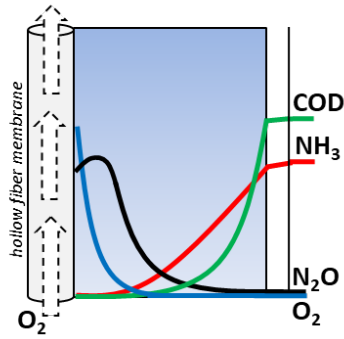
(a)

Co-diffusional



(b)

Membrane aerated
(Counter-diffusional)



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