Regulation of key N₂O production mechanisms during biological water treatment

Domingo-Felez, Carlos; Smets, Barth F.

Published in:
Current Opinion in Biotechnology

Link to article, DOI:
[10.1016/j.copbio.2019.03.006](https://doi.org/10.1016/j.copbio.2019.03.006)

Publication date:
2019

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Domingo-Felez, C., & Smets, B. F. (2019). Regulation of key N₂O production mechanisms during biological water treatment. *Current Opinion in Biotechnology*, 57, 119-126. <https://doi.org/10.1016/j.copbio.2019.03.006>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1   **Title**

2   Regulation of key N₂O production mechanisms during biological water treatment.

3   **Author list**

4   Carlos Domingo-Félez*, Barth F. Smets

5

6   Department of Environmental Engineering, Technical University of Denmark, Miljøvej 115, 2800 Kgs.
7   Lyngby, Denmark

8

9   * Corresponding author:

10   Carlos Domingo-Félez, Phone: +45 4525 1472, Fax: +45 4593 2850, E-mail: cadf@env.dtu.dk

---

11   **Highlights**

- 12   -   AOB, AOA and denitrifiers regulate biological and abiotic N₂O production.
- 13   -   Genomic and phenotypic variability of N-transforming guilds affect N₂O pathways.
- 14   -   Isotopocule tools cannot unambiguously elucidate N₂O pathway contributions yet.

15

Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas emitted during biological treatment of residual waters and can contribute significantly to the carbon footprint of the overall treatment, potentially offsetting energy-positive strategies. N_2O production is mediated by three known biological pathways and through abiotic reactions, driven by biologically-generated substances such as hydroxylamine and nitrite. The contributions of these different mechanism is determined by the environmental conditions and the resident microbial community. The newly discovered phenotypic diversity among aerobic ammonia oxidizers and the modularity of denitrifying species determines N_2O emissions. Isotopic methods can be used to quantify N_2O production pathways in water treatment systems, and mechanistic models can already predict N_2O emissions, but limitations on their accuracy and precision still exist.

Figure 1 - Model of biologically-driven N_2O production pathways during water treatment: aerobic ammonia oxidizing bacteria (left), abiotic reactions (middle), and canonical heterotrophic denitrifier (right). *cytI* represents $\text{cyt}_{\text{c}554}$ and $\text{mcyt}_{552\text{e}}$, *cyt2* represents $\text{cyt}_{\text{bc}1}$ and $\text{cyt}_{552\text{e}}$, *DH* NADH dehydrogenase. Other enzymes are described in the text. NO^*_{NN} Caranto and Lancaster 2017. $\text{N}_2\text{O}^*_{\text{NN}}$ Caranto *et al.* 2016. NN, ND, HD, Ab represent the four N_2O pathways mixed in the bulk: Nitrifier Nitrification, Nitrifier Denitrification, Heterotrophic Denitrification and Abiotic. Modified after Simon, Klotz 2013.

35 **Introduction**

36 Nitrous oxide (N_2O) and methane are greenhouse gases (GHGs) emitted during water treatment with high
37 radiative forcing compared to CO_2 . GHGs are a burden for planet Earth, and Life Cycle Assessments of
38 water technologies highlight their significant impact on the urban water system [1].

39 Sewage transport and treatment was suggested to contribute to 3.2% of the global anthropogenic N_2O
40 emissions (ca. 10% if manure, landfill leachates and industrial nitrogenous effluents are included) [2,3].
41 However, recent whole-plant measurements indicate an underestimation of the water treatment contribution
42 to global N_2O emissions [4]. The carbon footprint of wastewater treatment is highly sensitive to GHG
43 emissions, with a burden between 44 and 71%, compared to 2-28% of electricity consumption in
44 Scandinavian wastewater treatment plants [5]. For a novel activated sludge technology N_2O emissions (5.1-
45 6.6% of oxidized nitrogen) represented 83% of the total operational CO_2 footprint, and a 50% decrease of
46 N_2O emissions was necessary for a CO_2 neutral footprint [6]. Overall, while energy neutral and energy self-
47 sufficient WWTPs abound [7], carbon neutral WWTPs are rare [8]. Here we highlight: key factors that
48 regulate N_2O production mechanisms in water treatment systems, analytical tools to quantify the microbial
49 producing pathways, and mathematical models developed to capture N_2O dynamics and to design N_2O
50 emission mitigation strategies.

51 **Genomic potential of microbial communities driving N_2O production**

52 A state-of-the-art overview of the biological and abiotic N_2O -producing reactions in nitrogen removing
53 technologies was presented by Todt and Dörsch [9]: microbial communities have the genomic potential to
54 produce N_2O as a by-product of nitrification or as an intermediate during heterotrophic denitrification
55 (Figure 1).

56 Aerobic ammonia oxidizing bacteria (AOB) obtain energy from the oxidation of ammonia (NH_3) to nitrite
57 (NO_2^-) over hydroxylamine (NH_2OH) with molecular oxygen (O_2) as electron acceptor. NH_2OH oxidation is
58 catalysed by the HAO enzyme and releases four electrons, three in the oxidation to nitric oxide (NO), which
59 is proposed as the obligate intermediate in NH_2OH oxidation [10], and one more as NO is oxidized to NO_2^- .
60 Two of these electrons sustain NH_3 oxidation by the AMO enzyme and the other two are used anabolic
61 processes [11].

62 AOB can produce N_2O from the incomplete oxidation of NH_2OH to NO_2^- via HNO or NO; this is referred to
63 as nitrifier nitrification (NN). In addition, AOB can reduce NO_2^- at low dissolved oxygen (DO) conditions.
64 This process is encoded by the NO_2^- - and NO-reducing enzymes (NIR, NOR) and is termed nitrifier
65 denitrification (ND) [12]. Hink et al., (2017) suggests that the ND pathway is a route for dissipation of

66 electrons, when present at high supply rates, but not as an energy conserving process [13]; and it was also
67 suggested as a detoxification route at inhibitory NO_2^- concentrations [12].

68 Different AOB genera (*Nitrosomonas* and *Nitrospira*, r/K-strategists) display physiological differences
69 such as specific growth rate, substrate affinities, and specific N_2O production rates. AOBs possess a diverse
70 enzymology, which lead to propose alternate N_2O producing pathways beyond NirK, NorB and CYT P460
71 [14,15]. *N. communis* lacks NirK and was able to reduce NO_2^- to N_2O supporting the presence of alternate
72 NIRs, *Nitrosomonas* sp. Is79A3 and *N. ureae* Nm10 reduce NO with alternative pathways to the CYTc NO
73 reductase, and *N. multiformis* lacks CYT P460 still producing N_2O from aerobic NH_2OH oxidation [16].
74 Microrespirometric assays of five pure cultures of AOB showed positive NO and N_2O production by all
75 AOB strains associated to both NH_4^+ and NH_2OH oxidation. The dynamics and yields of NO and N_2O
76 production under aerobic and anoxic conditions varied greatly, and help associate hypothesized N_2O
77 production mechanisms to their genomic diversity [16]. Hence, the AOB-driven N_2O production of a NH_4^+ -
78 removing system depends on the environmental conditions and the AOB diversity.

79 Ammonia oxidizing archaea (AOA), found in more oligotrophic environments than AOB, have a different
80 enzymology, lacking HAO, CycB, and NORs [17]. N_2O production pathways, confirmed via ^{15}N -labeling
81 studies, require NO as an apparent obligate intermediate [18], while AOBs do not [15]. As genomes of AOA
82 do not encode for typical NOR denitrification, a chemical reaction of NH_2OH with NO was proposed as the
83 source of N_2O [18], with overall lower N_2O yields than AOB [19]. The biologically-driven abiotic N_2O
84 production from ammonia oxidation intermediates (NH_2OH , NO) is ubiquitous among all ammonia
85 oxidizers, but the contribution to the total N_2O yield remains to be characterized. The genomes of recently
86 discovered complete ammonia oxidizing *Nitrospira* do not encode genes related to NO_x production,
87 suggesting minimal N_2O production [20].

88 Heterotrophic denitrifying bacteria use NO_3^- , NO_2^- , NO and N_2O as electron acceptors in their respiratory
89 metabolism at low oxygen tension. Most denitrifiers can also respire oxygen. Canonical denitrifiers carry out
90 the four-step nitrate respiration using NAR, NIR, NOR and NOS enzymes [21], with different energy yields
91 per electron consumed in each step [22]. The electron flow from carbon oxidation is distributed to two
92 competing branches: nitrate reductase and cytochrome *c*, yielding non-consecutive reduction steps
93 influenced by the presence of other terminal acceptors [23]. While denitrifiers can carry genes for a complete
94 denitrification pathway, often only some genes are present [24,25] that can result in N_2O sources or sinks:
95 Co-occurrence of *nar*, *nir* and *nor* without *nos* would yield a net N_2O producing strain, while microbes
96 carrying only *nosZ* are N_2O reducers; the latter have been identified [26]. Hence, whether a denitrifying
97 community is a net N_2O source or sink may be governed by the diversity and relative abundance of the *nosZ*
98 gene versus the *nar*, *nir* and *nor* genes [27]. The *nosZ* gene consists of two clades; and microbes in clade II

99 showed higher growth yields and substrate affinities, but lower maximum consumption rates compared to
100 clade I [28,29]. Among both clades, of specific interest is the high variation in DO inhibition of the N_2O
101 reduction step, with Monod constant ranging between 0.005 and 0.9 mg/L [30–32]. The NOS activity adapts
102 transiently via lag phases to inhibitory oxic conditions [30], increasing the anoxic time necessary for a
103 system to remove accumulated N_2O [30]. This mechanism was recently proposed as a bet-hedging strategy
104 of denitrifiers for cell-diversification leading to phenotypic heterogeneity [33].

105 Independently from the main driving process (e.g., nitrification or denitrification) and the environmental
106 conditions (e.g., aerobic or anaerobic), biotically-driven abiotic N_2O production should not be ruled out [34].
107 A relevant reaction is the N-nitrosation of NH_2OH by HNO_2 [35], which may become significant at low pH
108 or under high NO_2^- conditions during ammonia oxidation [36,37]. Other abiotic reactions with N-compounds
109 and metals were reviewed by Zhu-Barker (2015) [34]. However, the kinetic characterization of abiotic N_2O
110 production is still an urgent need to predict the relative contribution of each N_2O pathway, with very few
111 kinetic parameters reported [36,38,39].

112 **Environmental regulation of N_2O production**

113 The environmental conditions *and* the microbial community determine the performance, including N_2O
114 production, of a biological treatment system [40]. Volumetric N_2O production rates ($\text{mgN}_2\text{O/L}\cdot\text{h}$) provide
115 little information as they are VSS-dependent. Specific rates ($\text{mgN}_2\text{O/gVSS}\cdot\text{h}$) are more informative [41], but
116 remain community-dependent. As N_2O production is associated with certain microbial guilds (e.g. AOB in
117 nitrification dominated systems), we suggest that comparison between systems is facilitated by reporting
118 N_2O rates normalized to the responsible biomass fractions (e.g., $\text{mgN}_2\text{O/gVSS}_{\text{AOB}}\cdot\text{h}$, where gVSS_{AOB} is
119 calculated using either experimental approaches (e.g. via qPCR or qFISH) or estimated from thermodynamic
120 and stoichiometric relationships). In addition, N_2O emission factors are more informative when related to
121 substrate depletion and not substrate load, to avoid underestimation of N_2O emissions from systems with low
122 N-removing efficiency (e.g. $\text{mgN}_2\text{O/mgNH}_4^+_{\text{rem}}$, $\text{mgN}_2\text{O/mgNO}_3^-_{\text{rem}}$). Initial studies of N_2O emissions from
123 water treatment systems correlated N_2O production rates to operational parameters such as solids retention
124 time (SRT), NH_4^+ load, or temperature, but these correlations are likely too simplistic for predicting purposes
125 [42]. The current understanding of the metabolic pathways should help to identify what controls the rate of
126 each N_2O pathway at the operating conditions. For example, nitrifying studies correlate N_2O production
127 associated to the ND pathway to NO_2^- concentrations at low DO. The correlation also indicates limiting
128 NO_2^- levels and excess supply of electrons. However, at the onset of anoxia the limiting substrate becomes
129 the electron donor pool, and not NO_2^- . Hence, in alternating aerated systems both electron donor and
130 acceptor can govern N_2O production from the same pathway.

131 While the internal electron pools cannot be directly quantified, the obligate NH_4^+ oxidation intermediates
132 NH_2OH and NO can be monitored [37], and hence specific monitoring of NH_2OH and NO might be
133 informative to ascertain the relative NN and ND contributions. NH_2OH oxidation is the energy-yielding
134 process and the NH_2OH concentration from AOB pure cultures or engineered systems is typically low
135 ($<0.1\text{mgN/L}$) [37]. A low concentration indicates a quick turnover, and would result in limited NH_2OH loss
136 through diffusion out of the cell. Aerobic NH_2OH oxidation to NO_2^- yields more N_2O than NH_3 oxidation, as
137 electrons required to activate AMO are not required and can increase the NO_x reduction [16,36,43]. Under
138 anoxic conditions NH_2OH can be auto-oxidized to N_2O via NO , with the released electrons channelled to
139 reduce NO_2^- (if present) and NO [10,43]. If we consider NH_2OH the electron donor of the NN and ND
140 pathways (or the internal electron pool derived from NH_2OH oxidation), the NH_2OH oxidation process in
141 both the NN and ND pathways is DO-independent. The relative availability of electron acceptors, O_2 and
142 NO_2^- , would regulate the NN and ND contributions. Overall, we suggest that NH_2OH and NO are more
143 informative than NH_4^+ and NO_2^- to understand the regulation mechanisms of N_2O production.

144 Transient NH_3 or O_2 levels both (or each) increase N_2O production from NH_4^+ -removing systems [44]. Based
145 on literature findings, we propose that the efficiency of AOBs at channelling electrons to anabolic processes
146 will determine the electron availability for the NN and ND mechanisms. An NH_3 overload would increase
147 the NH_3 turnover into NH_2OH and requires a sudden dissipation of electrons equivalents generated during
148 NH_2OH oxidation, observed to not accumulate to high concentrations. Similarly, in systems with alternate
149 oxic and anoxic conditions, the onset of anoxia halts NH_3 oxidation but not NH_2OH oxidation. In both cases,
150 when the cell could not incorporate the released electrons equivalents into the anabolism from the internal
151 pool, other available acceptors such as NO_2^- or NO could be reduced, explaining the increasing N_2O
152 production [44,45]. The proteomic analysis of *N. europaea* also indicated that imbalances between anabolic
153 and catabolic electron fluxes manifested in the production of nitrogenous intermediates [46].

154 The effect of pH on N_2O dynamics is complex. In general, pH can affect observed biological activity at
155 multiple hierarchical levels (including gene transcription, translation, post-translational, enzyme activity),
156 that are often not individually discernible. The maximum enzymatic activities are pH-dependent: NH_3
157 oxidation and N_2O reduction are favoured at alkaline pH levels, while HNO_2 reduction is favoured at slightly
158 acidic pH [47,48]. In addition, several molecules during N conversion have acid/base properties and their
159 speciation will be affected across the physiologically-relevant pH range ($\text{NH}_4^+/\text{NH}_3$, $\text{HNO}_2/\text{NO}_2^-$). NH_3 is
160 considered the substrate of AOB [48], but the substrate/inhibitor effect has not been clearly differentiated
161 from the pH-effect on the maximum specific activity. Accumulation of the unionized species HNO_2 , and not
162 NO_2^- , leads to higher N_2O emissions from both autotrophic and heterotrophic pathways. However, it is
163 unclear whether HNO_2 or a low-pH maximum specific nitrite reduction rate govern the higher ND and HD
164 rates observed at lower pH [49,50]. Within the abiotic N_2O -producing reactions, acidification enhanced the

165 N₂O yield of a *Nitrosospira*-dominated community due to the higher HNO₂ availability of the hybrid N₂O-
166 forming reaction of NH₂OH and HNO₂ [51].

167 The carbon composition of the water stream shapes the diversity of the microbial community [52,53]. The
168 metabolic pathways to oxidize organic carbon sources differ: the tricarboxylic acid cycle to oxidize acetate is
169 common among denitrifying bacteria, but methanol degradation requires other metabolic pathways present in
170 a small fraction of denitrifiers [54]. Ribera-Guardia *et al.* observed in lab-scale tests with denitrifying sludge
171 that individual dosing of acetate, ethanol and methanol lead to different electron consumption and
172 distribution profiles: ethanol favoured N₂O reduction compared to NO₃⁻ and NO₂⁻ reduction, acetate lead to
173 N₂O accumulation due to relative faster NO₃⁻ and NO₂⁻ reduction rates, and methanol oxidation was
174 significantly slower than ethanol or acetate [55].

175 Microbes with storage metabolism such as Phosphate-Accumulating Organisms (PAO) and Glycogen-
176 Accumulating Organisms (GAO) also possess denitrification capabilities. PAOs show higher denitrifying
177 rates and lower fractions of N₂O accumulation per N-reduced than GAOs [56]. A high N₂O yield (60-80%)
178 benefits novel strategies where energy is recovered through N₂O decomposition such as the Coupled
179 Aerobic–anoxic Nitrous Decomposition Operation (CANDO) [57]. Biomass storing polyhydroxybutyrate
180 maximized the NO₂⁻ to N₂O reduction yield, simultaneous reducing the C/N requirement for N-removal [57].

181 Hence, the N₂O production potential of a denitrifying community depends on the COD composition, the
182 concentration of N-intermediates, and the microbial diversity, which determine the competition between
183 each denitrifying step for the electron flow.

184 **N₂O pathway identification**

185 The N-transformations of electron acceptors from NO₂⁻ to NO and to N₂O of the ND and HD pathways are
186 indistinguishable, and in mixed communities the interactions and exchange of substrates difficult the
187 identification of N₂O producers (e.g. cross feeding of NO, NO₂⁻ between microorganisms). If only bulk N₂O
188 measurements are made, the relative contribution of different pathways could only be made if target
189 enzymatic processes are inhibited (e.g. by addition of allylthiourea to inhibit NH₄⁺ oxidation, or of acetylene
190 to inhibit bacterial NH₄⁺ oxidation and N₂O reduction). However, use of inhibitors is rarely specific, and
191 would also inhibit other metabolic processes, possibly altering intermediates of N₂O production rates.

192 Experimental methods based on relative isotopocule abundances (i.e. all isotopic variants) aim at quantifying
193 the contribution of each pathway. N₂O isotopologues differ in the atomic weight (¹⁴N or ¹⁵N, ¹⁶O or ¹⁸O),
194 and/or location of the N and O atoms in the asymmetric linear N₂O molecule (i.e., isotopomers). No single
195 isotopic method can elucidate, unambiguously, the contribution of each pathway, but they are
196 complementary [12].

197 Stable isotope methods combine addition of ^{15}N and ^{18}O tracers ($^{15}\text{NH}_4^+$, $^{15}\text{NO}_2^-$, $^{15}\text{NO}_3^-$, $^{18}\text{O-O}_2$), and
198 calculate pathway contributions based on isotopologue abundances. Only dual methods ($^{15}\text{N}/^{18}\text{O}$) distinguish
199 NN from ND and HD pathways in WWTPs. The isolation of the NN pathway is only possible if the rapid
200 oxygen exchange between H_2O and NO_2^- is be quantified. In incubations with partial-nitrification/anammox
201 biomass, after calculating the rapid ^{18}O dilution in NO_2^- formation, $^{46}\text{N}_2\text{O}$ represented the NN contribution
202 [58]. However, the use of isotopic tracers is limited to lab-scale batch incubations for economic reasons.

203 Differently from stable isotopic tracers, natural abundance methods quantify the N and O isotopic signature
204 in the N_2O molecule ($\delta^{15}\text{N}$, $\delta^{18}\text{O}$). In the N case the difference of the delta ($^{15}\text{N}/^{14}\text{N}$ isotope ratio relative to
205 a standard) between central (α) and terminal (β) N atoms, is termed Site Preference (SP). The exact SP
206 signature values for each N_2O production pathway are not elucidated (e.g. ND -1.7‰ – 0.1‰, HD -5.1‰ – -
207 0.5‰) [59], and the similarity in enzymes and molecules between the ND and nitrifying-coupled HD, make
208 the ND and HD ^{15}N isotopic values to not be unambiguously distinguishable [12]. SP measurements with
209 pure cultures do not guarantee that SP signatures for specific pathways are identified. On the other hand,
210 assays of purified enzymes, like HAO or NOR, may generate SP signatures of the NN and HD pathways –
211 provided signatures are enzyme-specific (HAO *N. europaea* 36.6‰, HAO *N. oceanii* 36.2‰) [60]. Clearly,
212 if two pathways possess similar SP signatures the contributions of the two pathways cannot be elucidated.
213 Ostrom *et al.* highlighted analytical limitations of SP measurements, where a the lack of isotopically
214 characterized N_2O standards for adequate sample normalization exists, and provided suggestions on best-
215 practices [61]. Moreover, no consensus exist on measurement protocols, that lead to high variability (10‰)
216 of the SP value of a reference gas measured among analytical laboratories [62]. Although the relative
217 contribution of ND and HD cannot be distinguished by SP values [12,58], SP-based studies can help identify
218 N_2O pathway dynamics in defined situations: in autotrophic nitrogen removing communities where the HD
219 contribution is expected to be low [59], or by combining SP and $\delta^{18}\text{O}$ values to calculate N_2O reduction to N_2
220 [63], or tracer studies with ^{15}N models to contrast individual pathway reactions with the observed molecular
221 $\text{N}_2/\text{N}_2\text{O}$ dynamics [64].

222 **N_2O mitigation strategies**

223 The mathematical description of N_2O production aims to develop and validate mitigation strategies. In
224 empirical models N_2O emissions are fit to operational factors (e.g. pH value, temperature, feeding and
225 aeration strategy, etc.) via multivariate statistical techniques, highlighting specific effects and combined
226 influences to which N_2O emissions are most sensitive [65,66]. Polynomial Box-Behnken models found
227 optimum operating conditions for N_2O mitigation from deammonification (pH = 7.8, single feeding,
228 continuous aeration), or nitrifying granular sludge (pH = 7.1, temperature = 22.3 °C, aeration rate = 0.20
229 m^3/h). However, optimum conditions may vary with sludge characteristics or operational configurations, and

230 as N₂O measurements quantify net rates (i.e. the result of simultaneous production and consumption); black-
231 box models do not distinguish between a lower consumption rate and a higher production rate. The black-
232 box modelling approach is powerful for its simplicity and can help develop mechanistic models.

233 The engineering design of N₂O mitigation strategies for novel N-removing technologies already benefits
234 from the current knowledge of N₂O pathway regulation. Nitritation/Denitritation and Partial-
235 Nitritation/Anammox systems are cost-effective alternatives to nitrification/denitrification, but are also
236 hotspots for N₂O production due to higher NO₂⁻ and lower DO concentrations. Steadier ammonium removal
237 rates are assumed to avoid imbalances in the internal electron pool two strategies and their implementation
238 have proven successful: by intermittent-feeding the NH₃ shock-loads decrease [67], and increasing the
239 aeration frequency leads to both lower aerobic removal rates and lower NO₂⁻ accumulation [41]. Increasing
240 DO levels decreased the contribution from denitrifying pathways, and external carbon dosage successfully
241 consumed N₂O produced from the onset of anoxia [67]. The newly classified N₂O reducers (*nosZ* clade II)
242 were enriched on a biofilter by feeding the gaseous N₂O stream from a WWTP, substantially decreasing N₂O
243 emissions [68].

244 Mitigation strategies validated with mechanistic models will be more robust than based on empirical
245 correlation models. Mechanistic models describe a complex process such as N₂O production via a
246 combination of variables through mathematical equations. N₂O models differ on the number of processes
247 and/or variables described, and the complexity of their mathematical rates.

248 Metabolic network models for single-species incorporate all the electron transport reactions known, and are
249 substantiated by the increasing genomic information. The complex parameterization of metabolic models is
250 still a drawback, and for AOBs only the *N. europaea* exists [45]. Integrative modelling combined with
251 dynamic flux balance analysis allows for dynamic simulations [69], which was previously limited to only
252 steady-state estimations [45].

253 ASM-based grey-box models are widely used in water treatment technologies. Microbial guilds are grouped
254 by functionalities and N₂O production is implemented as an extension of N-removing models of AOB and
255 HB. Several N₂O models consider the three biological pathways (NN, ND, HD) but differ on the number of
256 variables and processes described [43]: some structures simplify the N₂O-producing reactions by not
257 including intermediates such as NO or NH₂OH, or by shortening denitrifying pathways (NO₂⁻ reduction to
258 N₂O over NO) [70]. N₂O models are calibrated with TN datasets and additional N₂O measurements,
259 however, the direct precursors of N₂O are typically not validated. While NH₂OH levels in N-removing
260 systems are low (< 0.2 mgN/L) N₂O models have overestimated accumulation of 27% of the NH₃ oxidized as
261 NH₂OH in a biofilm [9], or up to 6 mgN/L in the effluent [71]. A model considering the ND and HD
262 pathways predicted N₂O dynamics from nitrifying batch tests. However, for the same overall N₂O fit the NO

emissions and N₂O pathway contributions varied greatly when NO affinities were varied within literature range (NO = 0.2 – 4.0% NO/N₂O, ND = 1 – 56%) [72]. The simultaneous calibration of intermediates such as NO or NH₂OH will increase the reliability of N₂O simulations and pathway contributions [43,70]. Due to the overparameterized nature of ASM-based N₂O models during model calibrations not all parameters are identifiable, and pathway contributions can only be validated in lab-scale systems. N₂O models for full-scale WWTP rely on bulk N₂O measurements or ex-situ isotopic tests [73]. Hence, the precision of best-fit N₂O simulations and pathway contributions should be treated cautiously.

The accuracy of N₂O model predictions is addressed in WWTP models, but only few studies have applied analysed the uncertainty (e.g. confidence intervals) [43,74], which is a severe weakness. We suggest that N₂O precision analysis together with systematic calibration protocols (e.g. analysis of residuals, parameter identifiability, parameter subset selection) will increase the confidence of future control-based mitigation strategies.

Conclusions

Recent pure-culture studies have focused on deciphering the metabolic pathways for N₂O production; yet, under the same environmental conditions members of the same guild display different NO and N₂O production rates. Biologically-driven abiotic N₂O production should not be overlooked, but its kinetics remain to be described. N₂O mitigation strategies informed by knowledge of biological mechanisms are being implemented. Novel mechanistic N₂O models considering three biological pathways can predict bulk emissions from WWT units accurately, but quantifying model precision needs further research. Recent studies have benefited from combining model predictions and isotopocule datasets, which can be used to validate the modelled predictions of pathway contributions and bulk emissions.

Thus, the combination of molecular ecological, computational and isotopic analysis approaches seems essential to improving our understanding of N₂O pathways in water treatment systems, and knowledge gleaned from other environmental systems (e.g. soils) should be incorporated.

Acknowledgements

This research was funded by the Danish Agency for Science, Technology and Innovation through the Research Project LaGas (12-132633), the Danish Council for Independent Research through Project N2Oman (17H01893) and the project HEPWAT, supported by the Danish Ministry of Environment and Food. The authors have no conflict of interest to declare, and are grateful to the reviewers for substantially improving the quality of the manuscript.

294   **References**

295   Papers of particular interest, published within the period of review have been highlighted as:

- 296   •   of special interest
- 297   • [34] – Review of abiotic reactions mediating N₂O production in soil environments: interactions of N-
298   substrates and metals.
- 299   • [43] – Calibration of an N₂O model with special focus on the parameter estimation procedure and the high
300   uncertainty of N₂O emissions.
- 301   • [55] – Electron competition occurs between the four denitrifying steps, and with varying degrees
302   depending on the carbon source.
- 303   • [58] – Dual tracer method (15N/18O) analysis of partial nitrification-anammox biomass shows a DO
304   regulation of N₂O production pathways (NN, ND) and low heterotrophic contribution.
- 305   ••   of outstanding interest
- 306   •• [9] – Overview of N₂O production pathways and mechanisms in suspended cultures and biofilm systems.
- 307   •• [12] – New advances in understanding the nitrifier denitrification pathway. An update from 2001.
- 308   •• [45] – The metabolic effects of oxic/anoxic cycling on *Nitrosomonas europaea* are more correlated to
309   proteomic analysis than transcriptomic.
- 310   •• [16] – Microrespirometric assays on five AOB pure cultures show genomic and phenotypic differences.
- 311   •• [59] – Review on isotope technologies for N₂O production pathways, focusing on wastewater treatment.
- 312
- 313   1.   Lane JL, de Haas DW, Lant PA: **The diverse environmental burden of city-scale urban water**
314   **systems.** *Water Res* 2015, **81**:398–415.
- 315   2.   Desloover J, Vlaeminck SE, Clauwaert P, Verstraete W, Boon N: **Strategies to mitigate N2O**
316   **emissions from biological nitrogen removal systems.** *Curr Opin Biotechnol* 2012, **23**:474–82.
- 317   3.   Short MD, Daikeler A, Peters GM, Mann K, Ashbolt NJ, Stuetz RM, Peirson WL: **Municipal**
318   **gravity sewers: an unrecognised source of nitrous oxide.** *Sci Total Environ* 2014, **468–469**:211–8.
- 319   4.   Delre A, Mønster J, Scheutz C: **Greenhouse gas emission quantification from wastewater**
320   **treatment plants, using a tracer gas dispersion method.** *Sci Total Environ* 2017, **605–606**:258–
321   268.
- 322   5.   Delre A, ten Hoeve M, Scheutz C: **Site-specific carbon footprints of Scandinavian wastewater**
323   **treatment plants, using the life cycle assessment approach.** *J Clean Prod* 2019, **211**:1001–1014.

- 324 6. Desloover J, De Clippeleir H, Boeckx P, Du Laing G, Colsen J, Verstraete W, Vlaeminck SE: **Floc-**
 325 **based sequential partial nitrification and anammox at full scale with contrasting N₂O emissions.**
 326 *Water Res* 2011, **45**:2811–21.
- 327 7. Yan P, Qin R, Guo J, Yu Q, Li Z, Chen Y, Shen Y, Fang F: **Net-Zero-Energy Model for**
 328 **Sustainable Wastewater Treatment.** *Environ Sci Technol* 2017, **51**:1017–1023.
- 329 8. Gustavsson DJI, Tumlin S: **Carbon footprints of Scandinavian wastewater treatment plants.**
 330 *Water Sci Technol* 2013, **68**:887.
- 331 9. Todt D, Dörsch P: **Mechanism leading to N₂O production in wastewater treating biofilm**
 332 **systems.** *Rev Environ Sci Bio/Technology* 2016, **15**:355–378.
- 333 10. Caranto JD, Lancaster KM: **Nitric oxide is an obligate bacterial nitrification intermediate**
 334 **produced by hydroxylamine oxidoreductase.** *Proc Natl Acad Sci* 2017,
 335 doi:10.1073/pnas.1704504114.
- 336 11. Vajrala N, Martens-Habbena W, Sayavedra-Soto L a, Schauer A, Bottomley PJ, Stahl D a, Arp DJ:
 337 **Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea.**
 338 *Proc Natl Acad Sci* 2013, **110**:1006–1011.
- 339 12. Wrage-Mönnig N, Horn MA, Well R, Müller C, Velthof G, Oenema O: **The role of nitrifier**
 340 **denitrification in the production of nitrous oxide revisited.** *Soil Biol Biochem* 2018, **33**:1723–
 341 1732.
- 342 13. Hink L, Lycus P, Gubry-Rangin C, Frostegård Å, Nicol GW, Prosser JI, Bakken LR: **Kinetics of NH**
 343 **3 -oxidation, NO-turnover, N₂ O-production and electron flow during oxygen depletion in**
 344 **model bacterial and archaeal ammonia oxidisers.** *Environ Microbiol* 2017, **19**:4882–4896.
- 345 14. Kozłowski JA, Price J, Stein LY: **Revision of N₂O-Producing Pathways in the Ammonia-**
 346 **Oxidizing Bacterium Nitrosomonas europaea ATCC 19718.** *Appl Environ Microbiol* 2014,
 347 **80**:4930–4935.
- 348 15. Caranto JD, Vilbert AC, Lancaster KM: **Nitrosomonas europaea cytochrome P460 is a direct link**
 349 **between nitrification and nitrous oxide emission.** *Proc Natl Acad Sci* 2016, **113**:14704–14709.
- 350 16. Kozłowski JA, Kits KD, Stein LY: **Comparison of Nitrogen Oxide Metabolism among Diverse**
 351 **Ammonia-Oxidizing Bacteria.** *Front Microbiol* 2016, **7**:1–9.
- 352 17. Kozłowski JA, Stieglmeier M, Schleper C, Klotz MG, Stein LY: **Pathways and key intermediates**
 353 **required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and**
 354 **Thaumarchaeota.** *ISME J* 2016, **10**:1836–1845.
- 355 18. Stieglmeier M, Mooshammer M, Kitzler B, Wanek W, Zechmeister-Boltenstern S, Richter A,
 356 Schleper C: **Aerobic nitrous oxide production through N-nitrosating hybrid formation in**
 357 **ammonia-oxidizing archaea.** *ISME J* 2014, **8**:1135–46.
- 358 19. Hink L, Nicol GW, Prosser JI: **Archaea produce lower yields of N₂ O than bacteria during**

- 359 **aerobic ammonia oxidation in soil.** *Environ Microbiol* 2016, **00**:1–28.
- 360 20. Palomo A, Pedersen AG, Fowler SJ, Dechesne A, Sicheritz-Pontén T, Smets BF: **Comparative**
 361 **genomics sheds light on niche differentiation and the evolutionary history of comammox**
 362 **Nitrospira.** *ISME J* 2018, doi:10.1038/s41396-018-0083-3.
- 363 21. Berks BC, Ferguson SJ, Moir JWB, Richardson DJ: **Enzymes and associated electron transport**
 364 **systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions.** *Biochim*
 365 *Biophys Acta - Bioenerg* 1995, **1232**:97–173.
- 366 22. Strohm TO, Griffin B, Zumft WG, Schink B: **Growth yields in bacterial denitrification and nitrate**
 367 **ammonification.** *Appl Environ Microbiol* 2007, **73**:1420–1424.
- 368 23. Kucera I, Dadak V, Dobry R: **The distribution of redox equivalents in the anaerobic respiratory**
 369 **chain of Paracoccus denitrificans.** *Eur J Biochem* 1983, **130**:359–364.
- 370 24. Graf DRH, Jones CM, Hallin S: **Intergenomic Comparisons Highlight Modularity of the**
 371 **Denitrification Pathway and Underpin the Importance of Community Structure for N₂O**
 372 **Emissions.** *PLoS One* 2014, **9**:e114118.
- 373 25. Lycus P, Lovise Bøthun K, Bergaust L, Peele Shapleigh J, Reier Bakken L, Frostegård Å:
 374 **Phenotypic and genotypic richness of denitrifiers revealed by a novel isolation strategy.** *ISME J*
 375 2017, **11**:2219–2232.
- 376 26. Jones CM, Spor A, Brennan FP, Breuil M-C, Bru D, Lemanceau P, Griffiths B, Hallin S, Philippot L:
 377 **Recently identified microbial guild mediates soil N₂O sink capacity.** *Nat Clim Chang* 2014,
 378 **4**:801–805.
- 379 27. Sanford RA, Wagner DD, Wu Q, Chee-Sanford JC, Thomas SH, Cruz-García C, Rodríguez G,
 380 Massol-Deyá A, Krishnani KK, Ritalahti KM, et al.: **Unexpected nondenitrifier nitrous oxide**
 381 **reductase gene diversity and abundance in soils.** *Proc Natl Acad Sci U S A* 2012, **109**:19709–14.
- 382 28. Yoon S, Nissen S, Park D, Sanford RA, Löffler FE: **Nitrous Oxide Reduction Kinetics Distinguish**
 383 **Bacteria Harboring Clade I NosZ from Those Harboring Clade II NosZ.** *Appl Environ Microbiol*
 384 2016, **82**:3793–3800.
- 385 29. Domeignoz-Horta LA, Putz M, Spor A, Bru D, Breuil MC, Hallin S, Philippot L: **Non-denitrifying**
 386 **nitrous oxide-reducing bacteria - An effective N₂O sink in soil.** *Soil Biol Biochem* 2016, **103**:376–
 387 379.
- 388 30. Suenaga T, Riya S, Hosomi M, Terada A: **Biokinetic characterization and activities of N₂O-**
 389 **reducing bacteria in response to various oxygen levels.** *Front Microbiol* 2018, **9**:1–10.
- 390 31. Morley N, Baggs EM, Dörsch P, Bakken LR: **Production of NO, N₂O and N₂ by extracted soil**
 391 **bacteria, regulation by NO₂(-) and O₂ concentrations.** *FEMS Microbiol Ecol* 2008, **65**:102–12.
- 392 32. Figueroa-González I, Quijano G, Laguna I, Muñoz R, García-Encina PA: **A fundamental study on**
 393 **biological removal of N₂O in the presence of oxygen.** *Chemosphere* 2016, **158**:9–16.

- 394 33. Lycus P, Soriano-Laguna MJ, Kjos M, Richardson DJ, Gates AJ, Milligan DA, Frostegård Å,
395 Bergaust L, Bakken LR: **A bet-hedging strategy for denitrifying bacteria curtails their release of**
396 **N₂O**. *Proc Natl Acad Sci* 2018, doi:10.1073/pnas.1805000115.
- 397 34. Zhu-Barker X, Cavazos AR, Ostrom NE, Horwath WR, Glass JB: **The importance of abiotic**
398 **reactions for nitrous oxide production**. *Biogeochemistry* 2015, **126**:251–267.
- 399 35. Spott O, Russow R, Stange CF: **Formation of hybrid N₂O and hybrid N₂ due to**
400 **codenitrification: First review of a barely considered process of microbially mediated N-**
401 **nitrosation**. *Soil Biol Biochem* 2011, **43**:1995–2011.
- 402 36. Terada A, Sugawara S, Hojo K, Takeuchi Y, Riya S, Harper WF, Yamamoto T, Kuroiwa M, Isobe K,
403 Katsuyama C, et al.: **Hybrid Nitrous Oxide Production from a Partial Nitrifying Bioreactor:**
404 **Hydroxylamine Interactions with Nitrite**. *Environ Sci Technol* 2017, **51**:2748–2756.
- 405 37. Liu S, Han P, Hink L, Prosser JI, Wagner M, Bruggemann N: **Abiotic conversion of extracellular**
406 **NH₂OH contributes to N₂O emission during ammonia oxidation**. *Environ Sci Technol* 2017,
407 doi:10.1021/acs.est.7b02360.
- 408 38. Buchwald C, Grabb K, Hansel CM, Wankel SD: **Constraining the role of iron in environmental**
409 **nitrogen transformations: Dual stable isotope systematics of abiotic NO₂-reduction by Fe(II)**
410 **and its production of N₂O**. *Geochim Cosmochim Acta* 2016, **186**:1–12.
- 411 39. Su Q, Domingo-Félez C, Jensen MM, Smets BF: **Abiotic nitrous oxide (N₂O) production shows**
412 **strong pH dependence, but contributes little to overall N₂O emissions in biological nitrogen**
413 **removal systems**. *Unpubl Work* 2018,
- 414 40. Song K, Suenaga T, Hamamoto A, Satou K, Riya S, Hosomi M, Terada A: **Abundance,**
415 **transcription levels and phylogeny of bacteria capable of nitrous oxide reduction in a municipal**
416 **wastewater treatment plant**. *J Biosci Bioeng* 2014, **xx**.
- 417 41. Domingo-Félez C, Mutlu AG, Jensen MM, Smets BF: **Aeration Strategies To Mitigate Nitrous**
418 **Oxide Emissions from Single-Stage Nitritation/Anammox Reactors**. *Environ Sci Technol* 2014,
419 **48**:8679–8687.
- 420 42. Kampschreur MJ, Temmink H, Kleerebezem R, Jetten MSM, van Loosdrecht MCM: **Nitrous oxide**
421 **emission during wastewater treatment**. *Water Res* 2009, **43**:4093–103.
- 422 43. Domingo-Félez C, Calderó-Pascual M, Sin G, Plósz BG, Smets BF: **Calibration of the**
423 **comprehensive NDHA-N₂O dynamics model for nitrifier-enriched biomass using targeted**
424 **respirometric assays**. *Water Res* 2017, **126**:29–39.
- 425 44. Chandran K, Stein LY, Klotz MG, van Loosdrecht MCM: **Nitrous oxide production by lithotrophic**
426 **ammonia-oxidizing bacteria and implications for engineered nitrogen-removal systems**.
427 *Biochem Soc Trans* 2011, **39**:1832–7.
- 428 45. Yu R, Perez-Garcia O, Lu H, Chandran K: **Nitrosomonas europaea adaptation to anoxic-oxic**

- cycling: Insights from transcription analysis, proteomics and metabolic network modeling. *Sci Total Environ* 2018, **615**:1566–1573.
46. Jiang D, Khunjar WO, Wett B, Murthy SN, Chandran K: **Characterizing the Metabolic Trade-Off in *Nitrosomonas europaea* in Response to Changes in Inorganic Carbon Supply.** *Environ Sci Technol* 2015, **49**:2523–31.
47. Blum J-M, Su Q, Ma Y, Valverde-Pérez B, Domingo-Félez C, Jensen MM, Smets BF: **The pH dependency of N-converting enzymatic processes, pathways and microbes: effect on net N₂O production.** *Environ Microbiol* 2018, **20**:1623–1640.
48. Simon J, Klotz MG: **Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations.** *Biochim Biophys Acta - Bioenerg* 2013, **1827**:114–135.
49. Shiskowski D, Mavinic D: **The influence of nitrite and pH (nitrous acid) on aerobic-phase, autotrophic N₂O generation in a wastewater treatment bioreactor.** *J Environ Eng Sci* 2006, **283**:273–283.
50. Thomsen JK, Geest T, Cox RP: **Mass spectrometric studies of the effect of pH on the accumulation of intermediates in denitrification by *Paracoccus denitrificans*.** *Appl Environ Microbiol* 1994, **60**:536–541.
51. Frame CH, Lau E, Nolan EJ, Goepfert TJ, Lehmann MF: **Acidification Enhances Hybrid N₂O Production Associated with Aquatic Ammonia-Oxidizing Microorganisms.** *Front Microbiol* 2017, **7**:2104.
52. Hallin S, Throback IN, Dicksved J, Pell M: **Metabolic profiles and genetic diversity of denitrifying communities in activated sludge after addition of methanol or ethanol.** *Appl Environ Microbiol* 2006, **72**:5445–5452.
53. Lu H, Chandran K, Stensel D: **Microbial ecology of denitrification in biological wastewater treatment.** *Water Res* 2014, **64**:237–254.
54. Madigan MT, Martinko JM, Stahl D, Clark DP: *Brock Biology of Microorganisms*. Pearson; 2010.
55. Ribera-Guardia A, Kassotaki E, Gutierrez O, Pijuan M: **Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community.** *Process Biochem* 2014, **49**:2228–2234.
56. Ribera-Guardia A, Marques R, Arangio C, Carvalheira M, Oehmen A, Pijuan M: **Distinctive denitrifying capabilities lead to differences in N₂O production by denitrifying polyphosphate accumulating organisms and denitrifying glycogen accumulating organisms.** *Bioresour Technol* 2016, **219**:106–113.
57. Gao H, Liu M, Griffin JS, Xu L, Xiang D, Scherson YD, Liu WT, Wells GF: **Complete Nutrient Removal Coupled to Nitrous Oxide Production as a Bioenergy Source by Denitrifying Polyphosphate-Accumulating Organisms.** *Environ Sci Technol* 2017, **51**:4531–4540.

- 464 58. Ma C, Jensen MM, Smets BF, Thamdrup B: **Pathways and Controls of N₂O Production in**
465 **Nitrification–Anammox Biomass.** *Environ Sci Technol* 2017, **51**:8981–8991.
- 466 59. Duan H, Ye L, Erler D, Ni B-J, Yuan Z: **Quantifying nitrous oxide production pathways in**
467 **wastewater treatment systems using isotope technology – a critical review.** *Water Res* 2017,
468 **122**:96–113.
- 469 60. Yamazaki T, Hozuki T, Arai K, Toyoda S, Koba K, Fujiwara T, Yoshida N: **Isotopomeric**
470 **characterization of nitrous oxide produced by reaction of enzymes extracted from nitrifying**
471 **and denitrifying bacteria.** *Biogeosciences* 2014, **11**:2679–2689.
- 472 61. Ostrom NE, Gandhi H, Coplen TB, Toyoda S, Böhlke JK, Brand WA, Casciotti KL, Dyckmans J,
473 Giesemann A, Mohn J, et al.: **Preliminary assessment of stable nitrogen and oxygen isotopic**
474 **composition of USGS51 and USGS52 nitrous oxide reference gases and perspectives on**
475 **calibration needs.** *Rapid Commun Mass Spectrom* 2018, **32**:1207–1214.
- 476 62. Mohn J, Wolf B, Toyoda S, Lin C, Liang M, Brüggemann N, Wissel H, Steiker AE, Dyckmans J,
477 Szwec L, et al.: **Interlaboratory assessment of nitrous oxide isotopomer analysis by isotope ratio**
478 **mass spectrometry and laser spectroscopy: current status and perspectives.** *Rapid Commun*
479 *Mass Spectrom* 2014, **28**:1995–2007.
- 480 63. Lewicka-Szczebak D, Augustin J, Giesemann A, Well R: **Quantifying N₂O reduction to N₂ based**
481 **on N₂O isotopocules – validation with independent methods (helium incubation and ¹⁵N gas**
482 **flux method) Dominika.** *Biogeosciences* 2017, **14**:711–732.
- 483 64. Müller C, Laughlin RJ, Spott O, Rütting T: **Quantification of N₂O emission pathways via a ¹⁵N**
484 **tracing model.** *Soil Biol Biochem* 2014, **72**:44–54.
- 485 65. Leix C, Drewes JE, Ye L, Koch K: **Strategies for enhanced deammonification performance and**
486 **reduced nitrous oxide emissions.** *Bioresour Technol* 2017, **236**:174–185.
- 487 66. Liu R-T, Wang X-H, Zhang Y, Wang M-Y, Gao M-M, Wang S-G: **Optimization of operation**
488 **conditions for the mitigation of nitrous oxide (N₂O) emissions from aerobic nitrifying granular**
489 **sludge system.** *Environ Sci Pollut Res* 2016, **23**:9518–9528.
- 490 67. Peng L, Carvajal-Arroyo JM, Seuntjens D, Prat D, Colica G, Pintucci C, Vlaeminck SE: **Smart**
491 **operation of nitrification/denitrification virtually abolishes nitrous oxide emission during**
492 **treatment of co-digested pig slurry centrate.** *Water Res* 2017, **127**:1–10.
- 493 68. Yoon H, Song MJ, Yoon S: **Design and Feasibility Analysis of a Self-Sustaining Biofiltration**
494 **System for Removal of Low Concentration N₂O Emitted from Wastewater Treatment Plants.**
495 *Environ Sci Technol* 2017, **51**:10736–10745.
- 496 69. Mellbye BL, Giguere AT, Murthy GS, Bottomley PJ, Sayavedra-Soto LA, Chaplen FWR: **Genome-**
497 **Scale, Constraint-Based Modeling of Nitrogen Oxide Fluxes during Coculture of Nitrosomonas**
498 **europaea and Nitrobacter winogradskyi.** *mSystems* 2018, **3**:1–13.

- 499 70. Pocquet M, Wu Z, Queinnec I, Sperandio M: **A two pathway model for N₂O emissions by**
500 **ammonium oxidizing bacteria supported by the NO/N₂O variation.** *Water Res* 2016, **88**:948–959.
- 501 71. Wan X, Baeten JE, Volcke EIP: **Effect of operating conditions on N₂O emissions from one-stage**
502 **partial nitrification-anammox reactors.** *Biochem Eng J* 2018, doi:10.1016/j.bej.2018.12.004.
- 503 72. Domingo-Félez C, Pellicer-Nàcher C, Petersen MS, Jensen MM, Plósz BG, Smets BF: **Heterotrophs**
504 **are key contributors to nitrous oxide production in activated sludge under low C-to-N ratios**
505 **during nitrification-Batch experiments and modeling.** *Biotechnol Bioeng* 2017, **114**:132–140.
- 506 73. Peng L, Ni B-J, Erler D, Ye L, Yuan Z: **The effect of dissolved oxygen on N₂O production by**
507 **ammonia-oxidizing bacteria in an enriched nitrifying sludge.** *Water Res* 2014, **66**:12–21.
- 508 74. Mannina G, Cosenza A, Viviani G, Ekama GA: **Sensitivity and uncertainty analysis of an**
509 **integrated ASM2d MBR model for wastewater treatment.** *Chem Eng J* 2018, **351**:579–588.
- 510