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Optimization of the aeration strategies in a deammonification sequencing batch reactor for efficient nitrogen removal and mitigation of N₂O production

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1 Optimization of the aeration strategies in a
2 deammonification sequencing batch reactor for
3 efficient nitrogen removal and mitigation of N₂O
4 production

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11 KEYWORDS. Aeration control; Anammox; Granular sludge; Mathematical modelling;
12 Nitrous oxide production

13
14 ABSTRACT. In deammonification systems, nitrite-oxidizing bacteria (NOB)
15 suppression and nitrous oxide (N₂O) mitigation are two important operational
16 objectives. To carry out this multivariable analysis of response, a comprehensive
17 model for the N cycle was developed and evaluated against experimental data from a

18 laboratory-scale deammonification granular sludge sequencing batch reactor.
19 Different aeration strategies were tested and the manipulated variables comprised the
20 dissolved oxygen (DO) set point in the aerated phase, aeration on/off frequency (F)
21 and the ratio (R) between the non-aerated and aerated phase durations. Experimental
22 results showed that a high ammonium utilization rate (AUR) in relation to the low nitrate
23 production rate (NPR) ($\text{NPR/AUR} = 0.07\text{-}0.08$) and limited N_2O emissions ($E_{\text{N}_2\text{O}} < 2\%$)
24 could be achieved at the DO set point = $0.7 \text{ mg O}_2/\text{L}$, R ratio = 2 and F frequency =
25 $6\text{-}7 \text{ h}^{-1}$. Under specific operational conditions (biomass concentration, $\text{NH}_4^+\text{-N}$ loading
26 rate and temperature), simulation results confirmed the feasible aeration strategies for
27 the trade-offs between the NOB suppression and N_2O emission. The intermittent
28 aeration regimes led to frequent shifts in the predominating N_2O production pathways,
29 i.e. hydroxylamine (NH_2OH) oxidation (aerated phase) vs. autotrophic denitrification
30 (non-aerated phase). The inclusion of the extracellular polymeric substances
31 mechanism in the model explained the observed activity of heterotrophs, especially
32 *Anaerolineae*, and granule formation.

33

34 1. INTRODUCTION

35 Deammonification is an effective and energy efficient process for nitrogen removal
36 in wastewater treatment plants (WWTPs). The process can be performed in many
37 configurations by two functional microorganisms, including ammonia oxidizing

38 bacteria (AOB) and anaerobic ammonia oxidation (anammox) bacteria. Those
39 microorganisms coexist and interact with heterotrophs and nitrite oxidizing bacteria
40 (NOB). In particular, the activity of NOB should be suppressed due to the competition
41 for either dissolved oxygen (DO) (with AOB) or nitrite (NO_2^- -N) (with anammox
42 bacteria). Among different methods proposed for NOB suppression¹⁻³, tight DO
43 control is regarded as the most effective approach^{4,5}.

44 Sequencing batch reactors (SBRs) with granular sludge are common
45 deammonification systems, in which the DO set point and aeration mode (continuous
46 vs. intermittent) are important control variables. The intermittent aeration mode is
47 controlled by two manipulated variables, including the aeration on/off frequency (F),
48 representing the number of the aeration phases per hour, and the ratio (R) between
49 the non-aerated and aerated phase durations. The intermittent aeration allows to
50 outcompete NOB by AOB due to a lag phase necessary for NOB to respond to the
51 transition from anoxic to aerobic conditions or potential inhibition of NOB by the
52 intermediate product, i.e. hydroxylamine (NH_2OH)⁶. In order to enhance NOB
53 suppression, the intermittent aeration with either high DO set points⁷ or lower DO set
54 points³ has been proposed. Cao et al.¹ provided the following explanation for this
55 apparent inconsistency. Under high DO levels (>1.5 mg O_2/L), the growth rate of r-
56 strategists *Nitrosomonas*-AOB is higher than that of r-strategists *Nitrobacter*-NOB. On
57 the contrary, lower DO levels (<1.0 mg O_2/L) may favor *Nitrosomonas*-AOB and

58 partially suppress NOB by selecting only K-strategists NOB (*Nitrospira*) rather than r-
59 strategists NOB (*Nitrobacter*).

60 An optimized intermittent aeration strategy may also comprise a higher aeration
61 on/off frequency ⁸, which could be beneficial for both NOB suppression and reduction
62 of energy consumption ⁹. However, under DO-limited and nitrite-elevated conditions,
63 the sustainability of deammonification systems may be questioned due to a potentially
64 significant nitrous oxide (N₂O) production ¹⁰. Simultaneous high nitrogen removal
65 efficiency and low N₂O production depend on many factors, e.g. wastewater
66 composition, granule size, the presence of organic substrates, nitrogen loading rate,
67 aeration pattern, pH and temperature ¹¹⁻¹⁴. Moreover, an appropriate aeration control
68 may also minimize N₂O production in deammonification systems ^{11,15,16}. However, no
69 specific operational strategy has been proposed yet for improving nitrogen removal
70 performance while simultaneously mitigating N₂O production ^{13,17}.

71 A few model-based studies have illustrated the effects of aeration patterns on
72 specific aspects of the nitrogen cycle, including either NOB suppression ^{18,19} or N₂O
73 mitigation in deammonification systems ¹³. However, no models have been applied to
74 carry out a multivariable analysis of response in those systems for efficient nitrogen
75 removal performance, NOB suppression and mitigation of N₂O production. It should
76 be noted that in the case of granular deammonification systems, selection of a
77 conceptual model becomes important. Basically, there are two different (but
78 numerically equivalent) approaches, including biofilm models and “apparent kinetics”

79 ²⁰. The advantage of the latter approach is that typical monitoring data can be used for
80 model calibration ²¹.

81 In this study, a comprehensive model for the N cycle was developed and evaluated
82 against comprehensive experimental data from a laboratory-scale deammonification
83 granular system. Subsequently, the model was used as a tool for the understanding
84 and integrated optimization of deammonification systems (process performance vs.
85 sustainability). The model-based analysis was supported by microbiological
86 investigations with the 16S rDNA Illumina High-Throughput Sequencing to identify
87 microorganisms present in the studied system. The main innovation and contribution
88 of this study is the multivariate analysis of BNR performance, NOB suppression and
89 N₂O mitigation by manipulating the DO set point and intermittent aeration control
90 settings (durations and proportions of on/off periods).

91

92 **2. MATERIALS AND METHODS**

93 **2.1. Laboratory setup and monitoring.** A bench-scale SBR with a working volume of
94 10 L was inoculated with biomass originated from a full-scale sidestream
95 deammonification SBR system in Plettenberg (Germany) ²². The detailed description
96 of laboratory setup and monitoring (including liquid N₂O concentrations) can be found
97 in the Supporting Information (SI, Section S.1).

98 **2.2. Experimental design and data collection.** Over the entire cultivation period of
99 314 days, three series of experiments (test trials) were carried out under various

100 operational conditions (scenarios) in terms of the aeration pattern, biomass
101 concentration, hydraulic retention time (HRT) and $\text{NH}_4^+\text{-N}$ loading rate (NLR) (Table 1).
102 In test trial no. 1, a continuous aeration pattern (scenario 1) was compared with three
103 intermittent aeration patterns (scenarios 2-4). In the latter scenarios, different DO set
104 points, F frequencies and R ratios were used (Table 1). Subsequently, four different R
105 ratios were evaluated in test trial no. 2 with the doubled biomass concentration
106 (scenarios 5-8). Next, a higher F frequency was tested under different R ratios in test
107 trial no. 3 (scenarios 9-14) with the biomass concentration increased fourfold in
108 comparison with test trial no. 1. The length of a single operational cycle of the studied
109 SBR was variable (5-24 h) and the cycle in each scenario consisted of four phases as
110 described in the footnote to Table 1. The stable operation was reached between each
111 test trial and a fixed number of cycles were considered in each scenario (Table 1).
112 During the experiments, mixed liquor samples were withdrawn in the reaction phase
113 every 30 min (scenario 1), 15-45 min (scenarios 2-3), 2 h (scenarios 4 and 9-14), and
114 6 h (scenarios 5-8). The samples were immediately filtered through 1.2 μm pore-size
115 nitrocellulose membrane filters (Whatman, Kent, UK) under vacuum pressure and
116 analyzed for ammonium ($\text{NH}_4^+\text{-N}$), nitrate ($\text{NO}_3^-\text{-N}$), and nitrite ($\text{NO}_2^-\text{-N}$)
117 concentrations. The detailed description of the analytical methods can be found in the
118 SI (section S.2).

119 **Table 1.** Summary of the operational conditions (aeration pattern, biomass concentration, HRT and NLR) in the studied SBR during
 120 the entire cultivation period

Days	Modeling phase	Test trial no.	Scenario no.	Aeration on/off (min)	R ratio	F frequency	DO set point (mg O ₂ /l)	HRT (d)	Number of cycles	Influent NH ₄ ⁺ -N (mg N/L)	NLR (mg N/(L·d))	MLVSS/MLSS (mg/L)	Test objectives	
1-4	Calibration	1*	1	Continuous	-	-	0.3		4				Continuous aeration vs. different intermittent aeration patterns. Moderate NH ₄ ⁺ -N load and low biomass concentration	
			2	15/45	3	1.0	1.0		4	528 ± 121	634 ± 145	993 ± 184/ 1530 ± 42		
			3	15/45	3	1.0	0.5		4					
			4	8/22	2.7	2.0	0.5		4					
5-154	Stable operation*	8/22	2.7	2.0	0.5	0.83		528 ± 121	634 ± 145				-	150 days of stable operation
155-158	Recalibration	2**	5	5/10	2	4.0			1				Intermittent aeration with a different length of the non-aerated phase following the aerated phase. High NH ₄ ⁺ -N load and moderate biomass concentration	
			6	5/15	3	3.0			1			2054 ± 76/		
			7	5/20	4	2.4	0.7	2.5		1	846 ± 46	339 ± 18		2481 ± 114
			8	5/25	5	2.0			1					
159-	Stable operation**	5/20	4	2.4	0.7	2.5			846 ± 46	339 ± 18	-	150 days of stable operation		
309-314	Validation	3***	9	5/5	1	6.0			3				Intermittent aeration with higher aeration frequency. High NH ₄ ⁺ -N load and high biomass concentration	
			10	5/10	2	4.0			3			3850		
			11	5/15	3	3.0	0.7	0.83		3	846 ± 46	1016 ± 55		±235/ 5847 ± 173
			12	3/3	1	10.0			3					
			13	3/6	2	6.7			3					

14	3/9	3	5.0	3
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121

122 Note:

123 * total operational cycle of 5 h, including 4 phases, i.e. 30 min of feeding (2.5 L), 4 h of reaction, 10 min of sedimentation and 20
124 min of decantation (2.5 L).

125 ** total operational cycle of 24 h, including 4 phases, i.e. 30 min of feeding (4 L), 23 h of reaction, 2 min of sedimentation and 28
126 min of decantation (4 L).

127 *** total operational cycle of 8 h, including 4 phases, i.e. 30 min of feeding (4 L), 7 h of reaction, 3 min of sedimentation and 27 min
128 of decantation (4 L)



129

130 **2.3. Process performance indicators.** The efficiency of nitrogen removal and N₂O
 131 emission were used as indicators to compare process performance of the analyzed
 132 scenarios. With respect to the first indicator, the AOB and NOB activities were
 133 measured by the specific rates of ammonium utilization (AUR, mg N/(g VSS·h)) and
 134 nitrate production (NPR, mg N/(g VSS·h)), respectively. The NPR/AUR ratio was
 135 assumed to represent the normalized NOB activity in comparison with the AOB
 136 activity. However, it should be emphasized that both rates could in practice be
 137 influenced by the activities of other microorganisms, i.e. heterotrophs (NPR) and
 138 anammox bacteria (NPR and AUR).

139 With respect to the second indicator, the N₂O emission factor (E_{N_2O} (%)) may be
 140 normalized with either amount of treated wastewater, PE year, N load removed or
 141 influent N load. In this study, the latter approach was assumed and E_{N_2O} was
 142 calculated by dividing the mass of N₂O emitted (M_{N_2O} (mg N/(L·d))) by the NLR (mg
 143 N/(L·d)) (Eq. 1):

$$144 \quad E_{N_2O} = \frac{M_{N_2O}}{NLR} \cdot 100\% \quad (1)$$

145 The mass M_{N_2O} (mg N/(L·d)) was calculated by integrating the N₂O stripping rates
 146 (r_{N_2O} (mg N/(L·d))) over the unit time (dt (d)) and dividing by the overall reaction time
 147 (t (d)) (Eq. 2):

$$148 \quad M_{N_2O} = \frac{\int r_{N_2O} \cdot dt}{t} \quad (2)$$

149 The rate r_{N_2O} (mg N/(L·d)) consists of two components related to aerated and non-
150 aerated conditions. The details of r_{N_2O} calculations can be found elsewhere ²³.

151 **2.4. Microbial examination.** *2.4.1. DNA extraction.* The microbial community
152 structure in the studied SBR was examined during each modelling phase, including
153 the calibration phase (CAL), recalibration phase (REC) after 150 days of cultivation,
154 and during the validation phase (VAL) after 300 days of cultivation. The samples were
155 withdrawn from the reactor and transferred into a 15 ml DNase-free tube. The
156 supernatant was removed and the tube was filled with biomass twice to thicken the
157 sludge concentration. The samples were stored at -25°C prior to DNA extraction. For the
158 DNA extraction, the sludge sample was initially centrifuged at 10000 g for 10 min and
159 washed with phosphate buffer. The centrifugation process was repeated and 200 mg
160 of the pellet of the sample was collected for the DNA extraction reaction, which was
161 carried out using the FastDNA™ SPIN KIT (MP Biomedicals, USA) following the
162 manufacturer's manual. DNA acquired from purification was used for the Illumina Next
163 Generation Sequencing protocol.

164 *2.4.2. High-throughput DNA sequencing.* High-throughput Illumina sequencing
165 targeting the V3-V4 region of the 16S rRNA gene was performed with S-d-Bact-0341-
166 b-S-17 and S-d-Bact-0785-a-A-21 primers ²⁴ and NEBNext®High-Fidelity 2X PCR
167 Master Mix (Bio Labs inc., USA) following the manufacturer's manual. The detailed
168 Illumina Next Generation Sequencing protocol can be found in the SI (section S.3).
169 Taxonomic differences between the metagenomes were analyzed using Statistical

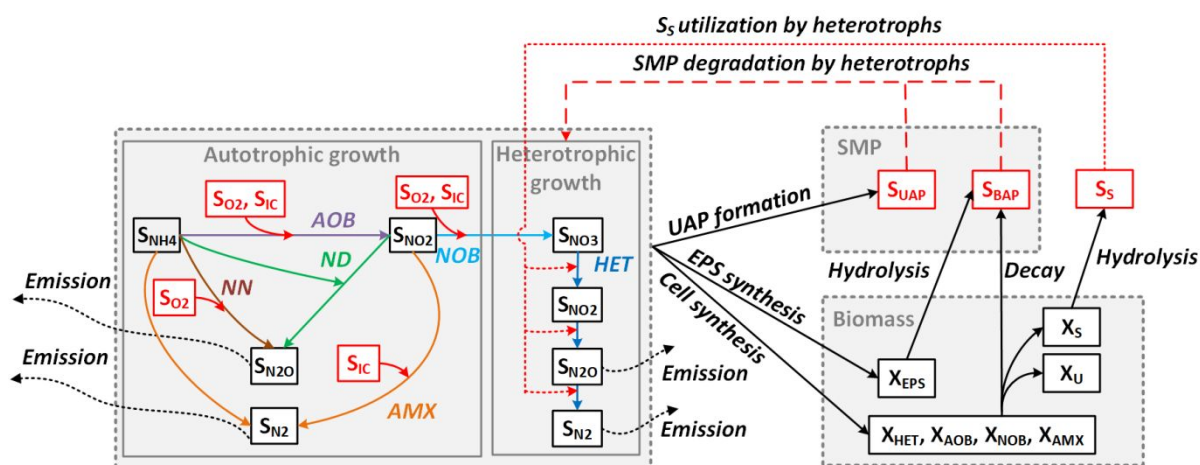
170 Analysis of Metagenomic Profiles (STAMP v. 2.1.3)²⁵ and visualized as heatmaps. A
171 genetic distance between the samples was tested by the ANOVA test, followed by the
172 post-hoc Tukey-Kramer test at 0.95 significance, and visualized as a dendrogram built
173 up with the unweighted pair group method with the arithmetic mean algorithm
174 (UPGMA). The data sets were uploaded to the MetaGenome Rapid Annotation
175 Subsystems Technology (MG-RAST) to enable public access to the files under the
176 accession numbers: mgm4784320.3 (CAL), mgm4784321.3 (REC) and
177 mgm4898872.3 (VAL).

178 **2.5. Modelling approach.** *2.5.1. Model development.* A conceptual model of the
179 biochemical transformations in the studied SBR is shown in Figure 1. The N₂O
180 production/consumption mechanisms (along with the gas-liquid transfer) were
181 adopted from Zaborowska et al.²³, including NH₂OH oxidation (NN) and autotrophic
182 denitrification (ND) mediated by AOB and three-step heterotrophic denitrification (HD)
183 with NO₂⁻-N and N₂O as intermediates. For modeling, the only source of electron
184 donors for autotrophic denitrification was NH₄⁺-N²⁶. Due to the high abundance of
185 heterotrophic biomass (42-68%) present in the studied system fed only with inorganic
186 substrates, the heterotrophic growth was also considered in the model. It has earlier
187 been reported that extracellular polymeric substances (EPS) and soluble microbial
188 products (SMP) could play a vital role in heterotrophic growth in anammox biofilm
189 systems^{27,28}. However, only one study investigated the effect of heterotrophic growth
190 on autotrophic nitrogen removal in granular systems²⁹, without considering the new

191 kinetics of microbial products formation from anammox. In the present study, the
 192 model incorporated heterotrophic growth on (i) readily biodegradable substrate (S_S)
 193 produced from the biomass decay, and (ii) SMPs, specifically biomass associated
 194 products (S_{BAP}) and utilization associated products (S_{UAP}). With respect to formation
 195 of SMPs, the following assumptions were made:

- 196 - S_{UAP} was formed during growth of autotrophs (AOB, NOB)²⁷, and autotrophs
 197 (anammox) and heterotrophs on S_S ²⁸.
- 198 - S_{BAP} was produced from X_{EPS} hydrolysis and biomass decay²⁸.

199 The extended model was implemented in GPS-X 8.0 simulation platform
 200 (Hydromantis, Canada), using a special utility called "Model Developer", based on the
 201 standard notation (Petersen-Gujer matrix) of ASM1³⁰. Further details regarding the
 202 model development can be found in the SI (Section S.4), including the definition of the
 203 state variables, stoichiometric matrix, process rate equations, and kinetic and
 204 stoichiometric parameters (Tables S1-S4).



206

207 **Figure 1.** The conceptual model of biochemical transformations in the studied SBR
208 (fed only with inorganic substrates) (“S_{NO2}”, “S_{N2O}” and “S_{N2}” for autotrophs and
209 heterotrophs could simultaneously be used by both groups of microorganisms).

210 *2.5.2. Model layout and initial biomass composition.* The GPS-X model layout
211 consisted of an SBR with a wastewater influent object. Because the interest of the
212 study was focused on the liquid phase concentrations, the granular sludge was
213 modelled using the “apparent” kinetics approach^{20,21}. The aeration patterns were
214 controlled by setting the DO set point on/off periods in “Timer Controller” and
215 manipulated by adjusting the PI control module (controller sampling time = 20 s;
216 proportional gain = 500; integral time = 0.0001 d). The DO controller manipulated the
217 field oxygen mass transfer coefficient (K_La) directly to match the desired DO set point
218 and calculated the airflow rate. Typical examples of the predicted and measured DO
219 concentrations are shown in Figure S1. The N₂O emission during the aerated phase
220 (air-stripping) was influenced by the K_La coefficient and estimated airflow rate²³. The
221 initial active biomass composition in each modelling phase (CAL, REC and VAL) was
222 assumed based on the results of the metagenomics analysis (Table S5). The share of
223 the remaining bacteria, either unclassified or other, were 31.1% (CAL), 45.7% (REC),
224 41.1% (VAL) (Table S5). These bacteria, not supposed to be responsible for the
225 considered biochemical reactions, were incorporated in the model as the slowly
226 biodegradable substrate (X_S , 92% of the remaining biomass) and unbiodegradable
227 particulates from cell decay (X_U , 8% of the remaining biomass). That assumption was

228 made based on the typical decay stoichiometry of biomass in the ASM1³⁰. The SMP
229 mechanisms were investigated assuming the initial SMP (S_{UAP} , S_{BAP}) and EPS (X_{EPS})
230 concentrations to be 0 mg COD/L and 0%, respectively.

231 *2.5.3. Model calibration and validation.* Figure S2 shows the four-step protocol of
232 model calibration and validation with the data from test trials no. 1-3, including
233 preliminary simulation, model calibration, recalibration, and validation. The detailed
234 description of these steps can be found in the SI (Section S.5). The conversion rates
235 of COD and N compounds were calculated in the model and visualized in the Sankey
236 graphs. A more advanced uncertainty analysis of N₂O production in the studied
237 system, based on the combination of global sensitivity analysis and Generalized
238 Likelihood Uncertainty Estimation methodology, can be found elsewhere³¹.

239 *2.5.4. Model-based optimization of intermittent aeration strategies.* In order to
240 simultaneously maximize the nitrogen removal performance and minimize N₂O
241 emissions in the deammonification SBR, different intermittent aeration strategies were
242 analyzed with the validated model at a high biomass concentration (MLVSS \approx 4000
243 mg/L) (test trial no. 3). The three operating parameters of interest, including the DO
244 set point in the aerated phase, F frequency, and R ratio were manipulated within the
245 following ranges: DO = 0.3-2.1 mg O₂/L (interval of 0.2 mg O₂/L), F = 0.5-10 h⁻¹
246 (interval of 0.5 h⁻¹), and R = 0.5-5.0 (interval of 0.5). Altogether, 2000 automatic
247 simulation runs were carried out by executing Python 3.7 scripts within the GPS-X
248 interface. The 2D-response contour plots of the three operational parameters on the

249 AOB activity, NOB activity and N₂O emissions were generated in MATLAB R2019b
250 (The MathWorks, Inc., USA). The feasible parameter ranges were scattered in the red
251 region, including all the red scatters when the AUR > 12 mg N/(g VSS·h), NPR/AUR <
252 0.1 and E_{N₂O} < 2%.

253 *2.5.5. Model limitations. Limitations to the applications.* The model structure is
254 applicable to the systems treating real wastewater (reject water) without any further
255 modifications or extensions, but model calibration/validation would be required for
256 each specific case. As a consequence, the regions of the optimal aeration settings
257 may also change.

258 *Limitations to the optimization of operational parameters.* The aeration strategy was
259 analyzed under specific operational conditions in terms of three manipulated variables,
260 including the DO set point, F frequency and R ratio. However, the region of the optimal
261 aeration settings may change while considering other operational parameters, such
262 as the biomass concentration, NH₄⁺-N loading rate and temperature.

263 *Limitations to the apparent kinetics.* The “apparent kinetics” approach was used as
264 a model concept for granular sludge in the studied SBR. However, due to the specific
265 characteristics of the granules (e.g. size distribution, density, porosity, etc.), the results
266 of the present study may not be directly applicable to other systems. For example, in
267 a continuously aerated system, the process rate of anammox bacteria would be
268 strongly suppressed, which requires different kinetic parameters or higher
269 concentrations of anammox bacteria compared to a biofilm model.

270 *Limitations to describe alkalinity and pH.* As the pH was controlled in the studied
271 reactor, alkalinity was not considered in the developed model. The model can be
272 extended with alkalinity as a state variable when low pH conditions may occur. The
273 details of considering alkalinity in biokinetic models were described by Henze et al. ³⁰.

274 *Limitations to describe the lag phase for NOB.* The effect of the lag phase for NOB
275 to respond the transition from anoxic to aerobic conditions was only reflected in lower
276 DO affinities of NOB in comparison with AOB³².

277 *Limitations to describe autotrophic denitrification by AOB.* Since NH_2OH oxidation
278 was not specifically considered in the model, it was assumed that the overall $\text{NH}_4^+\text{-N}$
279 oxidation to $\text{NO}_2^-\text{-N}$ would transfer electrons to the internally stored AOB electron pool.
280 Those electrons could subsequently be used for AOB-mediated reduction of $\text{NO}_2^-\text{-N}$
281 to $\text{N}_2\text{O-N}$. Therefore, a prerequisite for that process would be a preceding aerobic
282 phase or continuous low-DO conditions ³³. Under prolonged anoxic conditions,
283 autotrophic denitrification by AOB could be stopped due to exhausting electrons from
284 the pool. That phenomenon could not be predicted as the transfer of electrons was not
285 considered in the model.

286 *Limitations to the calibration protocol.* Due to the limitation of the simulation platform
287 (GPS-X), the selected scenarios could not be calibrated simultaneously with the same
288 parameter set (i.e. the objective function taking both scenarios into account
289 simultaneously). Alternatively, the use of the mean value of the estimated parameters
290 was applied only when both scenarios were carefully calibrated with similar parameter

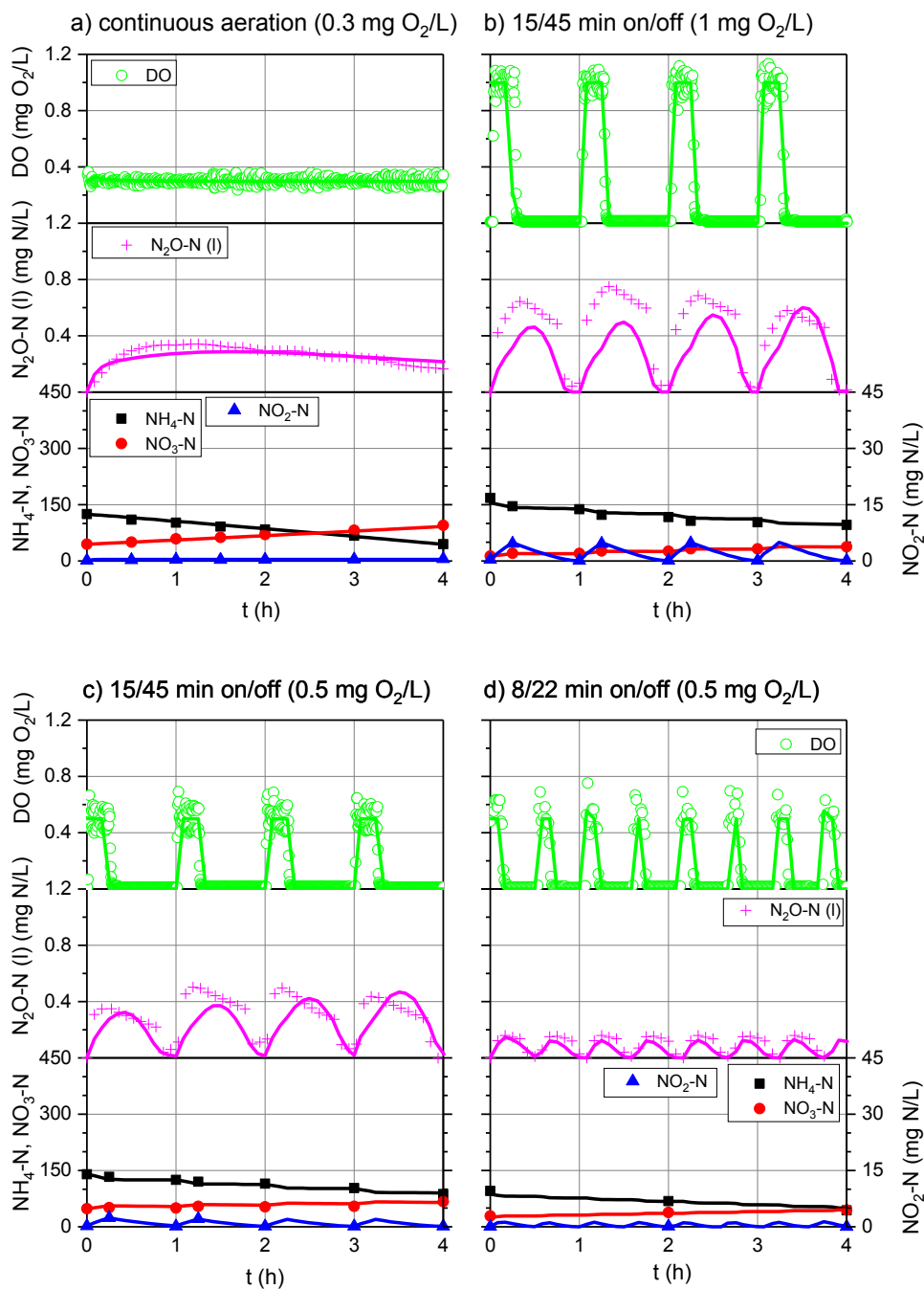
291 values. It was implicitly assumed that the model response to the parameters was
292 linear, after taking into consideration the different biomass compositions and
293 concentrations at each stage (CAL, REC and VAL). However, the selected approach
294 sacrificed the model accuracy for a faster calibration.

295

296 3. RESULTS AND DISCUSSION

297 **3.1. Nitrogen removal performance and N₂O production under different aeration**
298 **conditions.** The process performance indicators of the studied SBR are summarized
299 in Table S6, whereas the dynamic behavior of DO and inorganic nitrogen compounds
300 (N₂O-N, NH₄⁺-N, NO₃⁻-N, NO₂⁻-N) in the three trials are shown in Figures 2-4.

301 In test trial no. 1 with the moderate NH₄⁺-N load and low biomass concentration,
302 continuous aeration at the low DO set point (0.3 mg O₂/L) (scenario 1) apparently
303 favored the activity of AOB, which was reflected by the high AUR, but also insufficiently
304 suppressed NOB (NPR/AUR = 0.66). The switch to the intermittent aeration mode
305 (scenarios 2-3) explicitly improved the efficiency of nitrogen removal, primarily due to
306 suppressing NOB (NPR/AUR = 0.25-0.33), but considerably increased the liquid N₂O
307 concentrations. Further increasing the F frequency (scenario 4) indicated better
308 suppression of NOB activity and N₂O production while maintaining the AOB activity.



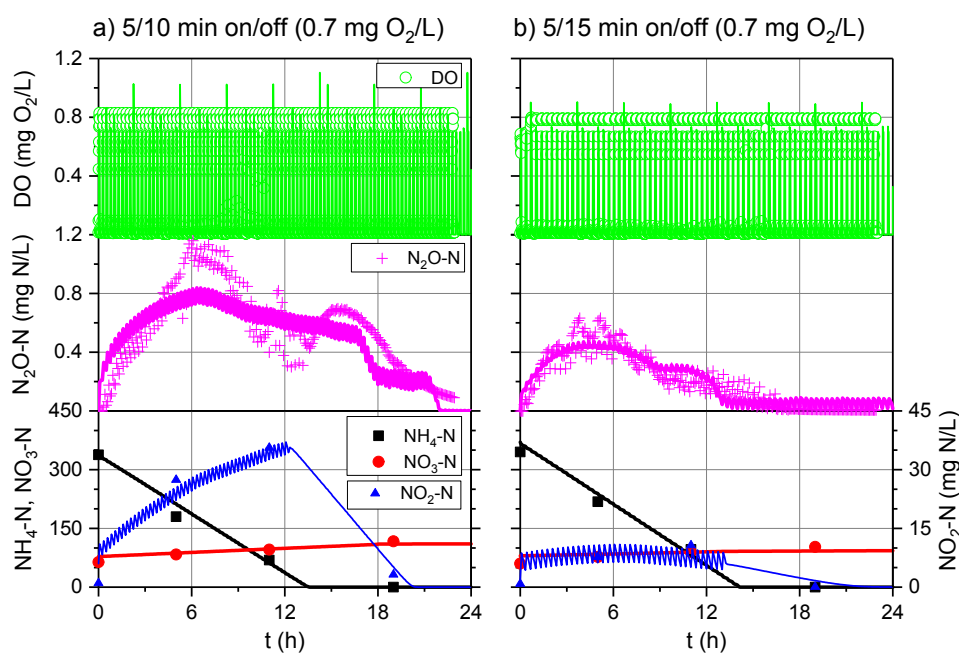
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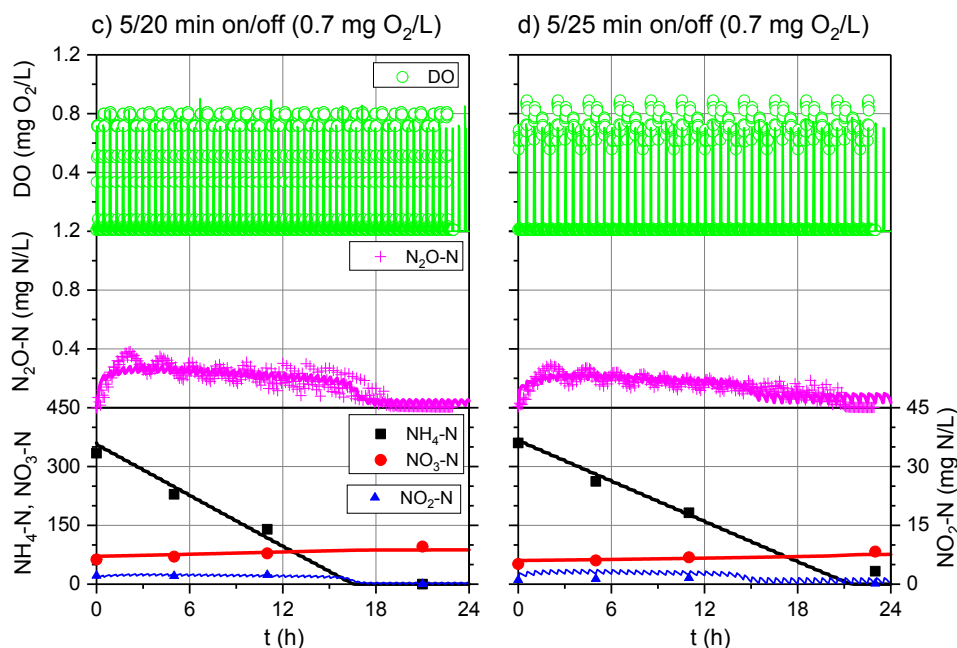
311 **Figure 2.** Measured data (scatters) vs. model predictions (solid lines) of DO and
 312 inorganic nitrogen compounds (N₂O-N, NH₄⁺-N, NO₃⁻-N, NO₂⁻-N) in test trial no. 1: (a)
 313 continuous aeration at DO = 0.3 mg O₂/L (SCE1), (b) intermittent aeration 15/45 min
 314 on/off at DO = 1.0 mg O₂/L (when on) (SCE2), (c) intermittent aeration 15/45 min on/off

315 at DO = 0.5 mg O₂/L (when on) (SCE3), (d) intermittent aeration 8/22 min on/off at DO
 316 = 0.5 mg O₂/L (when on) (SCE4).

317 Test trial no. 2 was carried out after 150 d of cultivation, at a higher biomass
 318 concentration (MLVSS ≈ 2000 mg/L) and constant DO set point of 0.7 mg O₂/L (Figure
 319 3). The increasing R ratios from 2 to 4 (scenarios 5-7) resulted in the improved NOB
 320 suppression (NPR/AUR = 0.16-0.09) without compromising the AOB activity (AUR =
 321 7.4-9.0 mg N/(g VSS·h)). Furthermore, the liquid N₂O concentrations and N₂O
 322 emission factors decreased due to enhanced anammox activities in the anoxic periods
 323 and reduced NO₂⁻-N accumulation (from 35 to 5 mg N/L) during the whole period. The
 324 extension of the non-aerated phase up to 25 min at R = 5 (scenario 8) allowed to
 325 mitigate the N₂O production, while maintaining the favorable NPR/AUR ratio (= 0.09).
 326 However, the overall process performance deteriorated due to the decreased AUR.



327



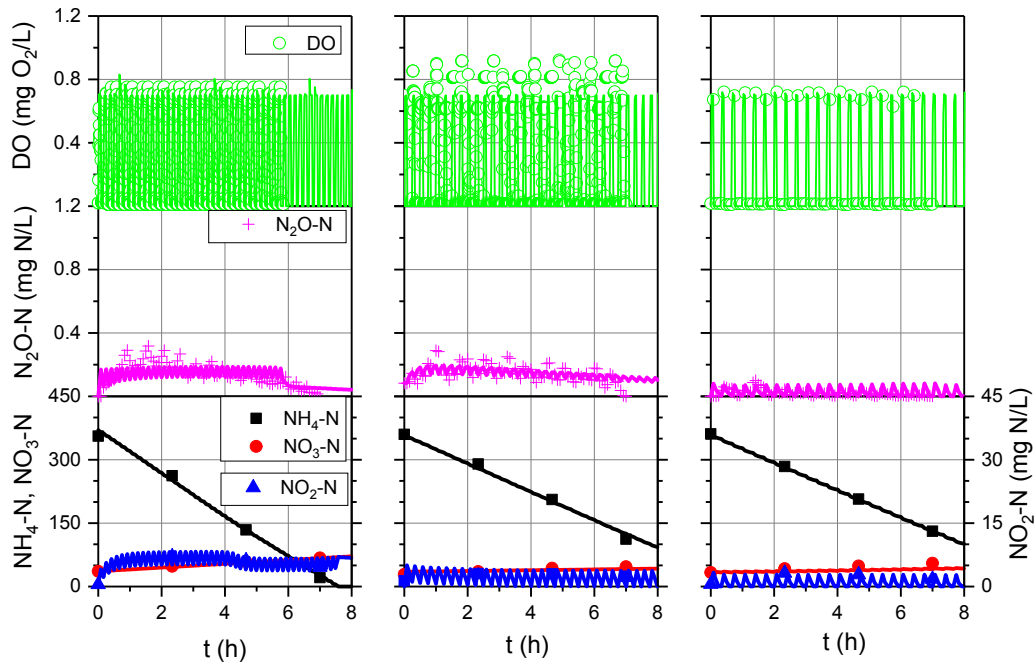
328

329 **Figure 3.** Measured data (scatters) vs. model predictions (solid lines) of DO and
 330 inorganic nitrogen compounds ($\text{N}_2\text{O-N}$, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$) in test trial no. 2: (a)
 331 intermittent aeration 5/10 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE5), (b)
 332 intermittent aeration 5/15 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE6), (c)
 333 intermittent aeration 5/20 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE7), (d)
 334 intermittent aeration 5/25 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE8).

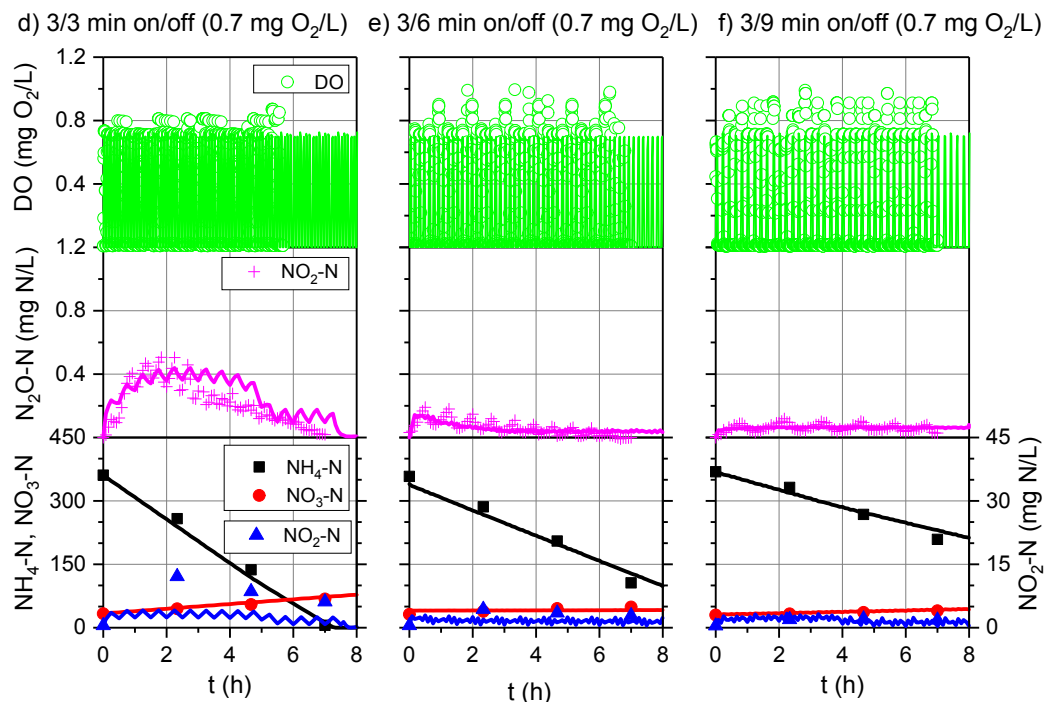
335 The final test trial no. 3 was carried out after over 300 days of cultivation, when the
 336 MLVSS reached approximately 4000 mg/L (Figure 4). The DO set point was kept
 337 constant at $0.7 \text{ mg O}_2/\text{L}$ and that trial focused on investigating the effect of aeration
 338 on/off frequencies at the R ratios ranging from 1 to 3. In all the scenarios, the
 339 NPR/AUR ratios were maintained in a very low and narrow range (0.07-0.09). The
 340 increased AURs ($13.0\text{-}18.1 \text{ mg N}/(\text{g VSS}\cdot\text{h})$) were obtained when $R \leq 2$ for both
 341 applied aerated phase durations (3 or 5 min). The higher aeration on/off frequency

342 (aerated phase of 3 min) slightly increased the AOB activity without compromising the
 343 NOB suppression. In general, the extended non-aerated phases resulted in the
 344 decreased liquid N_2O concentrations and emission factors. However, further
 345 increasing the R ratio (up to 3) negatively affected the AOB activity ($\text{AUR} = 8.9\text{--}9.0 \text{ mg}$
 346 $\text{N}/(\text{g VSS}\cdot\text{h})$) (scenarios 11 and 14).

a) 5/5 min on/off ($0.7 \text{ mg O}_2/\text{L}$) b) 5/10 min on/off ($0.7 \text{ mg O}_2/\text{L}$) c) 5/15 min on/off ($0.7 \text{ mg O}_2/\text{L}$)



347



348

349 **Figure 4.** Measured data (scatters) vs. model predictions (solid lines) of DO and
 350 inorganic nitrogen compounds ($\text{N}_2\text{O-N}$, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$) in test trial no. 3: (a)
 351 intermittent aeration 5/5 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE9), (b)
 352 intermittent aeration 5/10 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE10), (c)
 353 intermittent aeration 5/15 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE11), (d)
 354 intermittent aeration 3/3 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE12), (e)
 355 intermittent aeration 3/6 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE13), (f)
 356 intermittent aeration 3/9 min on/off at $\text{DO} = 0.7 \text{ mg O}_2/\text{L}$ (when on) (SCE14).

357 The intermittent aeration pattern (8/22 min on/off at DO set point of $0.5 \text{ mg O}_2/\text{L}$ in
 358 scenario 4), selected for the first stable operational period (5-154 d), was in
 359 accordance with the literature findings for deammonification systems^{9,22,34}. In the
 360 stable operation period (158-308 d), the intermittent aeration pattern (5/20 min on/off

361 at DO = 0.7 mg O₂/L) was selected to avoid insufficient NOB inhibition or decline the
362 AOB activity. The intermittent aeration pattern (3/6 min on/off at DO set point of 0.7
363 mg O₂/L in scenario 13) was ultimately selected to achieve the stable operation of the
364 system in terms of NOB suppression and N₂O mitigation (Table S6). Experimental
365 evidence of the efficient strategy was provided for a three-cycle simulation after
366 reaching the stable phase-dynamic conditions in 20 days in order to evaluate the long-
367 term efficiency of the selected strategy (Figure S3). The liquid N₂O concentrations
368 were stable around 0.14 mg N/L while the NOB activity was effectively suppressed
369 (NPR/AUR = 0.09).

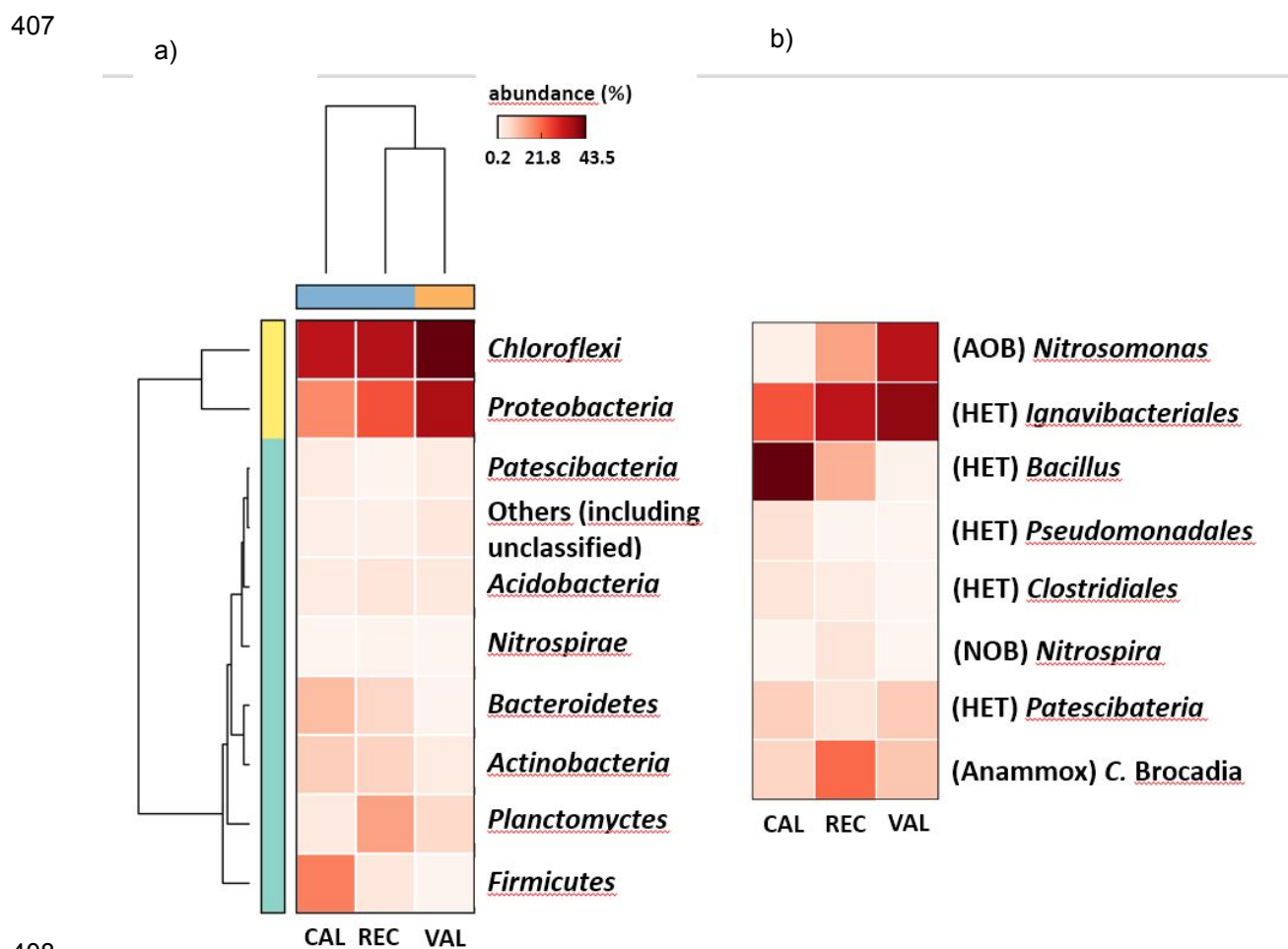
370 Literature data suggested that the anoxic phase duration between 5 and 20 min
371 could effectively delay the *Nitrospira*-NOB activity after the anoxic phase, regardless
372 of the aerated phase duration ^{1,9}. However, other researches proposed that the
373 minimum non-aerated phase duration of 15-20 min along with an aerated phase
374 duration no longer than 5-15 min would be essential to outcompete NOB ^{19,32}.
375 Furthermore, a higher aeration on/off frequency at low DO set points could efficiently
376 suppress the NOB activity, while maintaining high overall nitrogen removal
377 performance (70-80%) ^{8,9,22}. Although previous studies showed that low DO set points
378 favored NOB suppression due to a higher oxygen affinity of AOB compared to NOB,
379 recent studies suggested that the tight control of oxygen supply (durations and
380 proportions of on/off periods) rather than the DO set point may be critical for NOB
381 suppression. This can be attributed to a time lag of the NOB activity in adaption to

382 aerobic conditions and anammox bacteria outcompeting NOB for NO_2^- -N in anoxic
383 periods ^{7,35}. In the present study, it was found that an appropriate R ratio was more
384 influential than strict durations of the aeration on/off periods. For the acclimated
385 biomass (test trial no. 3), the optimal conditions were observed for $R \leq 2$, while
386 increasing the R ratio to 3 resulted in declining the AOB activity.

387 Integration of the N_2O production profiles during the aerated and non-aerated
388 phases revealed that approximately 50% of N_2O was produced during the aerated
389 phases, which was lower than the value (80%) reported by Blum et al. ¹¹. In
390 comparison with the continuous aeration, when almost all N_2O production was emitted
391 to the gas phase, approximately half of the produced N_2O was subsequently emitted
392 to the gas phase under the intermittent aeration conditions. The estimated N_2O
393 emission factors (Table S6) were within the range of literature data for
394 deammonification systems. e.g. 0.4-4.6% in terms of TN removed ^{11,15,17,36,37}. The vast
395 majority (approximately 98%) of N_2O was emitted during the aerated phases, which
396 was also close to the value (96%) reported by Blum et al. ¹¹. Potential contributions of
397 the specific biochemical pathways, including NH_2OH oxidation, autotrophic
398 denitrification and heterotrophic denitrification, are discussed in Section 3.4.

399 **3.2. Identification of the microbial community structure for setting the biomass**
400 **composition in the model.** The abundance and taxonomical affiliations of the particular
401 physiological bacterial groups in the biomass samples, collected during the calibration
402 (CAL), recalibration (REC) and validation (VAL) phases, are summarized in Table S5.

403 The dominant groups gradually shifted from heterotrophs to autotrophs (Figure 5)
 404 along with a decreasing microbial biodiversity (Figure S4). Among 67 bacterial species
 405 detected in the CAL sample, 44 were detected after 150 days of the cultivation period
 406 (REC), and only 18 at the end of the experiment (VAL).



408
 409 **Figure 5.** Heatmap of the differences between abundance of the bacterial phyla with
 410 the genetic distance between the samples (upper dendrogram) and between taxa (left
 411 dendrogram) (a) and representatives of the specific physiological bacteria groups (b)
 412 in the CAL, REC and VAL samples (The term “Others” refers to the sum of all
 413 classifications with less than 0.7% abundance and unclassified reads).

414 While microorganisms with the predominated heterotrophic metabolism decreased
415 their abundance from 68.0% to 47.4%, the abundance of autotrophic bacterial groups
416 explicitly tended to increase. The AOB were almost entirely represented by members
417 of the genus *Nitrosomonas*, which constituted 0.7% of the overall bacterial community
418 in the CAL sample. During the first 150 days of the cultivation period, their population
419 increased to 2.7% (REC) and continued to grow up to 9.0% in the final stage of the
420 experiment (VAL). This trend indicated a key role of r-strategist *Nitrosomonas* in
421 ammonium oxidation in the studied SBR.

422 NOB were exclusively represented by the genus *Nitrospira* from the phylum
423 *Nitrospirae*, which accounted for 0.2%, 0.7% and 0.1% in the CAL, REC and VAL
424 samples, respectively. In terms of r/K theory with respect to nitrifiers, the selected
425 dominant abundance of K-strategist *Nitrospira* could be attributed to two operational
426 factors, including low DO and NO₂⁻-N concentrations as well as diffusion limitation
427 within granules³⁸. In comparison with the known NOB, comammox bacteria (complete
428 ammonia oxidizers performing complete oxidation of ammonia to nitrate) were
429 discovered from the genus *Nitrospira*. These bacteria could be favored at low
430 temperature, low nitrogen substrate, and high DO conditions³⁹. In the studied SBR,
431 both AOB and NOB abundance increased during the cultivation period. Therefore, it
432 is difficult to unambiguously determine if some *Nitrospira*-NOB indeed performed the
433 metabolic comammox pathway, even though the potential was already reported in an
434 anammox-enriched SBR⁴⁰.

435 The phylum *Planctomycetes*, which includes anammox bacteria, constituted 3.0%
436 of the initial microbial population. However, only approximately 0.04% of DNA
437 sequences from the total number of reads were assigned to the typical anammox
438 bacteria from the genera *Candidatus Brocadia*. After first 150 days of the cultivation
439 period, the bacteria belonging to the phylum *Planctomycetes* comprised 14.0%.
440 *Candidatus Brocadia* related microorganisms reflected the dominant share of 8.7% in
441 the REC sample, however, their share decreased to 2.4% at the end of the experiment
442 (VAL sample).

443 **3.3. Quantitative assessment of model calibration and validation.** *3.3.1 Setting the*
444 *initial model inputs for simulations.* The initial biomass composition (X_{HET} , X_{AOB} , X_{NOB} ,
445 X_{AMX} , X_{S} , X_{U} , X_{EPS}) in the CAL, REC, and VAL phases was as follows: (i) 68%, 0.7%,
446 0.2%, 0.04%, 28.6%, 2.5%, 0% (ii) 42%, 2.7%, 0.7%, 8.7%, 42%, 3.7%, 0% and (iii)
447 47%, 9%, 0.1%, 2.4%, 38%, 3%, 0%, respectively. The microbial composition and
448 biomass concentrations were used as microbial input to reduce the model uncertainty.
449 The model kinetic parameter values, including the maximum specific growth rates and
450 key saturation/inhibition coefficients, are listed in Table S4. The default values were
451 adopted from literature and assumed based on the nitrogen conversion rates,
452 microbial input, r/K strategist explanation and diffusional explanation.

453 *3.3.2. Model calibration.* In test trial no. 1, scenario 2 was first calibrated as a
454 representative for the intermittent aeration pattern. 7 parameters were found
455 very/extremely influential ($S_{ij} \geq 1$) to NO_2^- -N and N_2O -N, i.e. $\eta_{g\text{HET}2}$, $\eta_{g\text{HET}3}$, $K_{\text{NO}2,\text{AOB}}$,

456 $K_{O,AOB}$, $K_{O,NOB}$, K_h and η_h (Table S7). Parameter estimation showed a good
457 agreement between the observations and model predictions ($|r|_{N_2O} > 0.8$) (Table S8).
458 However, based on the correlation matrix (Figure S5a), three of them were found
459 highly cross-correlated with more than one parameter and thus they were excluded
460 from estimation. After the iterative parameter estimation (Tables S9-S10) along with
461 correlation analysis (Figure S5b-c), altogether 4 correlation crossing parameters (
462 η_{gHET3} , $K_{NO_2,AOB}$, K_h and η_h) were excluded from the subset. The objective of the
463 iterative parameter estimation was to obtain the minimum number of identifiable
464 parameters (η_{gHET2} , $K_{O,AOB}$, $K_{O,NOB}$) that explain the data in scenario 2.

465 *3.3.3. Model recalibration.* Model recalibration was performed in scenario 6 to
466 achieve one unique parameter set for test trials no. 1-2. Based on the sensitivity
467 analysis for the prior parameter subset (Table S11), 2 previously excluded parameters
468 (η_{gHET3} and K_h) were added due to their very/extremely high influence ($S_{i,j} \geq 1$) to
469 NO_2^- -N and N_2O -N in scenario 6. The expansion of the subset (η_{gHET2} , η_{gHET3} , $K_{O,AOB}$,
470 $K_{O,NOB}$, K_h) increased the goodness-of-fit for both scenario 2 ($|r|_{N_2O} > 0.8$) and scenario
471 6 ($|r|_{N_2O} > 0.5$), and no highly-correlated parameter pairs were found in the latter
472 scenario (Figure S5d). Finally, the mean value from both scenarios was determined
473 as the final value of the adjusted parameters (Table S12).

474 *3.3.4. Model validation.* Model validation was performed with the adjusted
475 parameters in the remaining scenarios in test trial no. 3. Table S13 shows evaluation
476 of the model accuracy using Pearson's $|r|$, root of mean squared errors (RMSE) and

477 Nash-Sutcliff coefficient (NSE) in all the scenarios. Pearson's $|r|_{N_2O}$ varied in the range
478 0.69-0.94 (test trial no. 1), 0.34-0.73 (test trial no. 2) and 0.19-0.79 (test trial no. 3).
479 Apart from 4 unsatisfactory goodness-of-fit results (scenarios 8, 10, 11, 14, $|r|_{N_2O} <$
480 0.5), the N_2O predictions accurately fitted the observations in the remaining scenarios.
481 Generally, NH_4^+-N , $NO_3^- -N$, $NO_2^- -N$ were predicted better than N_2O . The RMSE and
482 NSE varied in the range 0.02-0.38 and -2.2-0.83 (except for the unsatisfactory
483 goodness-of-fit performance of scenario 11), respectively. The higher R ratio allowed
484 to decrease the liquid N_2O concentrations and the prediction accuracy decreased
485 when the liquid N_2O concentrations were maintained at a low level, thus causing the
486 unsatisfactory goodness-of-fit results. A comparison of RMSE and NSE with Pearson's
487 $|r|$ revealed that they poorly correlated, implying the necessity of more than one
488 criterion to improve the prediction accuracy. A global measure of model performance
489 and model uncertainty is discussed elsewhere ³¹.

490 The adjusted parameters were η_{gHET2} , η_{gHET3} , $K_{O,AOB}$, $K_{O,NOB}$ and K_h (Table
491 S12). The heterotrophic reduction factors for $NO_2^- -N$ and N_2O ($\eta_{gHET2} = 0.17$, η_{gHET3}
492 = 0.40) and the hydrolysis rate ($K_h = 2.6 \text{ d}^{-1}$) were within the literature range (η_{gHET2}
493 = 0.016-0.3, $\eta_{gHET3} = 0.075-0.81$, $K_h = 1.5-4.5 \text{ d}^{-1}$) ^{41,42}. The AOB oxygen affinity
494 constant ($K_{O,AOB} = 0.10 \text{ mg O}_2/\text{L}$) was lower than that of NOB ($K_{O,NOB} = 0.52 \text{ mg O}_2/\text{L}$)
495 and both values were typical for either r-strategist *Nitrosomonas*-AOB ($K_{O,AOB} = 0.03-$
496 $1.22 \text{ mg O}_2/\text{L}$) or K-strategist *Nitrospira*-NOB ($K_{O,NOB} = 0.5-0.6 \text{ mg O}_2/\text{L}$) ⁴³. The
497 relatively high oxygen affinity for K-strategist *Nitrospira* in comparison with r-strategist

498 *Nitrobacter* ($K_{O_2,NOB} = 0.43\text{-}1.1 \text{ mg O}_2/\text{L}$)¹ could also explain the difficulty of NOB
499 suppression under low DO conditions.

500 **3.4. Identification of the N₂O production pathways, NOB suppression mechanisms**
501 **and role of EPS.** The model predictions, shown in Figure 6a, suggest that the readily
502 biodegradable substrate (S_S), hydrolysed from the main biomass decay product (X_S),
503 was the main organic carbon source for heterotrophs. Both SMP components (UAP
504 and BAP) could hardly be utilized by heterotrophs due to the low generation rates of
505 SMP from biomass growth and low affinities of heterotrophs. The EPS pool tended to
506 accumulate thus decreasing the availability of organic carbon for the growth of
507 heterotrophs. As a consequence, the share of heterotrophic biomass decreased from
508 68% in the CAL phase to 42-47% in the REC/VAL phases. Such a model prediction is
509 consistent with the microbiological results, where representatives of *Anaerolineae*
510 from the *Chloroflexi* phylum increased their share from 25% (CAL) to 34% (VAL) in the
511 total bacterial community. That member of *Chloroflexi* is implicitly the microorganism
512 responsible for EPS production and plays an important role in the structural formation
513 of granules⁴⁴.

514 The finding of the present study is different from results of a theoretical modelling
515 study in an anammox biofilm system²⁸ which was validated based on earlier
516 experimental data⁴⁵. The authors proposed that heterotrophs primarily grew on UAP
517 produced mainly from anammox growth. This difference, compared to the present
518 study, explicitly results from the major biomass components, including anammox

519 bacteria (60%) and EPS (20%), whereas the fraction of heterotrophs remained
520 relatively small (10%).

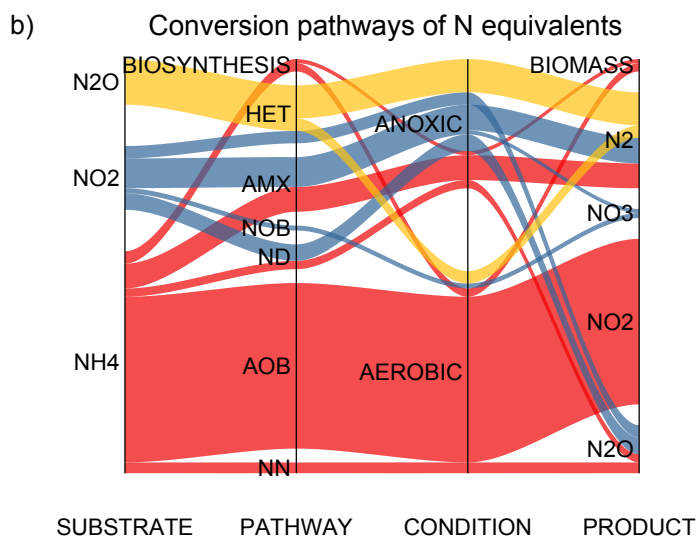
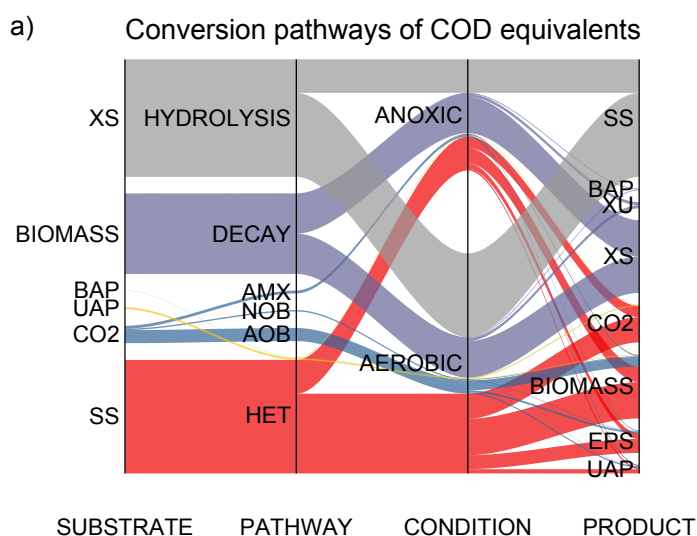
521 The model predictions revealed that NH_2OH oxidation (NN) and autotrophic
522 denitrification (ND) were the dominating pathways of N_2O production in the aerated
523 phase (80-100%) and non-aerated phase (60-90%), respectively. Heterotrophic
524 denitrification served as a sink for N_2O production under both aerated (20-30%) and
525 non-aerated phase (70-80%) (Figure 6b). For comparison, either NH_2OH oxidation or
526 autotrophic denitrification pathway has been reported as the dominant pathway in a
527 deammonification system under oxic conditions ($\text{DO} \geq 0.2 \text{ mg O}_2/\text{L}$) and a wide range
528 of the influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations ¹⁰. Even though the heterotrophic
529 denitrification pathway has been neglected in most deammonification modelling studies,
530 Wan et al. ¹³ showed that heterotrophic denitrification could act as a sink in deeper
531 layers (anoxic condition) of deammonification granules even without organic carbon in
532 the influent feed.

533 The N_2O production rate was dependent on the AUR in the aerated phase and $\text{NO}_2^-\text{-N}$
534 N concentration in the non-aerated phase (Figure S6), which is similar to the findings
535 of Blum et al. ¹¹. Intense aeration could result in higher N_2O production and emission
536 due to both higher AURs and stripping effect, as suggested by Castro-Barros et al. ¹⁵,
537 and weaker suppression of the NOB activity (as shown in scenarios 2-3). A longer
538 non-aerated phase could cause more N_2O consumption by heterotrophic

539 denitrification, but it could also lead to lower AURs and NPR/AURs (as shown in
540 scenarios 5-8).

541 Ma et al.¹⁹ attributed the mechanism of NOB suppression by intermittent aeration to
542 the periodic inhibition by FA (threshold of 0.2 mg N/L) corresponding to periodic pH
543 upshifts during the non-aerated phase. Higher aeration on/off frequencies could also
544 cause more often pH upshifts and enhance NOB suppression. However, in the present
545 study, the pH upshifts in relation to the intermittent aeration pattern were negligible as
546 the pH value was controlled in the range 7.5-7.9 by dosing NaOH. The consumption
547 of NH_4^+ -N in the reaction phase resulted in the overall decrease in the FA
548 concentration in the range 1-9 mg N/L and 0-21 mg N/L in test trial no. 1 and test trials
549 no. 2-3, respectively. The FA inhibition thresholds were reported in the range 0.64-4.3
550 mg N/L for NOB^{46,47}, and specifically 0.04-0.08 mg N/L for *Nitrospira*⁴⁸. Recent
551 studies have also shown contradictory results that FA concentrations in the range
552 18.1–25.0 mg N/L had a limited effect on NOB suppression⁴⁹, implicitly due to the
553 diverse NOB community. It should be noted that the different size of granules could
554 also have a potential effect on diffusion limitations and variations in the NOB activity.
555 However, this effect was not considered in the present study due to a relatively stable
556 granule size. In the present study, a similar FA decreasing trend under different R
557 ratios could not explain the stronger NOB suppression related to the longer non-
558 aerated phase. In addition, FNA inhibition on NOB was not considered because FNA
559 concentrations (< 0.002 mg N/L) were below the threshold values (0.02-0.1 mg N/L)

560 ⁴⁶ during all the experiments. However, Blum et al. ¹² suggested that pH may regulate
 561 the rates between N₂O production and consumption. Indeed, Kandars et al. ⁵⁰ showed
 562 that more N₂O could be reduced via complete denitrification at higher pH (7.5-7.6 vs.
 563 6.6-7.1). However, in the present study, such influence was avoided by controlling a
 564 narrow pH range (7.5-7.9).



567 **Figure 6.** Sankey graph showing the conversion pathways of COD equivalents (a) and
 568 N equivalents (b) at the end of the experimental period (based on the results of

569 scenario 13) (for higher sharpness, contributions smaller than 0.3% (a) and 1% (b)
570 were neglected).

571 **3.5. Model-based intermittent aeration strategies for performance optimization.** The
572 AOB activity, NOB activity and N₂O emissions, evaluated under different aeration
573 strategies when the DO set point, R ratio and F frequency were respectively fixed to
574 0.7 mg O₂/L, 2 and 6.5 h⁻¹, are shown in Figure S7, whereas the remaining scenarios
575 are shown in Figures S7-S9. The aeration strategies scattered in the red region were
576 feasible only when the DO set point, R ratio and F frequency were within the range
577 0.5-2.1 mg O₂/L, 1.5-3.5 and 3-10 h⁻¹, respectively. At the reference state (DO=0.7 mg
578 O₂/L, R=2 and F=6.5 h⁻¹), the predicted AUR, NPR/AUR and N₂O emission factor were
579 approximately 12 mg N/(g VSS·h), 0.05 and 1%, respectively, which was close to the
580 experimental findings (scenario 13) (13 mg N/(g VSS·h), 0.07 and 1% at DO=0.7 mg
581 O₂/L, R=2 and F=6.5 h⁻¹). The AOB activity could be increased to 14 mg N/(g VSS·h)
582 without compromising the NOB suppression (NPR/AUR < 0.1) and N₂O emission
583 (E_{N₂O} < 2%). This increase could result from either decreasing the R ratio to 1.5 (Figure
584 S7a-c), or increasing the DO set point to 1.3 mg O₂/L (Figure S7d-f). The intermittent
585 aeration with either higher DO set point and higher R ratio or lower DO set point and
586 lower R ratio was suggested to maintain AUR > 12 mg N/(g VSS·h), NPR/AUR < 0.1
587 and E_{N₂O} < 2% (Figure S7g-i).

588 As shown in Figures S7-S9, the R ratio and DO set point were crucial parameters
589 for performance optimization, however, with the opposite effects on NOB suppression

590 and N₂O mitigation. The AOB activity could be slightly improved by either decreasing
591 the R ratio or increasing the DO set point. In the meantime, a higher F frequency was
592 suggested to maintain the sufficient NOB suppression and N₂O mitigation (Figures S8-
593 S9). Similar parabolic-shaped optimal range of the R ratio and DO set point were found
594 when the fixed F frequency exceeded 3 h⁻¹ (Figure S10). The R ratio should tightly be
595 controlled when the DO set point was adjusted. Increasing the F frequency could further
596 expand the feasible region for both R ratio and DO set point.

597

598 ASSOCIATED CONTENT

599 **Supporting Information.**

600 The Supporting Information is available free of charge.

601 Experimental setup, model development, calibration and validation procedure,
602 definition of the state variables, stoichiometric matrix, kinetic rate equations, model
603 parameters, sensitivity analysis, correlation matrices and further metagenomics and
604 modelling results (PDF)

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608 **Notes**

609 The authors declare no competing financial interest.

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618 REFERENCES

- 619 (1) Cao, Y.; van Loosdrecht, M. C. M.; Daigger, G. T. Mainstream Partial Nitrification–Anammox in
620 Municipal Wastewater Treatment: Status, Bottlenecks, and Further Studies. *Appl. Microbiol.*
621 *Biotechnol.* **2017**, *101* (4), 1365–1383. <https://doi.org/10.1007/s00253-016-8058-7>.
- 622 (2) Duan, H.; Ye, L.; Lu, X.; Yuan, Z. Overcoming Nitrite Oxidizing Bacteria Adaptation through
623 Alternating Sludge Treatment with Free Nitrous Acid and Free Ammonia. *Environ. Sci. Technol.*
624 **2019**, *53*, 1937–1946. <https://doi.org/10.1021/acs.est.8b06148>.
- 625 (3) Feng, Y.; Lu, X.; Al-Hazmi, H.; Mąkinia, J. An Overview of the Strategies for the
626 Deammonification Process Start-up and Recovery after Accidental Operational Failures. *Rev.*
627 *Environ. Sci. Biotechnol.* **2017**, *16* (3), 541–568. <https://doi.org/10.1007/s11157-017-9441-2>.
- 628 (4) Miao, Y.; Zhang, L.; Li, B.; Zhang, Q.; Wang, S.; Peng, Y. Enhancing Ammonium Oxidizing
629 Bacteria Activity Was Key to Single-Stage Partial Nitrification-Anammox System Treating Low-
630 Strength Sewage under Intermittent Aeration Condition. *Bioresour. Technol.* **2017**, *231*, 36–44.
631 <https://doi.org/10.1016/j.biortech.2017.01.045>.
- 632 (5) Zhang, M.; Wang, S.; Ji, B.; Liu, Y. Towards Mainstream Deammonification of Municipal
633 Wastewater: Partial Nitrification-Anammox versus Partial Denitrification-Anammox. *Sci. Total*
634 *Environ.* **2019**, *692*, 393–401. <https://doi.org/10.1016/j.scitotenv.2019.07.293>.
- 635 (6) Noophan, P. L.; Figueroa, L. A.; Munakata-Marr, J. Nitrite Oxidation Inhibition by Hydroxylamine:
636 Experimental and Model Evaluation. *Water Sci. Technol.* **2004**, *50* (6), 295–304.
637 <https://doi.org/10.2166/wst.2004.0388>.
- 638 (7) Gao, H.; Scherson, Y. D.; Wells, G. F. Towards Energy Neutral Wastewater Treatment:
639 Methodology and State of the Art. *Env. Sci. Process. Impacts* **2014**, *16* (6), 1223–1246.

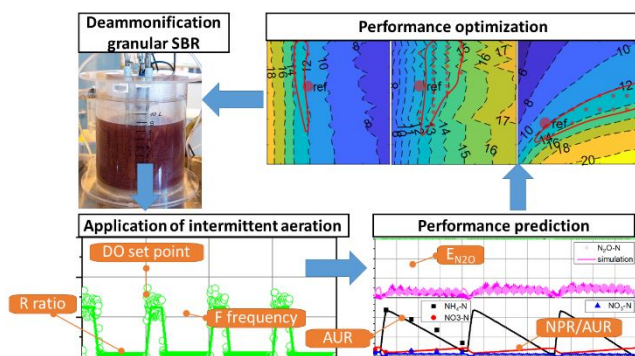
- 640 <https://doi.org/10.1039/C4EM00069B>.
- 641 (8) Han, M.; De Clippeleir, H.; Al-Omari, A.; Wett, B.; Vlaeminck, S. E.; Bott, C.; Murthy, S. Impact
642 of Carbon to Nitrogen Ratio and Aeration Regime on Mainstream Deammonification. *Water Sci.*
643 *Technol.* **2016**, *74* (2), 375–384. <https://doi.org/10.2166/wst.2016.202>.
- 644 (9) Miao, Y.; Zhang, L.; Yang, Y.; Peng, Y.; Li, B.; Wang, S.; Zhang, Q. Start-up of Single-Stage
645 Partial Nitrification-Anammox Process Treating Low-Strength Swage and Its Restoration from
646 Nitrate Accumulation. *Bioresour. Technol.* **2016**, *218*, 771–779.
647 <https://doi.org/10.1016/j.biortech.2016.06.125>.
- 648 (10) Ma, C.; Jensen, M. M.; Smets, B. F.; Thamdrup, B. Pathways and Controls of N₂O Production
649 in Nitritation–Anammox Biomass. *Environ. Sci. Technol.* **2017**, *51* (16), 8981–8991.
650 <https://doi.org/10.1021/acs.est.7b01225>.
- 651 (11) Blum, J.-M.; Jensen, M. M.; Smets, B. F. Nitrous Oxide Production in Intermittently Aerated
652 Partial Nitritation-Anammox Reactor: Oxidic N₂O Production Dominates and Relates with
653 Ammonia Removal Rate. *Chem. Eng. J.* **2018**, *335*, 458–466.
654 <https://doi.org/10.1016/j.cej.2017.10.146>.
- 655 (12) Blum, J.-M.; Su, Q.; Ma, Y.; Valverde-Pérez, B.; Domingo-Félez, C.; Jensen, M. M.; Smets, B.
656 F. The PH Dependency of N-Converting Enzymatic Processes, Pathways and Microbes: Effect
657 on Net N₂O Production: PH Dependency of N-Converting Enzymatic Processes, Pathways and
658 Microbes. *Environ. Microbiol.* **2018**. <https://doi.org/10.1111/1462-2920.14063>.
- 659 (13) Wan, X.; Baeten, J. E.; Volcke, E. I. P. Effect of Operating Conditions on N₂O Emissions from
660 One-Stage Partial Nitritation-Anammox Reactors. *Biochem. Eng. J.* **2019**, *143*, 24–33.
661 <https://doi.org/10.1016/j.bej.2018.12.004>.
- 662 (14) Massara, T. M.; Malamis, S.; Guisasola, A.; Baeza, J. A.; Noutsopoulos, C.; Katsou, E. A Review
663 on Nitrous Oxide (N₂O) Emissions during Biological Nutrient Removal from Municipal
664 Wastewater and Sludge Reject Water. *Sci. Total Environ.* **2017**, *596–597*, 106–123.
665 <https://doi.org/10.1016/j.scitotenv.2017.03.191>.
- 666 (15) Castro-Barros, C. M.; Daelman, M. R. J.; Mampaey, K. E.; van Loosdrecht, M. C. M.; Volcke, E.
667 I. P. Effect of Aeration Regime on N₂O Emission from Partial Nitritation-Anammox in a Full-
668 Scale Granular Sludge Reactor. *Water Res.* **2015**, *68*, 793–803.
669 <https://doi.org/10.1016/j.watres.2014.10.056>.
- 670 (16) Domingo-Félez, C.; Mutlu, A. G.; Jensen, M. M.; Smets, B. F. Aeration Strategies To Mitigate
671 Nitrous Oxide Emissions from Single-Stage Nitritation/Anammox Reactors. *Environ. Sci.*
672 *Technol.* **2014**, *48* (15), 8679–8687. <https://doi.org/10.1021/es501819n>.
- 673 (17) Leix, C.; Drewes, J. E.; Ye, L.; Koch, K. Strategies for Enhanced Deammonification Performance
674 and Reduced Nitrous Oxide Emissions. *Bioresour. Technol.* **2017**, *236*, 174–185.
675 <https://doi.org/10.1016/j.biortech.2017.03.182>.
- 676 (18) Corbalá-Robles, L.; Picioreanu, C.; van Loosdrecht, M. C. M.; Pérez, J. Analysing the Effects of
677 the Aeration Pattern and Residual Ammonium Concentration in a Partial Nitritation-Anammox
678 Process. *Environ. Technol.* **2016**, *37* (6), 694–702.
679 <https://doi.org/10.1080/09593330.2015.1077895>.
- 680 (19) Ma, Y.; Domingo-Félez, C.; Plósz, B. Gy.; Smets, B. F. Intermittent Aeration Suppresses Nitrite-
681 Oxidizing Bacteria in Membrane-Aerated Biofilms: A Model-Based Explanation. *Environ. Sci.*

- 682 *Technol.* **2017**, *51* (11), 6146–6155. <https://doi.org/10.1021/acs.est.7b00463>.
- 683 (20) Baeten, J. E.; Batstone, D. J.; Schraa, O. J.; van Loosdrecht, M. C. M.; Volcke, E. I. P. Modelling
684 Anaerobic, Aerobic and Partial Nitrification-Anammox Granular Sludge Reactors - A Review.
685 *Water Res.* **2019**, *149*, 322–341. <https://doi.org/10.1016/j.watres.2018.11.026>.
- 686 (21) Baeten, J. E.; van Loosdrecht, M. C. M.; Volcke, E. I. P. Modelling Aerobic Granular Sludge
687 Reactors through Apparent Half-Saturation Coefficients. *Water Res.* **2018**, *146*, 134–145.
688 <https://doi.org/10.1016/j.watres.2018.09.025>.
- 689 (22) Jardin, N.; Hennerkes, J. Full-Scale Experience with the Deammonification Process to Treat
690 High Strength Sludge Water – a Case Study. *Water Sci. Technol.* **2012**, *65* (3), 447.
691 <https://doi.org/10.2166/wst.2012.867>.
- 692 (23) Zaborowska, E.; Lu, X.; Makinia, J. Strategies for Mitigating Nitrous Oxide Production and
693 Decreasing the Carbon Footprint of a Full-Scale Combined Nitrogen and Phosphorus Removal
694 Activated Sludge System. *Water Res.* **2019**, *162*, 53–63.
695 <https://doi.org/10.1016/j.watres.2019.06.057>.
- 696 (24) Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F. O.
697 Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-
698 Generation Sequencing-Based Diversity Studies. *Nucleic Acids Res.* **2013**, *41* (1), 1–11.
699 <https://doi.org/10.1093/nar/gks808>.
- 700 (25) Parks, D. H.; Beiko, R. G. Identifying Biologically Relevant Differences between Metagenomic
701 Communities. *Bioinformatics* **2010**, *26* (6), 715–721.
702 <https://doi.org/10.1093/bioinformatics/btq041>.
- 703 (26) Lu, X.; D. S. Pereira, T.; Al-Hazmi, H. E.; Majtacz, J.; Zhou, Q.; Xie, L.; Makinia, J. Model-Based
704 Evaluation of N₂O Production Pathways in the Anammox-Enriched Granular Sludge Cultivated
705 in a Sequencing Batch Reactor. *Environ. Sci. Technol.* **2018**, *52* (5), 2800–2809.
706 <https://doi.org/10.1021/acs.est.7b05611>.
- 707 (27) Azari, M.; Le, A. V.; Lübken, M.; Denecke, M. Model-Based Analysis of Microbial Consortia and
708 Microbial Products in an Anammox Biofilm Reactor. *Water Sci. Technol.* **2018**, *77* (7), 1951–
709 1959. <https://doi.org/10.2166/wst.2018.081>.
- 710 (28) Liu, Y.; Sun, J.; Peng, L.; Wang, D.; Dai, X.; Ni, B.-J. Assessment of Heterotrophic Growth
711 Supported by Soluble Microbial Products in Anammox Biofilm Using Multidimensional Modeling.
712 *Sci. Rep.* **2016**, *6* (1), 1–11. <https://doi.org/10.1038/srep27576>.
- 713 (29) Mozumder, Md. S. I.; Picioreanu, C.; van Loosdrecht, M. C. M.; Volcke, E. I. P. Effect of
714 Heterotrophic Growth on Autotrophic Nitrogen Removal in a Granular Sludge Reactor. *Environ.*
715 *Technol.* **2014**, *35* (8), 1027–1037. <https://doi.org/10.1080/09593330.2013.859711>.
- 716 (30) Henze, M.; Gujer, W.; Mino, T.; van Loosedrecht, M. *Activated Sludge Models ASM1, ASM2,*
717 *ASM2d and ASM3; IWA TASK GROUP ON MATHEMATICAL MODELLING FOR DESIGN AND*
718 *OPERATION OF BIOLOGICAL WASTEWATER TREATMENT*, Ed.; IWA Publishing: UK, 2000.
- 719 (31) Lu, X.; Al-Hazmi, H.; Majtacz, J.; Mannina, G.; Makinia, J. The Influence of the Objective
720 Functions and a Priori Parameter Distribution on the Model Uncertainty of N₂O Production in a
721 Deammonification System. In *10th International Congress on Environmental Modelling and*
722 *Software*; Brussels, Belgium, 2020.
- 723 (32) Gilbert, E. M.; Agrawal, S.; Brunner, F.; Schwartz, T.; Horn, H.; Lackner, S. Response of Different

- 724 Nitrospira Species To Anoxic Periods Depends on Operational DO. *Environ. Sci. Technol.* **2014**,
725 *48* (5), 2934–2941. <https://doi.org/10.1021/es404992g>.
- 726 (33) Sabba, F.; Terada, A.; Wells, G.; Smets, B. F.; Nerenberg, R. Nitrous Oxide Emissions from
727 Biofilm Processes for Wastewater Treatment. *Appl. Microbiol. Biotechnol.* **2018**, *102* (22), 9815–
728 9829. <https://doi.org/10.1007/s00253-018-9332-7>.
- 729 (34) Miao, Y.; Peng, Y.; Zhang, L.; Li, B.; Li, X.; Wu, L.; Wang, S. Partial Nitrification-Anammox (PNA)
730 Treating Sewage with Intermittent Aeration Mode: Effect of Influent C/N Ratios. *Chem. Eng. J.*
731 **2018**, *334*, 664–672. <https://doi.org/10.1016/j.cej.2017.10.072>.
- 732 (35) Ma, B.; Bao, P.; Wei, Y.; Zhu, G.; Yuan, Z.; Peng, Y. Suppressing Nitrite-Oxidizing Bacteria
733 Growth to Achieve Nitrogen Removal from Domestic Wastewater via Anammox Using
734 Intermittent Aeration with Low Dissolved Oxygen. *Sci. Rep.* **2015**, *5* (1), 1–9.
735 <https://doi.org/10.1038/srep13048>.
- 736 (36) Connan, R.; Dabert, P.; Moya-Espinosa, M.; Bridoux, G.; Béline, F.; Magrí, A. Coupling of Partial
737 Nitrification and Anammox in Two- and One-Stage Systems: Process Operation, N₂O Emission
738 and Microbial Community. *J. Clean. Prod.* **2018**, *203*, 559–573.
739 <https://doi.org/10.1016/j.jclepro.2018.08.258>.
- 740 (37) Yang, J.; Trela, J.; Plaza, E. Nitrous Oxide Emissions from One-Step Partial Nitrification/Anammox
741 Processes. *Water Sci. Technol.* **2016**, *74* (12), 2870–2878. <https://doi.org/10.2166/wst.2016.454>.
- 742 (38) Arnaldos, M.; Amerlinck, Y.; Rehman, U.; Maere, T.; Van Hoey, S.; Naessens, W.; Nopens, I.
743 From the Affinity Constant to the Half-Saturation Index: Understanding Conventional Modeling
744 Concepts in Novel Wastewater Treatment Processes. *Water Res.* **2015**, *70*, 458–470.
745 <https://doi.org/10.1016/j.watres.2014.11.046>.
- 746 (39) Mehrani, M.-J.; Sobotka, D.; Kowal, P.; Ciesielski, S.; Makinia, J. The Occurrence and Role of
747 Nitrospira in Nitrogen Removal Systems. *Bioresour. Technol.* **2020**, *303*, 122936.
748 <https://doi.org/10.1016/j.biortech.2020.122936>.
- 749 (40) Ciesielski, S.; Czerwionka, K.; Sobotka, D.; Dulski, T.; Makinia, J. The Metagenomic Approach
750 to Characterization of the Microbial Community Shift during the Long-Term Cultivation of
751 Anammox-Enriched Granular Sludge. *J. Appl. Genet.* **2018**, *59* (1), 109–117.
752 <https://doi.org/10.1007/s13353-017-0418-1>.
- 753 (41) Mannina, G.; Cosenza, A.; Ekama, G. A. A Comprehensive Integrated Membrane Bioreactor
754 Model for Greenhouse Gas Emissions. *Chem. Eng. J.* **2018**, *334*, 1563–1572.
755 <https://doi.org/10.1016/j.cej.2017.11.061>.
- 756 (42) Pan, Y.; Ni, B.-J.; Lu, H.; Chandran, K.; Richardson, D.; Yuan, Z. Evaluating Two Concepts for
757 the Modelling of Intermediates Accumulation during Biological Denitrification in Wastewater
758 Treatment. *Water Res.* **2015**, *71*, 21–31. <https://doi.org/10.1016/j.watres.2014.12.029>.
- 759 (43) Park, M.-R.; Park, H.; Chandran, K. Molecular and Kinetic Characterization of Planktonic
760 *Nitrospira* Spp. Selectively Enriched from Activated Sludge. *Environ. Sci. Technol.* **2017**, *51* (5),
761 2720–2728. <https://doi.org/10.1021/acs.est.6b05184>.
- 762 (44) Speirs, L. B. M.; Rice, D. T. F.; Petrovski, S.; Seviour, R. J. The Phylogeny, Biodiversity, and
763 Ecology of the Chloroflexi in Activated Sludge. *Front. Microbiol.* **2019**, *10*, 2015.
764 <https://doi.org/10.3389/fmicb.2019.02015>.
- 765 (45) Ni, B.-J.; Rusalleda, M.; Smets, B. F. Evaluation on the Microbial Interactions of Anaerobic

- 766 Ammonium Oxidizers and Heterotrophs in Anammox Biofilm. *Water Res.* **2012**, *46* (15), 4645–
 767 4652. <https://doi.org/10.1016/j.watres.2012.06.016>.
- 768 (46) Park, S.; Bae, W. Modeling Kinetics of Ammonium Oxidation and Nitrite Oxidation under
 769 Simultaneous Inhibition by Free Ammonia and Free Nitrous Acid. *Process Biochem.* **2009**, *44*
 770 (6), 631–640. <https://doi.org/10.1016/j.procbio.2009.02.002>.
- 771 (47) Ushiki, N.; Jinno, M.; Fujitani, H.; Suenaga, T.; Terada, A.; Tsuneda, S. Nitrite Oxidation Kinetics
 772 of Two *Nitrospira* Strains: The Quest for Competition and Ecological Niche Differentiation. *J.*
 773 *Biosci. Bioeng.* **2017**, *123* (5), 581–589. <https://doi.org/10.1016/j.jbiosc.2016.12.016>.
- 774 (48) Blackburne, R.; Vadivelu, V. M.; Yuan, Z.; Keller, J. Kinetic Characterisation of an Enriched
 775 *Nitrospira* Culture with Comparison to *Nitrobacter*. *Water Res.* **2007**, *41* (14), 3033–3042.
 776 <https://doi.org/10.1016/j.watres.2007.01.043>.
- 777 (49) Zhang, F.; Yang, H.; Wang, J.; Liu, Z.; Guan, Q. Effect of Free Ammonia Inhibition on NOB
 778 Activity in High Nitrifying Performance of Sludge. *RSC Adv.* **2018**, *8* (56), 31987–31995.
 779 <https://doi.org/10.1039/C8RA06198J>.
- 780 (50) Kandars, L.; Yang, J.-J.; Baresel, C.; Zambrano, J. Full-Scale Comparison of N₂O Emissions
 781 from SBR N/DN Operation versus One-Stage Deammonification MBBR Treating Reject Water
 782 – and Optimization with PH Set-Point. *Water Sci. Technol.* **2019**, *79* (8), 1616–1625.
 783 <https://doi.org/10.2166/wst.2019.163>.

784 ABSTRACT ART.



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