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Postprint of: Al-Hazmi H., Xi L., Majtacz J., Kowal P., Xie L., Mąkinia J., Optimization of the Aeration Strategies in a Deammonification Sequencing Batch Reactor for Efficient Nitrogen Removal and Mitigation of N<sub>2</sub>O Production, ENVIRONMENTAL SCIENCE & TECHNOLOGY (2020), DOI: [10.1021/acs.est.0c04229](https://doi.org/10.1021/acs.est.0c04229)

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## Optimization of the aeration strategies in a deammonification sequencing batch reactor for efficient nitrogen removal and mitigation of N<sub>2</sub>O production

Journal:	<i>Environmental Science &amp; Technology</i>
Manuscript ID	es-2020-042297.R2
Manuscript Type:	Article
Date Submitted by the Author:	14-Dec-2020
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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 N<sub>2</sub>O  
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 (N<sub>2</sub>O) 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  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  emission. The intermittent  
28 aeration regimes led to frequent shifts in the predominating  $\text{N}_2\text{O}$  production pathways,  
29 i.e. hydroxylamine ( $\text{NH}_2\text{OH}$ ) 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 ( $\text{NO}_2^-$ -N) (with anammox  
42 bacteria). Among different methods proposed for NOB suppression<sup>1-3</sup>, tight DO  
43 control is regarded as the most effective approach<sup>4,5</sup>.

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 ( $\text{NH}_2\text{OH}$ )<sup>6</sup>. In order to enhance NOB  
53 suppression, the intermittent aeration with either high DO set points<sup>7</sup> or lower DO set  
54 points<sup>3</sup> has been proposed. Cao et al.<sup>1</sup> provided the following explanation for this  
55 apparent inconsistency. Under high DO levels ( $>1.5 \text{ mg O}_2/\text{L}$ ), 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 ( $<1.0 \text{ mg O}_2/\text{L}$ ) 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 <sup>8</sup>, which could be beneficial for both NOB suppression and reduction  
62 of energy consumption <sup>9</sup>. 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 (N<sub>2</sub>O) production <sup>10</sup>. Simultaneous high nitrogen removal  
65 efficiency and low N<sub>2</sub>O 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 <sup>11–14</sup>. Moreover, an appropriate aeration control  
68 may also minimize N<sub>2</sub>O production in deammonification systems <sup>11,15,16</sup>. However, no  
69 specific operational strategy has been proposed yet for improving nitrogen removal  
70 performance while simultaneously mitigating N<sub>2</sub>O production <sup>13,17</sup>.

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 <sup>18,19</sup> or N<sub>2</sub>O  
73 mitigation in deammonification systems <sup>13</sup>. 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 N<sub>2</sub>O 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 <sup>20</sup>. The advantage of the latter approach is that typical monitoring data can be used for  
80 model calibration <sup>21</sup>.

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 N<sub>2</sub>O 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) <sup>22</sup>. The detailed description  
96 of laboratory setup and monitoring (including liquid N<sub>2</sub>O 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  $\mu\text{m}$  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 <sub>2</sub> /l)	HRT (d)	Number of cycles	Influent NH <sub>4</sub> <sup>+</sup> -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 <sub>4</sub> <sup>+</sup> -N load and low biomass concentration
			2	15/45	3	1.0	1.0	0.83	4	528 ± 121	634 ± 145	993 ± 184/	
			3	15/45	3	1.0	0.5		4			1530 ± 42	
			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 <sub>4</sub> <sup>+</sup> -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 <sub>4</sub> <sup>+</sup> -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/	
			12	3/3	1	10.0			3			5847 ± 173	
			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 N<sub>2</sub>O  
 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 N<sub>2</sub>O 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 N<sub>2</sub>O 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 N<sub>2</sub>O 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 <sup>23</sup>.

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  $-25^{\circ}\text{C}$  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 <sup>24</sup> 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

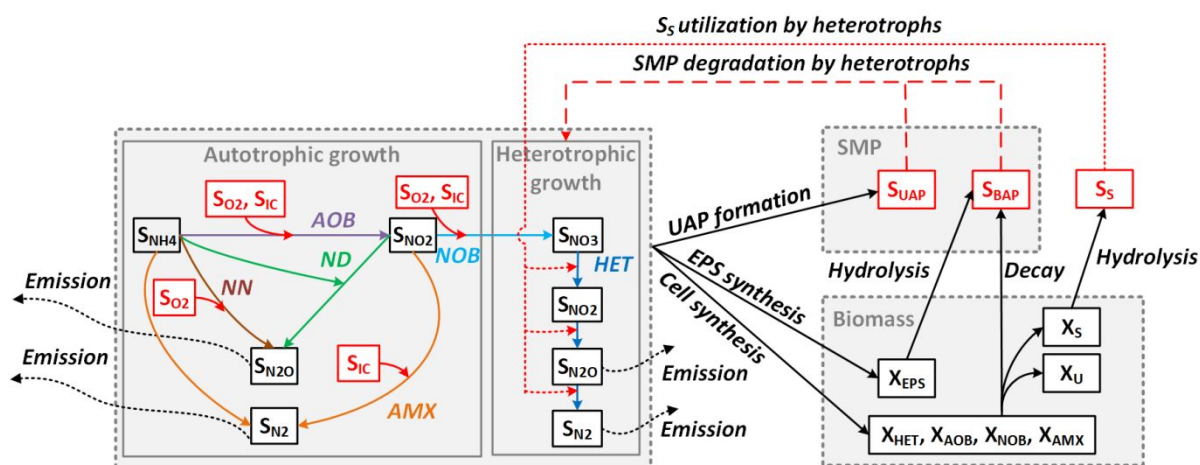
170 Analysis of Metagenomic Profiles (STAMP v. 2.1.3)<sup>25</sup> and visualized as heatmaps. A  
171 genetic distance between the samples was tested by the ANOVA test, followed by the  
172 post-hoc Tukey-Kramer test at 0.95 significance, and visualized as a dendrogram built  
173 up with the unweighted 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 N<sub>2</sub>O  
180 production/consumption mechanisms (along with the gas-liquid transfer) were  
181 adopted from Zaborowska et al.<sup>23</sup>, including NH<sub>2</sub>OH oxidation (NN) and autotrophic  
182 denitrification (ND) mediated by AOB and three-step heterotrophic denitrification (HD)  
183 with NO<sub>2</sub><sup>-</sup>-N and N<sub>2</sub>O as intermediates. For modeling, the only source of electron  
184 donors for autotrophic denitrification was NH<sub>4</sub><sup>+</sup>-N<sup>26</sup>. 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<sup>27,28</sup>. However, only one study investigated the effect of heterotrophic growth  
190 on autotrophic nitrogen removal in granular systems<sup>29</sup>, 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)<sup>27</sup>, and autotrophs  
 197 (anammox) and heterotrophs on  $S_S$ <sup>28</sup>.
- 198 -  $S_{BAP}$  was produced from  $X_{EPS}$  hydrolysis and biomass decay<sup>28</sup>.

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 ASM1<sup>30</sup>. 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<sub>NO2</sub>”, “S<sub>N2O</sub>” and “S<sub>N2</sub>” 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<sup>20,21</sup>. 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 N<sub>2</sub>O emission during the aerated phase  
220 (air-stripping) was influenced by the  $K_La$  coefficient and estimated airflow rate<sup>23</sup>. 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 ASM1<sup>30</sup>. 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 N<sub>2</sub>O production in the studied  
237 system, based on the combination of global sensitivity analysis and Generalized  
238 Likelihood Uncertainty Estimation methodology, can be found elsewhere<sup>31</sup>.

239 *2.5.4. Model-based optimization of intermittent aeration strategies.* In order to  
240 simultaneously maximize the nitrogen removal performance and minimize N<sub>2</sub>O  
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 O<sub>2</sub>/L (interval of 0.2 mg O<sub>2</sub>/L), F = 0.5-10 h<sup>-1</sup>  
246 (interval of 0.5 h<sup>-1</sup>), 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 N<sub>2</sub>O 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<sub>N<sub>2</sub>O</sub> < 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, NH<sub>4</sub><sup>+</sup>-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. <sup>30</sup>.

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 AOB<sup>32</sup>.

277 *Limitations to describe autotrophic denitrification by AOB.* Since  $\text{NH}_2\text{OH}$  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 <sup>33</sup>. 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

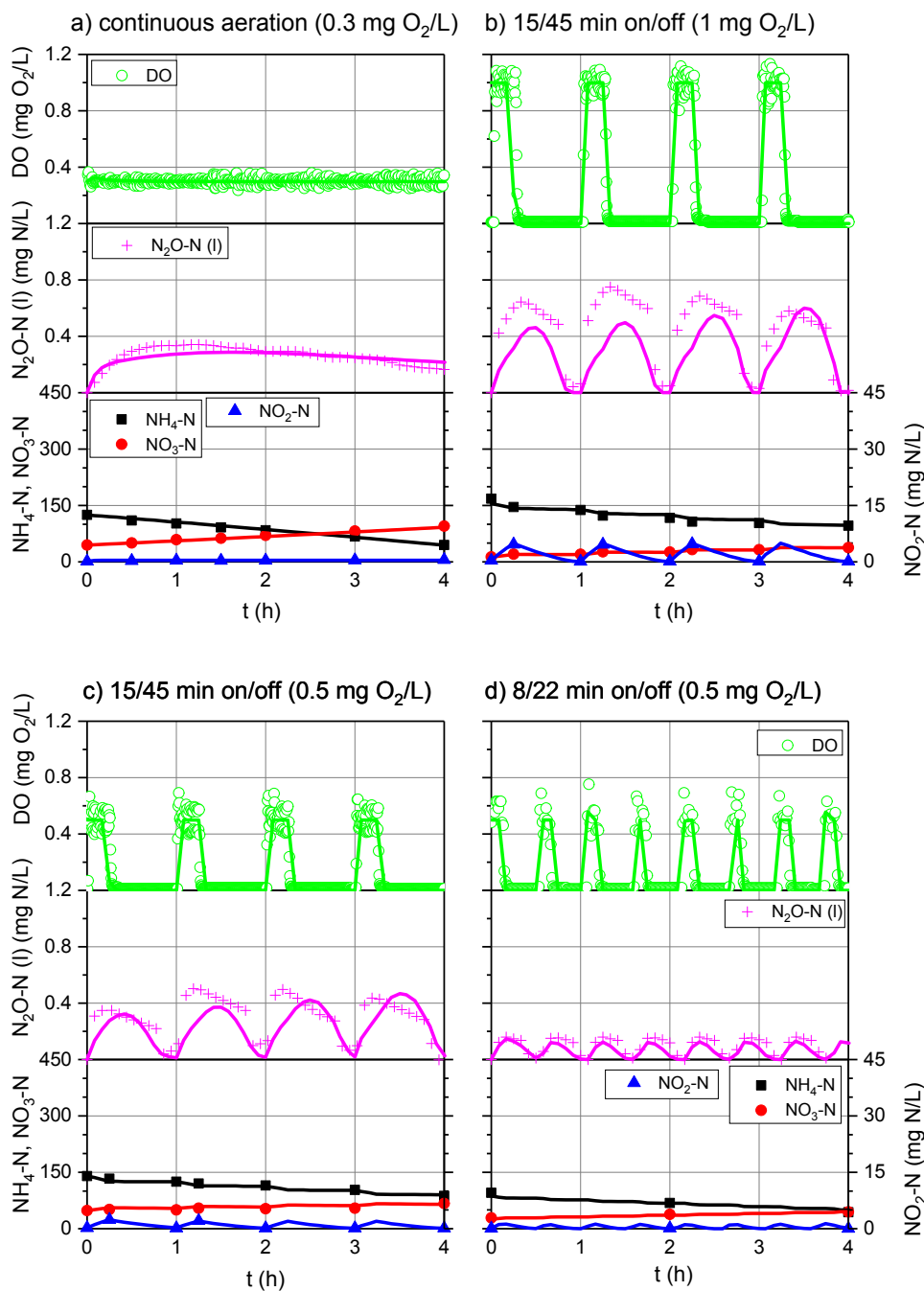
291 values. It was implicitly assumed that the model response to the parameters was  
292 linear, after taking into consideration the different biomass compositions and  
293 concentrations at each stage (CAL, REC and VAL). However, the selected approach  
294 sacrificed the model accuracy for a faster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

295

### 296 3. RESULTS AND DISCUSSION

297 **3.1. Nitrogen removal performance and N<sub>2</sub>O 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 (N<sub>2</sub>O-N, NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, NO<sub>2</sub><sup>-</sup>-N) in the three trials are shown in Figures 2-4.

301 In test trial no. 1 with the moderate NH<sub>4</sub><sup>+</sup>-N load and low biomass concentration,  
302 continuous aeration at the low DO set point (0.3 mg O<sub>2</sub>/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 N<sub>2</sub>O  
307 concentrations. Further increasing the F frequency (scenario 4) indicated better  
308 suppression of NOB activity and N<sub>2</sub>O production while maintaining the AOB activity.



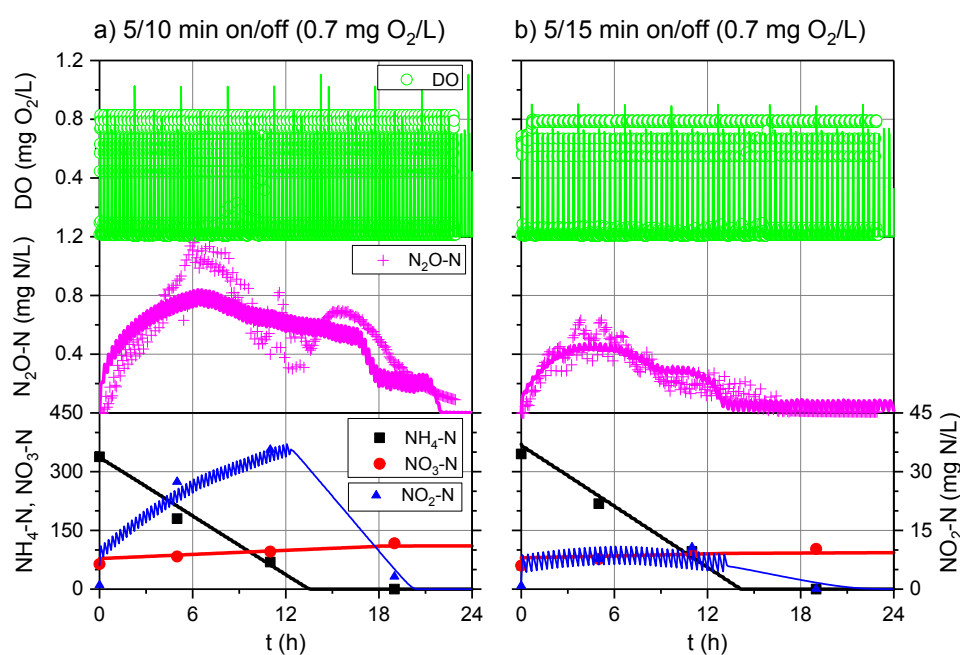
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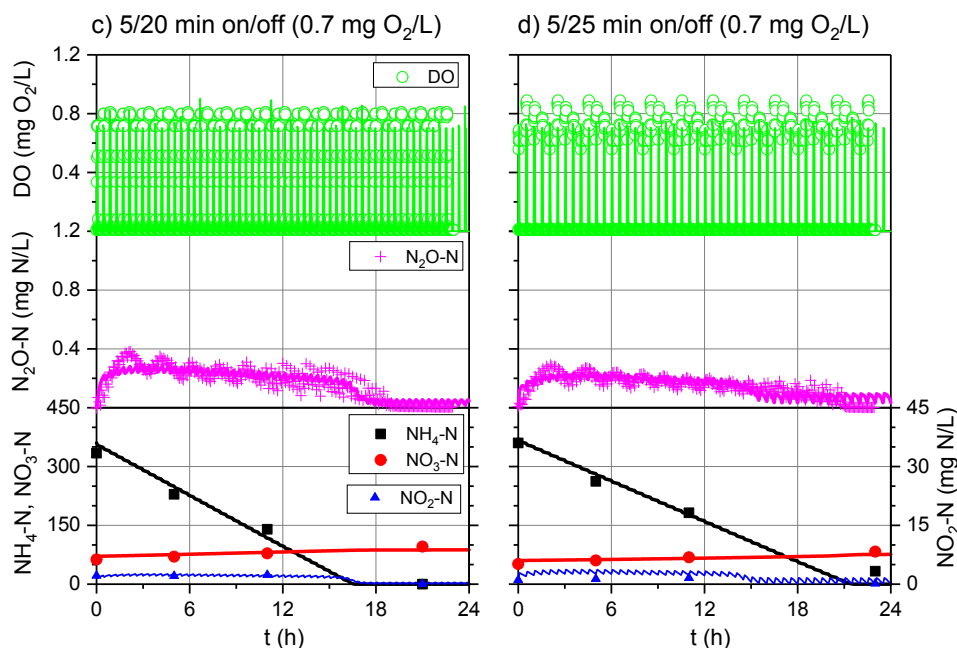
311 **Figure 2.** Measured data (scatters) vs. model predictions (solid lines) of DO and  
 312 inorganic nitrogen compounds (N<sub>2</sub>O-N, NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, NO<sub>2</sub><sup>-</sup>-N) in test trial no. 1: (a)  
 313 continuous aeration at DO = 0.3 mg O<sub>2</sub>/L (SCE1), (b) intermittent aeration 15/45 min  
 314 on/off at DO = 1.0 mg O<sub>2</sub>/L (when on) (SCE2), (c) intermittent aeration 15/45 min on/off

315 at DO = 0.5 mg O<sub>2</sub>/L (when on) (SCE3), (d) intermittent aeration 8/22 min on/off at DO  
 316 = 0.5 mg O<sub>2</sub>/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 O<sub>2</sub>/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 N<sub>2</sub>O concentrations and N<sub>2</sub>O  
 322 emission factors decreased due to enhanced anammox activities in the anoxic periods  
 323 and reduced NO<sub>2</sub><sup>-</sup>-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 N<sub>2</sub>O production, while maintaining the favorable NPR/AUR ratio (= 0.09).  
 326 However, the overall process performance deteriorated due to the decreased AUR.



327



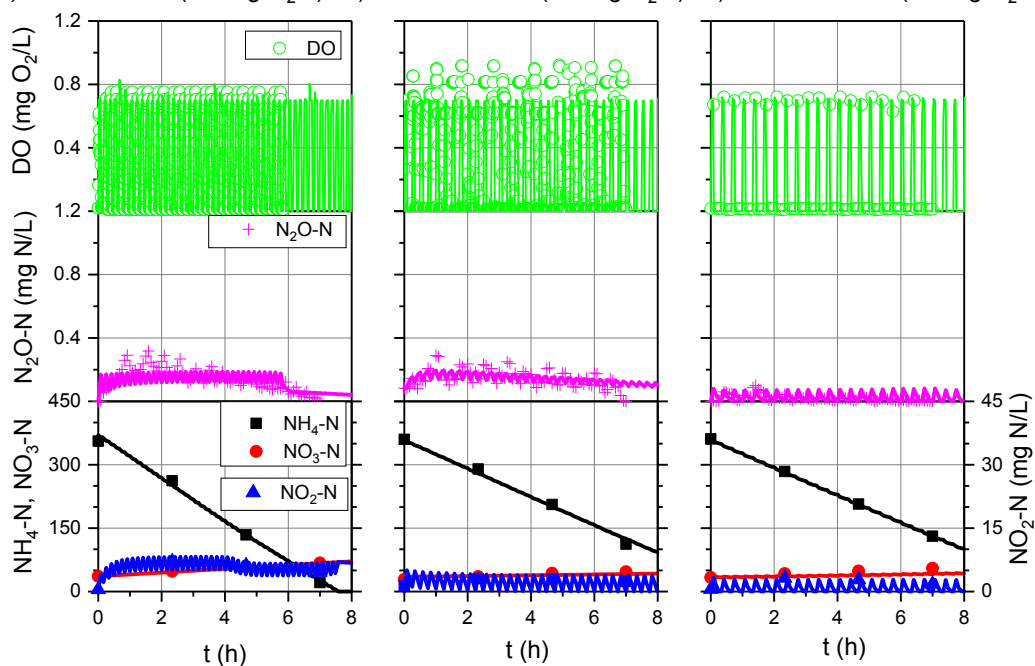
328

329 **Figure 3.** Measured data (scatters) vs. model predictions (solid lines) of DO and  
 330 inorganic nitrogen compounds (N<sub>2</sub>O-N, NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, NO<sub>2</sub><sup>-</sup>-N) in test trial no. 2: (a)  
 331 intermittent aeration 5/10 min on/off at DO = 0.7 mg O<sub>2</sub>/L (when on) (SCE5), (b)  
 332 intermittent aeration 5/15 min on/off at DO = 0.7 mg O<sub>2</sub>/L (when on) (SCE6), (c)  
 333 intermittent aeration 5/20 min on/off at DO = 0.7 mg O<sub>2</sub>/L (when on) (SCE7), (d)  
 334 intermittent aeration 5/25 min on/off at DO = 0.7 mg O<sub>2</sub>/L (when on) (SCE8).

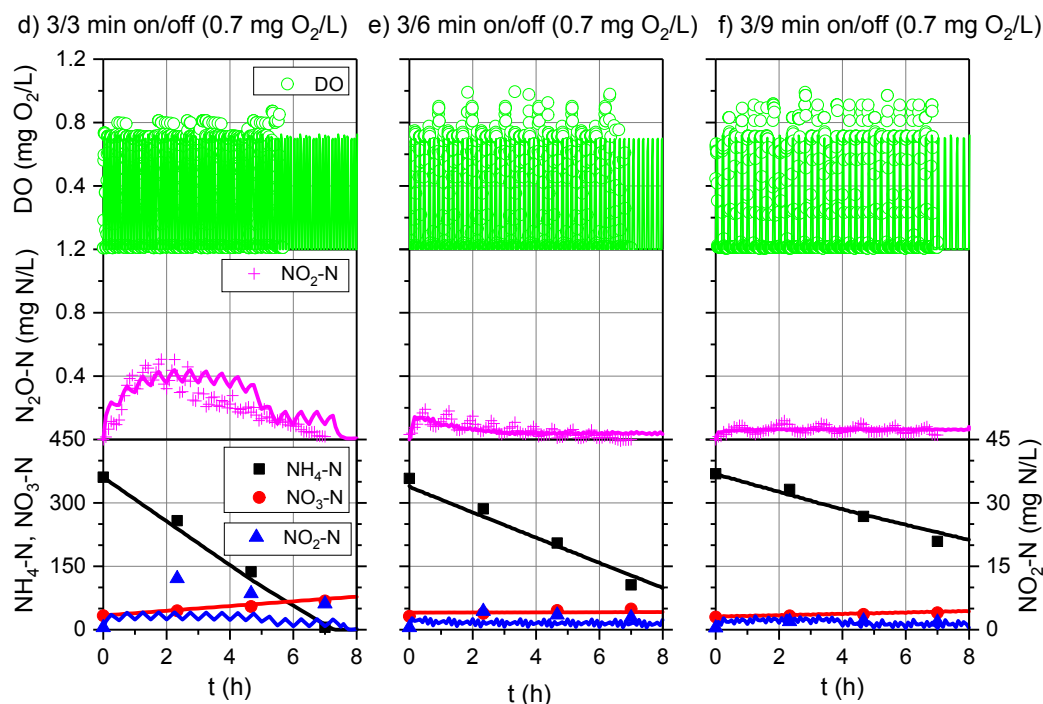
335 The final test trial no. 3 was carried out after over 300 days of cultivation, when the  
 336 MLVSS reached approximately 4000 mg/L (Figure 4). The DO set point was kept  
 337 constant at 0.7 mg O<sub>2</sub>/L and that trial focused on investigating the effect of aeration  
 338 on/off frequencies 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 (13.0-18.1 mg N/(g VSS·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  $\text{N}_2\text{O}$  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  $\text{DO}$  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<sup>9,22,34</sup>. In the  
 360 stable operation period (158-308 d), the intermittent aeration pattern (5/20 min on/off

361 at DO = 0.7 mg O<sub>2</sub>/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 O<sub>2</sub>/L in scenario 13) was ultimately selected to achieve the stable operation of the  
364 system in terms of NOB suppression and N<sub>2</sub>O 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 N<sub>2</sub>O 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 <sup>1,9</sup>. 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 <sup>19,32</sup>.  
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%) <sup>8,9,22</sup>. 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

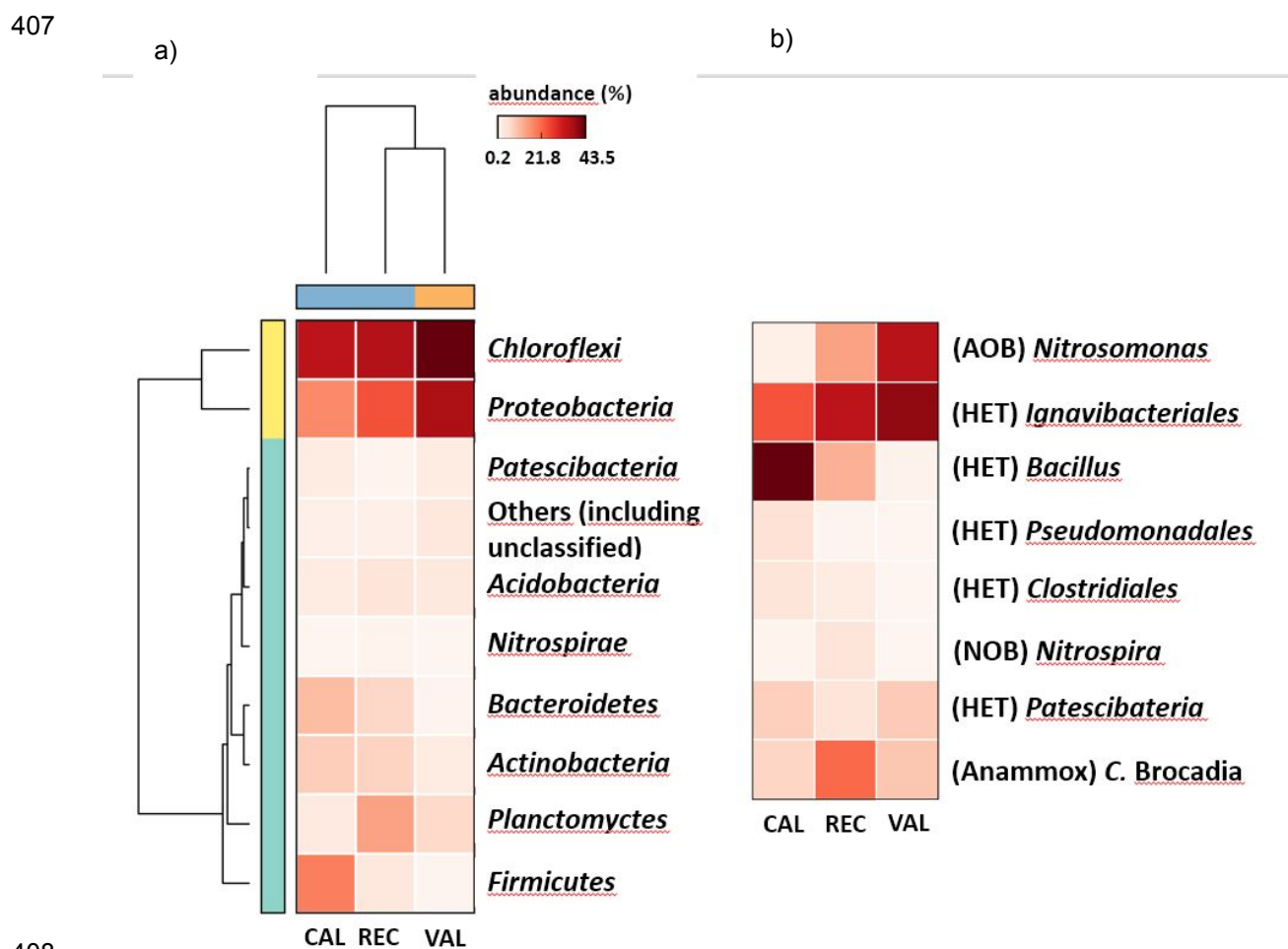


382 aerobic conditions and anammox bacteria outcompeting NOB for  $\text{NO}_2^-$ -N in anoxic  
383 periods <sup>7,35</sup>. In the present study, it was found that an appropriate R ratio was more  
384 influential than strict durations of the aeration on/off periods. For the acclimated  
385 biomass (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  $\text{N}_2\text{O}$  production profiles during the aerated and non-aerated  
388 phases revealed that approximately 50% of  $\text{N}_2\text{O}$  was produced during the aerated  
389 phases, which was lower than the value (80%) reported by Blum et al. <sup>11</sup>. In  
390 comparison with the continuous aeration, when almost all  $\text{N}_2\text{O}$  production was emitted  
391 to the gas phase, approximately half of the produced  $\text{N}_2\text{O}$  was subsequently emitted  
392 to the gas phase under the intermittent aeration conditions. The estimated  $\text{N}_2\text{O}$   
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 <sup>11,15,17,36,37</sup>. The vast  
395 majority (approximately 98%) of  $\text{N}_2\text{O}$  was emitted during the aerated phases, which  
396 was also close to the value (96%) reported by Blum et al. <sup>11</sup>. Potential contributions of  
397 the specific biochemical pathways, including  $\text{NH}_2\text{OH}$  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 NO<sub>2</sub><sup>-</sup>-N concentrations as well as diffusion limitation  
427 within granules<sup>38</sup>. 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 conditions<sup>39</sup>. 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 SBR<sup>40</sup>.

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 ( $X_{\text{HET}}$ ,  $X_{\text{AOB}}$ ,  $X_{\text{NOB}}$ ,  
445  $X_{\text{AMX}}$ ,  $X_{\text{S}}$ ,  $X_{\text{U}}$ ,  $X_{\text{EPS}}$ ) 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  $\text{NO}_2^-$ -N and  $\text{N}_2\text{O}$ -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  $\eta_h$  (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  $\eta_{gHET3}$ ,  $K_{NO_2,AOB}$ ,  $K_h$  and  $\eta_h$ ) were excluded from the subset. The objective of the  
463 iterative parameter estimation was to obtain the minimum number of identifiable  
464 parameters ( $\eta_{gHET2}$ ,  $K_{O,AOB}$ ,  $K_{O,NOB}$ ) 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 ( $\eta_{gHET3}$  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 ( $\eta_{gHET2}$ ,  $\eta_{gHET3}$ ,  $K_{O,AOB}$ ,  
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 <sup>31</sup>.

490 The adjusted parameters were  $\eta_{gHET2}$ ,  $\eta_{gHET3}$ ,  $K_{O,AOB}$ ,  $K_{O,NOB}$  and  $K_h$  (Table  
491 S12). The heterotrophic reduction factors for  $NO_2^-$ -N and  $N_2O$  ( $\eta_{gHET2} = 0.17$ ,  $\eta_{gHET3}$   
492 = 0.40) and the hydrolysis rate ( $K_h = 2.6 \text{ d}^{-1}$ ) were within the literature range ( $\eta_{gHET2}$   
493 = 0.016-0.3,  $\eta_{gHET3} = 0.075$ -0.81,  $K_h = 1.5$ -4.5  $\text{d}^{-1}$ ) <sup>41,42</sup>. 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  $\text{mg O}_2/\text{L}$ ) <sup>43</sup>. The  
497 relatively high oxygen affinity for K-strategist *Nitrospira* in comparison with r-strategist

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

500 **3.4. Identification of the N<sub>2</sub>O 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 granules<sup>44</sup>.

514 The finding of the present study is different from results of a theoretical modelling  
515 study in an anammox biofilm system<sup>28</sup> which was validated based on earlier  
516 experimental data<sup>45</sup>. 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  $\text{NH}_2\text{OH}$  oxidation (NN) and autotrophic  
522 denitrification (ND) were the dominating pathways of  $\text{N}_2\text{O}$  production in the aerated  
523 phase (80-100%) and non-aerated phase (60-90%), respectively. Heterotrophic  
524 denitrification served as a sink for  $\text{N}_2\text{O}$  production under both aerated (20-30%) and  
525 non-aerated phase (70-80%) (Figure 6b). For comparison, either  $\text{NH}_2\text{OH}$  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 <sup>10</sup>. Even though the heterotrophic  
529 denitrification pathway has been neglected in most deammonification modelling studies,  
530 Wan et al. <sup>13</sup> 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.

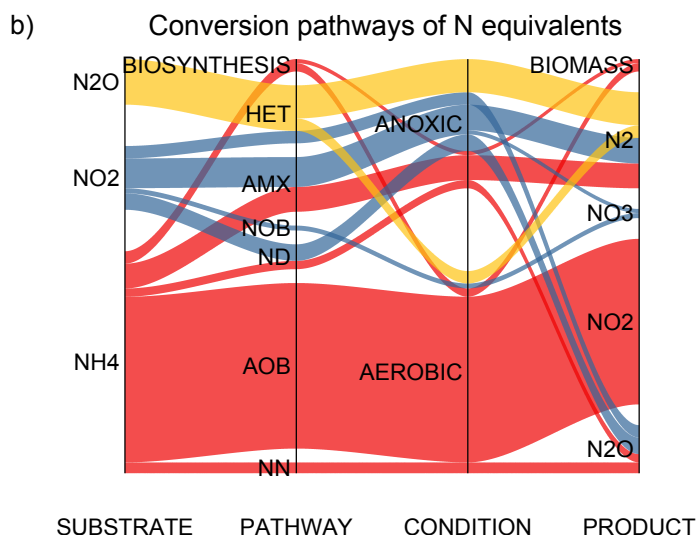
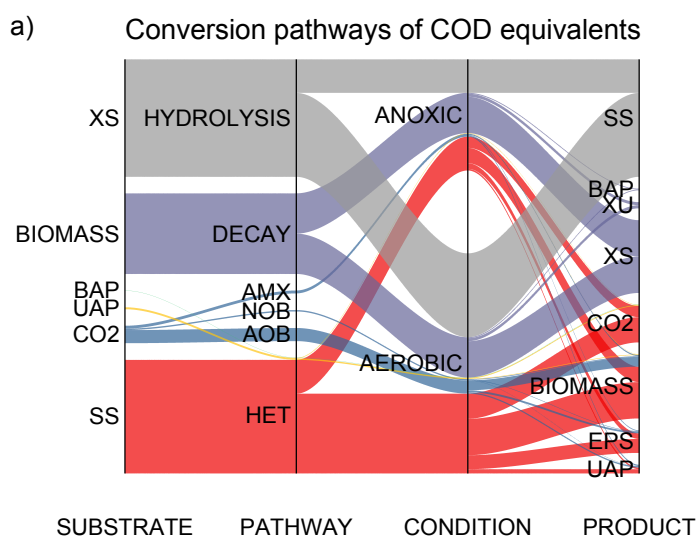
533 The  $\text{N}_2\text{O}$  production rate was dependent on the AUR in the aerated phase and  $\text{NO}_2^-\text{-N}$   
534 N concentration in the non-aerated phase (Figure S6), which is similar to the findings  
535 of Blum et al. <sup>11</sup>. Intense aeration could result in higher  $\text{N}_2\text{O}$  production and emission  
536 due to both higher AURs and stripping effect, as suggested by Castro-Barros et al. <sup>15</sup>,  
537 and weaker suppression of the NOB activity (as shown in scenarios 2-3). A longer  
538 non-aerated phase could cause more  $\text{N}_2\text{O}$  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.<sup>19</sup> 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  $\text{NH}_4^+\text{-N}$  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<sup>46,47</sup>, and specifically 0.04-0.08 mg N/L for *Nitrospira*<sup>48</sup>. 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 suppression<sup>49</sup>, 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 <sup>46</sup> during all the experiments. However, Blum et al. <sup>12</sup> suggested that pH may regulate  
 561 the rates between N<sub>2</sub>O production and consumption. Indeed, Kandars et al. <sup>50</sup> showed  
 562 that more N<sub>2</sub>O 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 N<sub>2</sub>O 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 O<sub>2</sub>/L, 2 and 6.5 h<sup>-1</sup>, 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 O<sub>2</sub>/L, 1.5-3.5 and 3-10 h<sup>-1</sup>, respectively. At the reference state (DO=0.7 mg  
578 O<sub>2</sub>/L, R=2 and F=6.5 h<sup>-1</sup>), the predicted AUR, NPR/AUR and N<sub>2</sub>O 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 O<sub>2</sub>/L, R=2 and F=6.5 h<sup>-1</sup>). The AOB activity could be increased to 14 mg N/(g VSS·h)  
582 without compromising the NOB suppression (NPR/AUR < 0.1) and N<sub>2</sub>O emission  
583 (E<sub>N<sub>2</sub>O</sub> < 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 O<sub>2</sub>/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<sub>N<sub>2</sub>O</sub> < 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 N<sub>2</sub>O 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 N<sub>2</sub>O 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<sup>-1</sup> (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.

## 610 ACKNOWLEDGMENT

611 This study has been financially supported by the Polish National Center for Research  
612 and Development under the project “Reduction of N<sub>2</sub>O emissions from wastewater  
613 treatment plants - measurements, modeling and process optimization (RENEMO)”  
614 from Polish-German Cooperation for Sustainable Development Programme  
615 (WPN/7/2013), Major Science and Technology Program for Water Pollution Control  
616 and Treatment, China (2017ZX07206-002) and International Exchange Program for  
617 Graduate Students, Tongji University (No. 201801024).

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784 ABSTRACT ART.

