

1 **The coexistence and competition of canonical and comammox nitrite oxidizing** 2 **bacteria in a nitrifying activated sludge system – experimental observations and** 3 **simulation studies**

4
5 **Mohamad-Javad Mehrani¹, Przemyslaw Kowal¹, Dominika Sobotka¹, Martyna Godzieba², Sławomir**
6 **Ciesielski², Jianhua Guo³, Jacek Makinia^{1*}**

7 ¹ Faculty of Civil and Environmental Engineering, Gdansk University of Technology, Narutowicza Street 11/12, 80-233 Gdansk,
8 Poland

9 ² Department of Environmental Biotechnology, Department of Environmental Biotechnology, University of Warmia and Mazury
10 in Olsztyn, Sloneczna 45G, 10-719 Olsztyn, Poland

11 ³Australian Centre for Water and Environmental Biotechnology (ACWEB, formerly AWMC), The University of Queensland, St.
12 Lucia, Queensland 4072, Australia

13 *Correspondence: Jacek Makinia (Email: jmakinia@pg.edu.pl, Tel: +48 58 347 19 54, Address: Gdansk University of
14 Technology, ul. Narutowicza 11/12, 80-233, Gdansk, Poland)

15 16 **Abstract**

17 The second step of nitrification can be mediated by nitrite oxidizing bacteria (NOB), i.e.
18 *Nitrospira* and *Nitrobacter*, with different characteristics in terms of the r/K theory. In this study,
19 an activated sludge model was developed to account for competition between two groups of
20 canonical NOB and comammox bacteria. Heterotrophic denitrification on soluble microbial
21 products was also incorporated into the model. Four 5-week washout trials were carried out at
22 dissolved oxygen-limited conditions for different temperatures (12°C vs. 20°C) and main
23 substrates (NH₄⁺-N vs. NO₂⁻-N). Due to the aggressive reduction of solids retention time (from 4
24 to 1 d), the biomass concentrations were continuously decreased and stabilized after two weeks at
25 a level below 400 mg/L. The collected experimental data (N species, biomass concentrations, and
26 microbiological analyses) were used for model calibration and validation. In addition to the
27 standard predictions (N species and biomass), the newly developed model also accurately
28 predicted two microbiological indicators, including the relative abundance of comammox

29 bacteria as well as nitrifiers to heterotrophs ratio. Sankey diagrams revealed that the relative
30 contributions of specific microbial groups to N conversion pathways were significantly shifted
31 during the trial. The contribution of comammox did not exceed 5% in the experiments with both
32 NH_4^+ -N and NO_2^- -N substrates. This study contributes to a better understanding of the novel
33 autotrophic N removal processes (e.g. deammonification) with nitrite as a central intermediate
34 product.

35

36 **Keywords:** Process Simulation; Comammox; *Nitrospira*; *Nitrobacter*; Two-step nitrification

37

38

39

40 1. INTRODUCTION

41 Although nitrification has been known since the end of the 19th century, the process of
42 understanding has changed dramatically in recent 30 years, which was reflected by evolving
43 descriptions in the Metcalf and Eddy handbook series (1990, 2003, 2014). The second stage of
44 nitrification (nitrite oxidation, nitrataion) has been receiving special attention in response to the
45 development of the novel shortcut nitrogen (N) removal processes, including deammonification
46 and a shortened pathway of nitrification-denitrification via nitrite (“nitrite shunt”). In those
47 processes, nitrite is a central component and effective suppression of nitrite oxidizing bacteria
48 (NOB) is required for successful performance. However, the knowledge of the metabolism of
49 NOB has been limited and NOB remains a “big unknown of the nitrogen cycle” (Daims et al.,
50 2016).

51
52 Traditionally, the genus *Nitrobacter* was considered the typical NOB representative (Metcalf and
53 Eddy, 1990), whereas more recently the genus *Nitrospira* has been accepted as a more common
54 NOB population (Metcalf and Eddy, 2014). The dominance of *Nitrospira* in the NOB population
55 of activated sludge systems has indeed been confirmed in numerous recent laboratory- and full-
56 scale studies (Keene et al., 2017; Li et al., 2020; Persson et al., 2017; Wu et al., 2019; Zheng et
57 al., 2019a). Mehrani et al., (2020) presented a comprehensive review study and meta-analysis on
58 the role of *Nitrospira* in the N removal systems.

59
60 These two genera (*Nitrobacter* and *Nitrospira*) reveal different characteristics in terms of the r/K
61 theory. *Nitrobacter* represents the r-strategists which grow faster at high concentrations of the
62 substrates (NO_2^- -N and dissolved oxygen (DO)), whereas *Nitrospira* is the K-strategist with high
63 substrate affinity at low concentration (Yu et al., 2020). Due to these differences, a more complex



64 suppression strategy would be required for NOB. In general, the controlled solids retention time
65 (SRT), combined with DO-limited conditions and high residual ammonia, have been
66 recommended (Regmi et al., 2014). However, low DO conditions (<1.0 mg O₂/L) can be
67 inefficient with respect to the suppression of K-strategist *Nitrospira* (Cao et al., 2017).

68
69 In addition to the competition between *Nitrobacter* and *Nitrospira*, recent findings suggest that
70 there are other critical issues to be considered for NOB suppression. These issues comprise the
71 occurrence of different NOB populations, such as *Ca. Nitrotoga* (Kitzinger et al. 2018) and
72 specific metabolic properties of some microorganisms, such as comammox-*Nitrospira*. In
73 particular, the discovery of comammox, i.e. complete ammonia oxidation, by a single *Nitrospira*-
74 type microorganism (Daims et al., 2015; van Kessel et al., 2015) has overturned “a century-old
75 dogma of nitrification research” (Koch et al., 2019). However, the actual role of comammox
76 bacteria in full-scale WWTPs is still ambiguous (Koch et al., 2019).

77
78 The dominance of specific groups of nitrifying bacteria results from selective pressures of the
79 operational conditions in the bioreactor, including substrate (NH₄⁺-N or NO₂⁻-N) concentration,
80 DO concentration, and temperature (Metcalf and Eddy, 2014). An efficient approach to the
81 investigation of the complex nitrifier competition would be a combination of dedicated physical
82 experiments with advanced microbiological analyses and mathematical modeling. Cao et al.
83 (2017) noted that the nitrification models should accommodate appropriately the competition
84 between AOB and NOB to understand factors influencing the competition between autotrophic
85 N-converting organisms. Two-step nitrification models have been continuously developed for
86 almost 60 years as summarized by (Mehrani et al., 2022). Such models were used for the
87 development of suppression strategies for NOB considered collectively as one group (Duan et al.,



88 2019; Kent et al., 2019; Ma et al., 2017; Pérez et al., 2014). Very recent theoretical advances
89 include the examination of the competition for different r/K strategist groups of AOB and NOB
90 (Yu et al., 2020; Yin et al., 2022) and the incorporation of comammox in the traditional two-step
91 model (Mehrani et al., 2021). However, no models have been applied in practice to investigate
92 the competition of different NOB groups under different substrate (limited vs. unlimited)
93 conditions.

94

95 The purpose of this study was to develop and validate a model describing the co-existence and
96 competition between comammox NOB and two groups of canonical NOB, termed NOB1
97 (*Nitrospira*) and NOB2 (*Nitrobacter*), revealing different r/K characteristics, in response to
98 decreasing SRTs under different substrate limitation conditions. The experimental data were
99 collected during four long-term washout experiments carried out at two temperatures (12°C vs.
100 20°C) with different substrates ($\text{NH}_4^+\text{-N}$ vs. $\text{NO}_2^-\text{-N}$). The effect of the substrate was also
101 investigated in terms of the behavior of comammox bacteria. Overall, it was hypothesized that
102 the newly developed model would provide a better explanation of the competition between the
103 different NOB groups. Such a model can be a diagnosis and optimization tool for practical
104 applications of the novel shortcut N removal processes under different $\text{NO}_2^-\text{-N}$ availabilities.

105

106 2. MATERIAL AND METHODS

107 2.1. Laboratory experiments and data collection for modeling

108 2.1.1. Long-term washout experiments with different nitrogen substrates

109 Four long-term washout trials were carried out under various laboratory conditions concerning
110 the nitrogen substrate and temperature (Table 1). For each experiment, new inoculum biomass
111 samples were obtained from a large municipal wastewater treatment plant (WWTP) in Swarzewo
112 (180 000 PE), located in northern Poland. The biological part of that plant, performing biological
113 nutrient removal, consists of six parallel sequencing batch reactors (SBRs). The effluent
114 standards have been set following the European Union Urban Wastewater Directive (91/21/EEC),
115 i.e., total N (TN) = 10 mg N/L and total P (TP) = 1 mg P/L.

116
117 The laboratory experiments were carried out in a fully automated plexiglass SBR with a working
118 volume of 10 L. The reactor was placed in a thermostatic water bath to keep the temperature
119 setpoints. Control systems were also installed for aeration and pH. A detailed description of the
120 laboratory setup can be found elsewhere (Mehrani et al., 2022).

121
122 In each experiment, the reactor was operated at three cycles per day (8 hours each), including
123 three phases: feeding (15 minutes), reaction (450 minutes), and decantation (15 minutes). The
124 latter phase was carried out while mixing, so the solids retention time (SRT) became equal to the
125 hydraulic retention time. The amount of waste activated sludge (WAS), removed during the
126 decantation phase, was progressively increasing. This resulted in a gradually decreasing SRT
127 from the initial 4 d to 1 d at the end of the trial. For all the experiments, the continuous aeration
128 mode was employed with the DO setpoint of 0.6 ± 0.1 mg/L, while the pH was kept at 7.5 ± 0.2 by
129 dosing NaOH (2M solution). The temperature setpoints were kept close to the actual process



130 conditions, i.e., 12°C (winter) and 20°C (summer). For a better understanding of the competition
131 between the NOB groups, the reactor was fed with either $\text{NH}_4^+\text{-N}$ or $\text{NO}_2^-\text{-N}$ as the sole inorganic
132 N substrate. The influent N loadings and detailed feed composition are shown in the SI (Figure
133 S1 and Table S1, respectively).

134
135 Mixed liquor samples were collected 3 times per week, then filtered and analyzed for different N
136 forms, including $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and $\text{NO}_2^-\text{-N}$, at the initial and the end of the reaction phase.
137 Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS)
138 concentrations were analyzed at the initial of the reaction phase. For microbiological analyses,
139 biomass samples were collected from the reactor in duplicate three times: at the beginning (0 d),
140 in the middle phase (20 d), and at the end of each trial (35 d). The samples from the initial and
141 middle phases were transferred to 50 mL Falcon-type tubes for sedimentation and thickening.
142 The terminal samples, due to the dilution of mixed liquor, were obtained by filtering 5 L of the
143 effluent through 0.22 μm pore size filters. The biomass samples were stored at -25°C prior to
144 DNA extraction.

145

146 **Table 1.** Operational parameters and conditions in the reactor during the washout experiments
147

Trial	Temperature (°C)	pH	DO (mg O ₂ /L)	Source of N in the feed	MLSS/MLVSS (mg/L)
T1	20	7.5±0.2	0.6±0.1	$\text{NH}_4^+\text{-N}$	2140/1500
T2	12	7.5±0.2	0.6±0.1	$\text{NH}_4^+\text{-N}$	1890/1450
T3	20	7.5±0.2	0.6±0.1	$\text{NO}_2^-\text{-N}$	1970/1520
T4	12	7.5±0.2	0.6±0.1	$\text{NO}_2^-\text{-N}$	2020/1440

148

149 **2.1.2. Chemical and microbiological analytical methods**

150 The analytical methods used by Dr. Lange and Shimadzu were based on the APHA Standard
151 Methods (2002). Cuvette tests in a Xion 500 spectrophotometer were used to determine NH_4^+ -N,
152 NO_3^- -N, and NO_2^- -N concentrations (Hach Lange, Germany). Before the analysis, mixed liquor
153 samples were filtered under vacuum pressure using a 1.2 m pore size nitrocellulose filter MFV-3
154 (Millipore, USA). The gravimetric technique was used to determine the MLSS concentration and
155 its volatile fraction (MLVSS) in line with the APHA Standard Methods (2002).

156
157 The DNA extraction was performed followed by FastDNA™ SPIN KIT (MP Biomedicals, USA)
158 based on the manufacturer's manual. Genomic DNA extracted from the duplicated samples was
159 pooled together. Following purification, the DNA was utilized for the Illumina Next Generation
160 Sequencing procedure. Similar to our previous studies (Al-Hazmi et al., 2021), high-throughput
161 Illumina sequencing of the V3-V4 regions of the 16S rRNA gene procedure and subsequent DNA
162 sequencing data processing and analysis were carried out.

163

164 **2.2. Organization of the modeling study**

165 The whole modeling study was arranged in five steps as shown in Figure 1, and each step was
166 described in the following sub-sections.

167

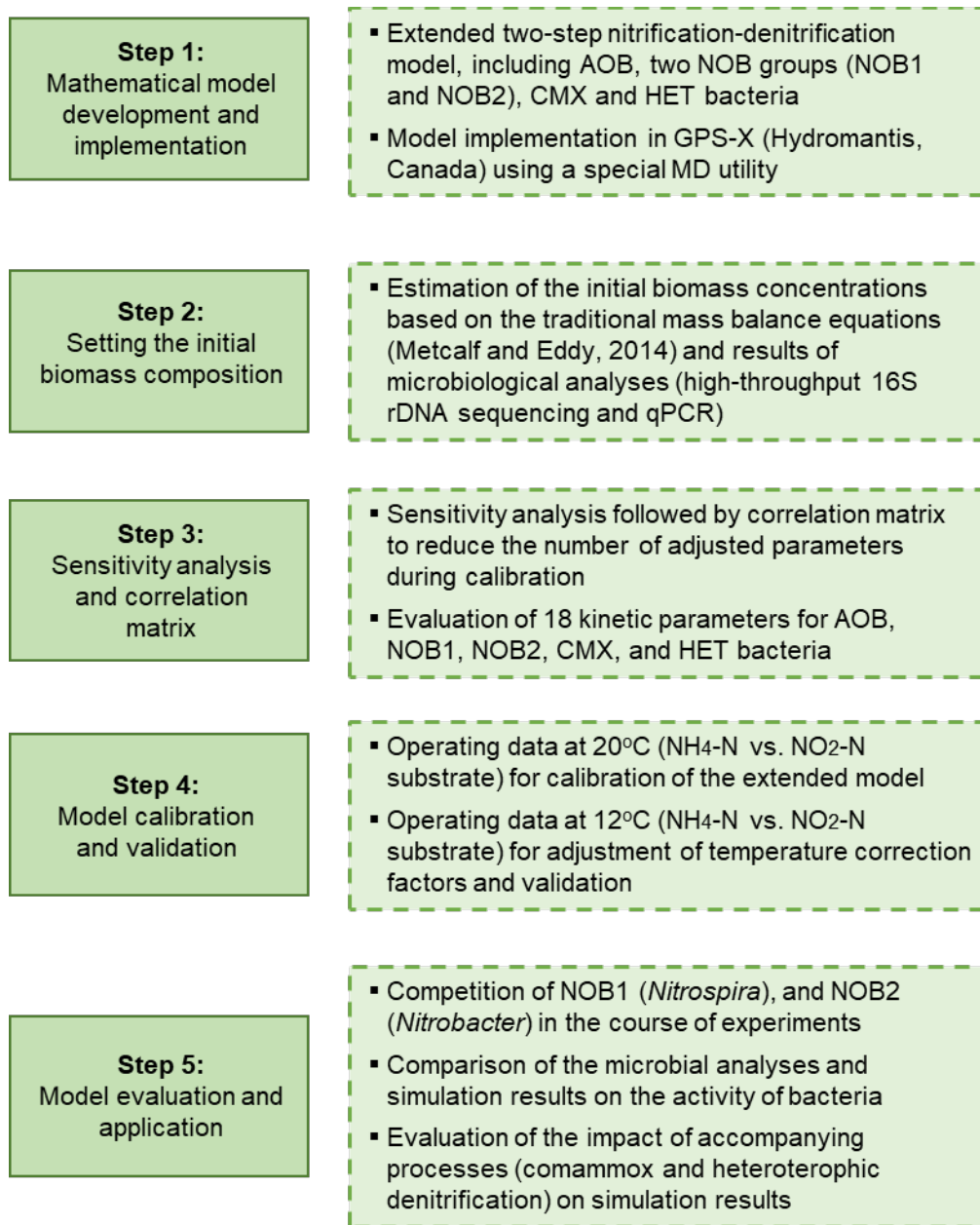


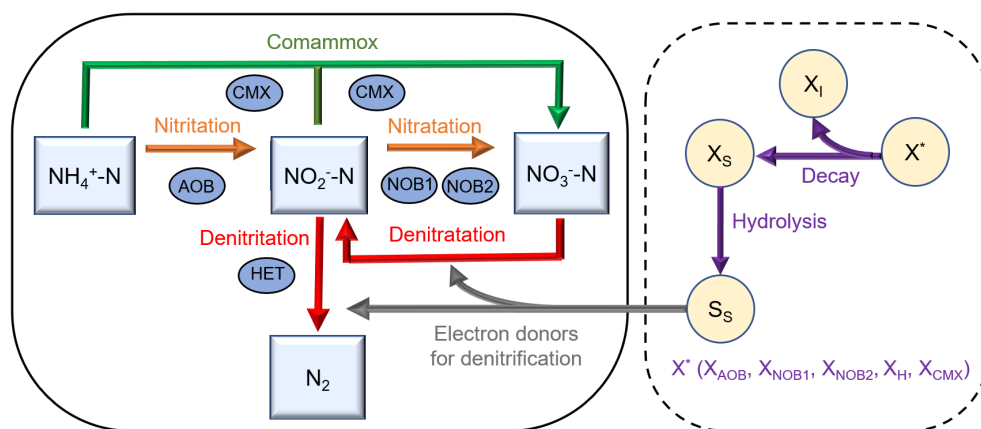
Figure 1. Flowchart diagram of the simulation procedure and model application for the competition of two NOB groups and interactions with comammox and heterotrophic denitrification

2.2.1. Mathematical model development and implementation

The microbial growth model, used in the present study, was based on the death-regeneration concept from the Activated Sludge Model No. 1 (ASM1) (Henze, 2000). In our previous studies, a two-step nitrification model was developed with further expansions to incorporate comammox (Mehrani et al., 2021) and heterotrophic denitrification on soluble microbial products (SMP)

178 (Mehrani et al., 2022). In the present study, NOB were divided into two subgroups depending on
 179 their different characteristics in terms of the r/K theory, i.e., *Nitrospira* as K-strategists vs.
 180 *Nitrobacter* as r-strategists (Figure 2b). Their behavior and competition were assessed during the
 181 washout experiments under different substrate availabilities (Table 2). It was assumed that the
 182 substrate affinity is higher for *Nitrobacter* than *Nitrospira* ($K_{NO_2,NB} > K_{NO_2,NS}$) allowing the latter
 183 to prevail under NO_2^- -N limited conditions (NH_4^+ -N feeding). On the other hand, *Nitrobacter* can
 184 outcompete *Nitrospira* under NO_2^- -N feeding due to a higher maximum specific growth rate
 185 ($\mu_{max,NB} > \mu_{max,NS}$).

186



187

188 **Figure 2.** A concept of the extended nitrification model considering two NOB groups and interactions with
 189 comammox and heterotrophic denitrification

190

191 The GPS-X software 8.0 (Hydromantis, Canada) was used as a simulation environment for
 192 implementing the model and running simulations. Internal GPS-X utilities were used for
 193 sensitivity analysis (Analyzer) and parameter optimization (Optimizer).

194

195

196

197 **Table 2.** Effect of the substrate conditions (limited vs. unlimited) on the different r/K NOB strategists

NOB substrate availability		<i>Nitrospira</i> (K-strategist)		<i>Nitrobacter</i> (r-strategist)
Limited (NH ₄ ⁺ -N feed)	↑	$\mu_{max,NS} \frac{S_{NO2}}{S_{NO2} + K_{NO2,NS}} \frac{S_O}{S_O + K_{O,NS}}$	↓	$\mu_{max,NB} \frac{S_{NO2}}{S_{NO2} + K_{NO2,NB}} \frac{S_O}{S_O + K_{O,NB}}$
Unlimited (NO ₂ ⁻ -N feed)	↓	$\mu_{max,NS} \frac{S_{NO2}}{S_{NO2} + K_{NO2,NS}} \frac{S_O}{S_O + K_{O,NS}}$	↑	$\mu_{max,NB} \frac{S_{NO2}}{S_{NO2} + K_{NO2,NB}} \frac{S_O}{S_O + K_{O,NB}}$

198

199 2.2.2. Setting the initial conditions (biomass composition and model parameters)

200 The mechanistic ASM-type models require a setting of the initial concentrations for specific
 201 groups of microorganisms. Based on the systematic protocol proposed in the previous study
 202 (Mehrani et al., 2022), a combination of mass balance calculations and microbiological analyses
 203 were applied to determine the initial concentrations of AOB, NOB (*Nitrospira*), comammox
 204 bacteria, and denitrifying heterotrophs. The abundances of *Nitrobacter* were below the detection
 205 limit in all the initial samples. Therefore, the initial concentrations of these bacteria were set at
 206 1% of *Nitrospira* initial concentration.

207

208 The initial values of kinetic and stoichiometric parameters were adopted from the literature (Koch
 209 et al., 2019; Yu et al., 2020; Metcalf and Eddy 2003; Hiatt and Grady, 2008; Roots et al. 2019).
 210 Subsequently, the kinetic parameters were subjected to sensitivity analysis (see Section 2.3).
 211 Three kinetic parameters, including K_O for AOB and NOB1 together with K_{NO2} for NOB1, were
 212 experimentally determined in batch tests (Mehrani et al., 2022). These parameters were not
 213 further adjusted during model calibration.

214

215

2.3. Sensitivity analysis and correlation matrix

Coupling local sensitivity analysis and the development of a correlation matrix allows reducing the number of parameters adjusted during model calibration. The analysis was carried out based on the results from trial T1 ($\text{NH}_4^+\text{-N}$ substrate) and trial T3 ($\text{NO}_2^-\text{-N}$ substrate). For trial T1, the different microbial groups (AOB, NOB1, CMX, and HET) were subjected to the sensitivity analysis concerning $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and $\text{NO}_2^-\text{-N}$ behavior. In trial T3, AOB were not considered due to the lack of substrate ($\text{NH}_4^+\text{-N}$) for these bacteria. Instead, NOB2 were included concerning $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ behavior. The classification of sensitivity coefficients, $S_{i,j}$, (from 0 to >2.5) was adopted from a recent study of Cao et al. (2020).

Subsequently, all pairs of the most sensitive kinetic parameters were evaluated by the correlation matrix. If the correlation coefficient for any pair is high enough, the calibration procedure can be simplified by adjusting only one of the two parameters (Zhu et al., 2015).

2.4. Model calibration and validation

The results of the trials at 20 °C (T1 and T3) with both substrates ($\text{NH}_4^+\text{-N}$ vs. $\text{NO}_2^-\text{-N}$) were used for model calibration, while the results of the two trials at 12 °C (T2 and T4) were used for validation. The Nelder-Mead simplex method with the maximum likelihood objective function was used for parameter estimation as described in detail in a previous study (Lu et al. 2019). The model efficiency was assessed using the conventional performance metrics, such as the determination coefficient (R^2), root mean square error (RMSE), and mean absolute error (MAE). In addition, the Janus coefficient (J^2) was calculated to evaluate a change in the model efficiency between the calibration and validation phases (Hauduc et al., 2015).

240 **2.5. Model evaluation and application**

241 After model validation, the competition between two NOB groups (*Nitrospira* and *Nitrobacter*)
242 under different substrate conditions ($\text{NH}_4^+\text{-N}$ and NO_2^-N) was investigated by comparing the
243 predicted process rates (mg N/L·d) in trials T1 and T3. Sankey diagrams were developed based
244 on the daily average rates at different phases of the experiment, i.e. 0 d (beginning), 20 d, and 35
245 d (end). The diagrams were used to identify the dominant N conversion pathways and assess the
246 effect of the accompanying processes (comammox, heterotrophic denitrification).

247

248

249 **3. RESULTS**

250 **3.1. Initial biomass composition**

251 In all the trials, the initial MLSS and MLVSS concentrations were approximately 2000 and 1500
252 mg/L, respectively (Table 1). The calculated initial concentrations of the specific microbial
253 groups (X_{AOB} , X_{NOB} , X_{CMX} , X_{HET}), estimated sequentially using the systematic protocol of
254 Mehrani et al. (2022), are shown in the SI (Table S3).

255

256 **3.2. Sensitivity analysis and correlation matrix**

257 Figure 3a,c shows the sensitivity coefficients, derived for all 18 kinetic coefficients (μ , K , b),
258 which were obtained based on the results from trial T1 ($\text{NH}_4^+\text{-N}$ substrate) and trial T3 (NO_2^-N
259 substrate). For trial T1, the different microbial groups (AOB, NOB1, CMX, and HET) were
260 subjected to the sensitivity analysis for the behavior of $\text{NH}_4^+\text{-N}$, NO_3^-N , and NO_2^-N . For trial
261 T3, NOB2 were considered instead of AOB, and the sensitivity analysis was performed based on
262 the behavior of NO_3^-N and NO_2^-N .

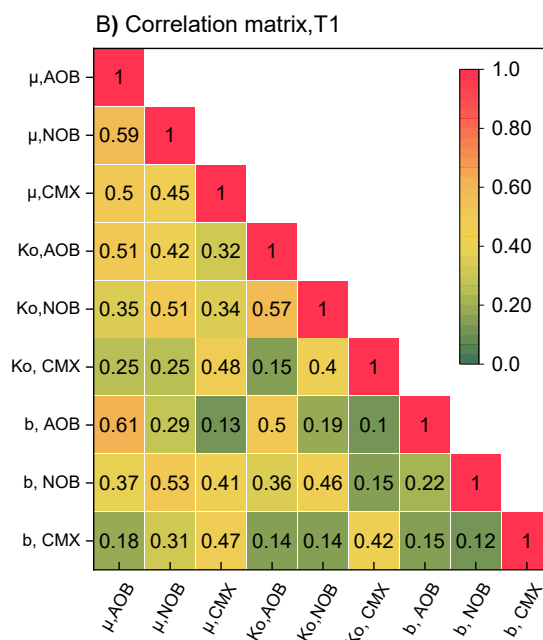
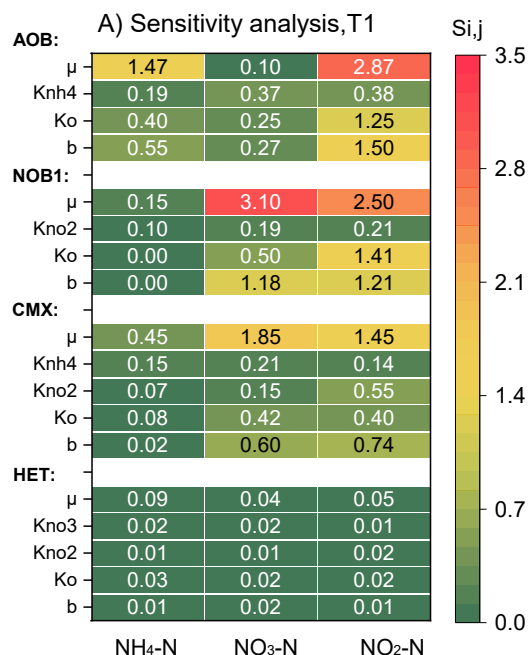
263

264 In general, for trial T1, the very influential coefficients ($S_{ij} \geq 2$) were μ_{AOB} and μ_{NOB1} concerning
265 the behavior of NO_2^-N , and μ_{NOB1} associated with the behavior of NO_3^-N . The μ_{AOB} had also a
266 substantial influence ($1 \leq S_{ij} < 2$) on the behavior of NH_4^+N , while μ_{CMX} was very influential (S_{ij}
267 ≥ 2) on the NO_3^-N behavior. The decay rates (b_{AOB} and b_{NOB1}) and DO half-saturation
268 coefficients (K_{OAOB} and K_{ONOB1}) were influential ($1 \leq S_{ij} < 2$) on the NO_2^-N behavior.

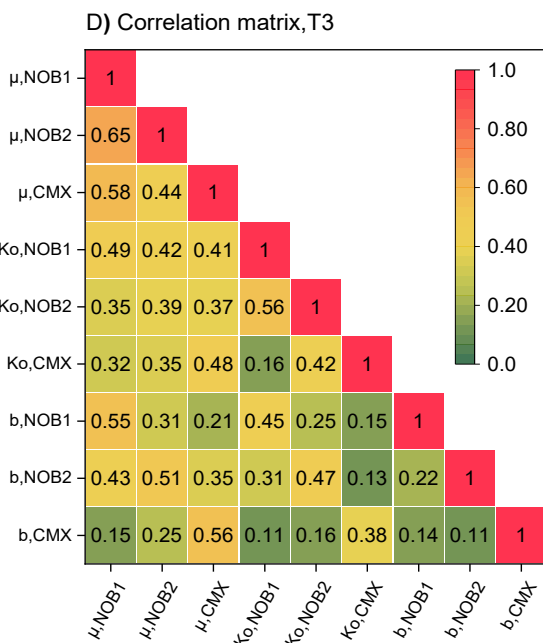
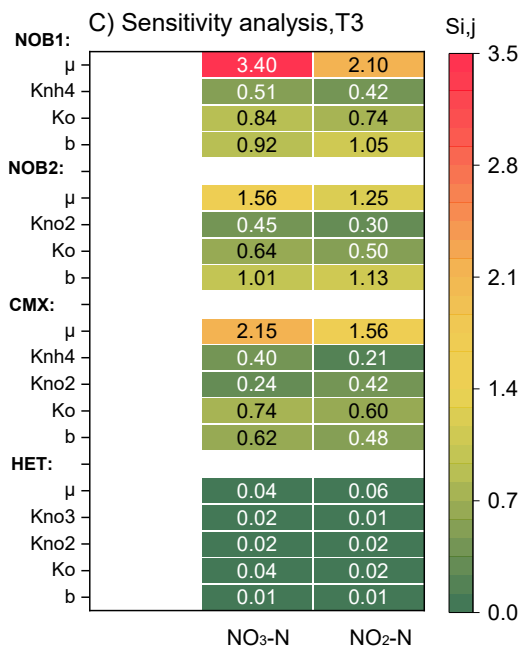
269
270 For trial T3, μ_{NOB1} and μ_{CMX} with $S_{ij} > 2$, followed by μ_{NOB2} with $S_{ij} = 1.8$, were the most
271 influential coefficients associated with the behavior of NO_3^-N . The decay rates (b_{NOB1} and b_{NOB2})
272 were also influential ($S_{ij} > 1$) with respect to the behavior of NO_2^-N and NO_3^-N .

273
274 Based on the results of sensitivity analysis for both trials, the very influential kinetic parameters
275 ($S_{ij} > 1$) were subjected to evaluation by a correlation matrix (Figure 3b,d). In general, the highest
276 correlation coefficients in both trials referred to the maximum growth rates (μ) and decay
277 coefficients (b) for all the nitrifier groups. Hence, for simplification of the calibration procedure,
278 b coefficients were omitted in further adjustments.

279



280



281

282

Figure 3. Sensitivity coefficients, $S_{i,j}$, and correlation matrix for the most sensitive kinetic parameters (μ , K , b) in

283

trial T1 (A-B) and trial T3 (C-D) (the effect of NOB2 in trial T1 was not considered due to very low sensitivity

284

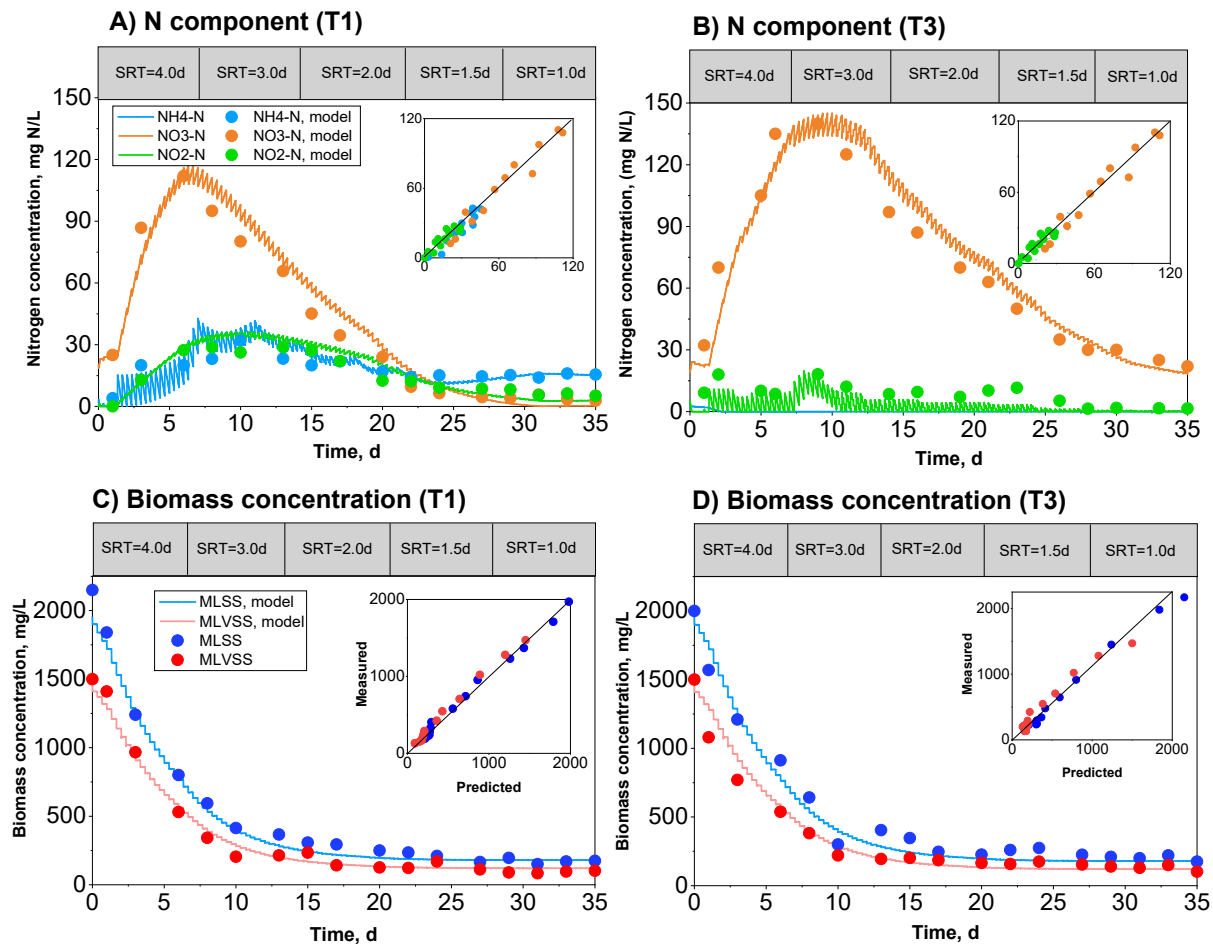
($S_{i,j} \leq 0.01$))

285

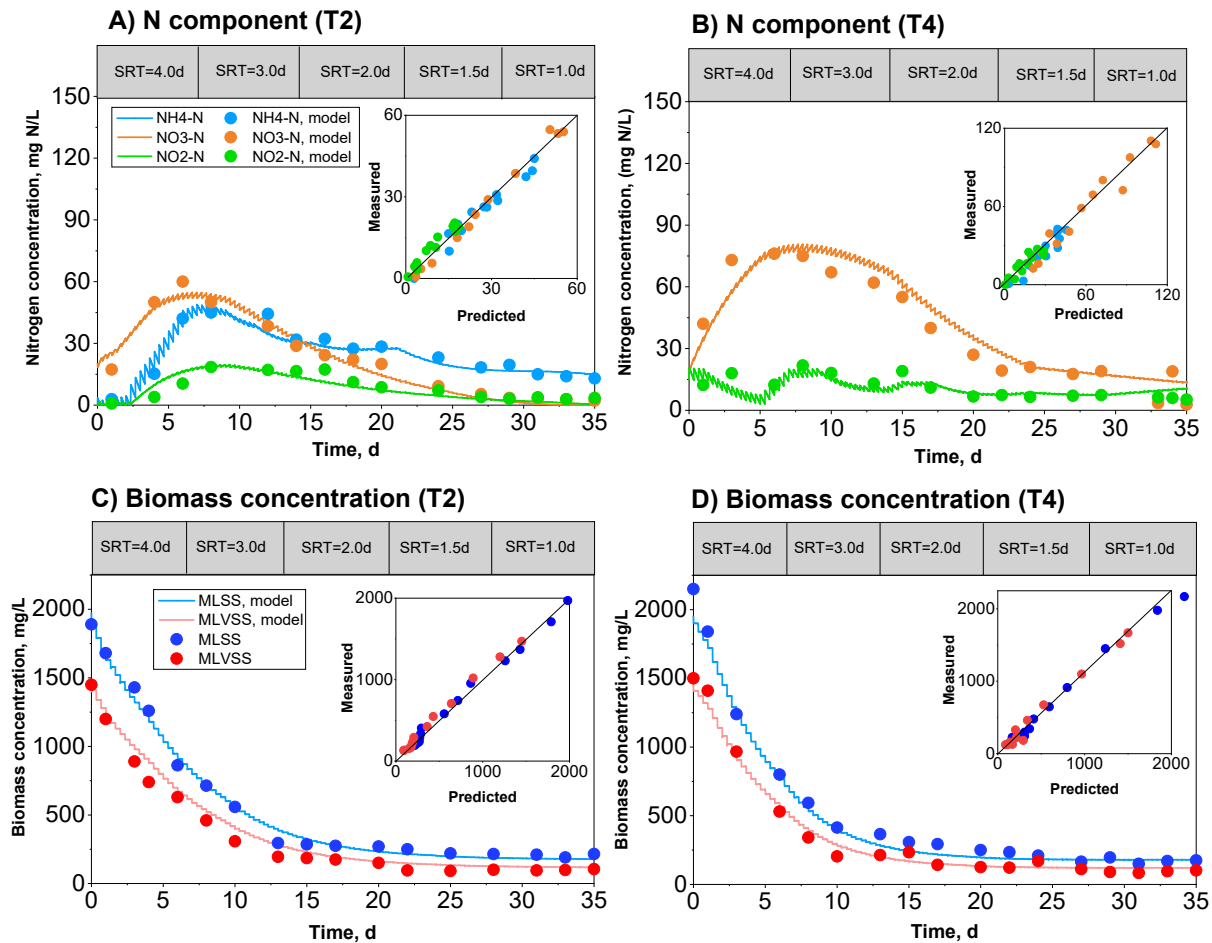
286

287 **3.3. Model calibration and validation**

288 The experimental observations and predicted behaviors of N species ($\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and
289 $\text{NO}_2^-\text{-N}$), and biomass concentrations for the calibrated model (trials T1 and T3) are shown in
290 Figure 4. Similar data for the validated model (trials T2 and T4) are shown in Figure 5. In all the
291 trials, the biomass concentrations (MLSS, MLVSS) were continuously decreased and stabilized
292 after two weeks at below 400 mg/L. Moreover, regardless of the substrate ($\text{NH}_4^+\text{-N}$ vs. $\text{NO}_2^-\text{-N}$),
293 the behavior of $\text{NO}_3^-\text{-N}$ was generally similar with a peak concentration occurring after one week.
294



295
296 **Figure 4.** Observed data vs. model predictions for the calibrated model: A) N components in trial T1, B) N
297 components in trial T3, C) Biomass concentrations in trial T1, D) Biomass concentrations in trial T3
298



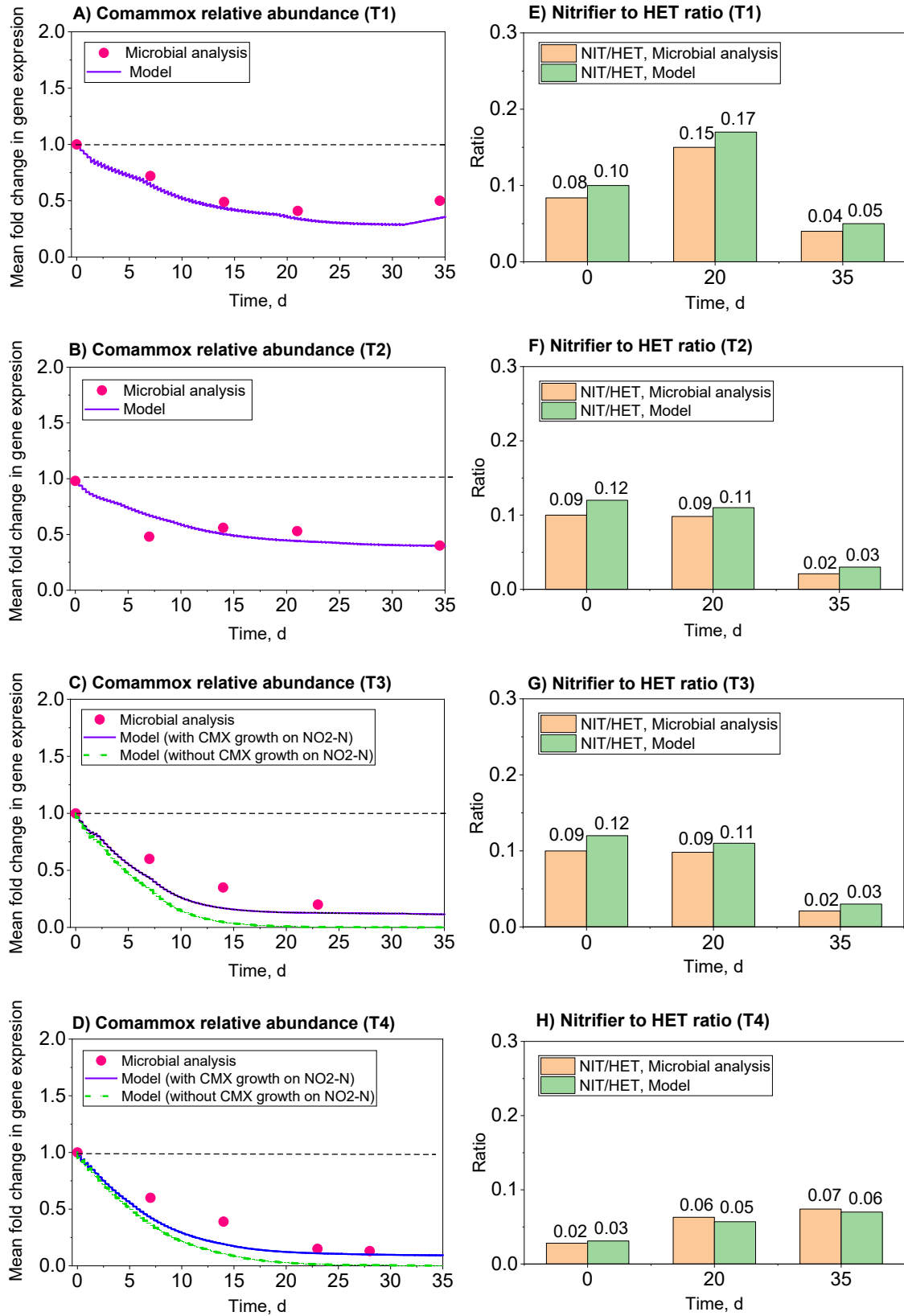
299
 300 **Figure 5.** Observed data vs. model predictions for the validated model: A) N components in T2, B) N components
 301 in T4, C) Biomass concentrations in T2, D) Biomass concentrations in T4

302
 303 In addition to the standard predictions (N species and biomass), the newly developed model also
 304 predicted two microbiological indicators, including the relative abundance of comammox
 305 bacteria and nitrifiers (NIT) to heterotrophs (HET) ratio (Figure 6). The relative abundance of
 306 comammox bacteria in the trials with $\text{NH}_4^+\text{-N}$ substrate (T1 and T2) decreased and stabilized at
 307 0.4-0.5 (Figure 6a-b), while in the trials with $\text{NO}_2^-\text{-N}$ substrate (T3 and T4), the abundance
 308 sharply decreased in the first week and stabilized at 0.1-0.2 at the end of trials (Figure 6c-d). The
 309 results with $\text{NO}_2^-\text{-N}$ substrate strongly suggest that comammox bacteria can grow on $\text{NO}_2^-\text{-N}$, as
 310 the model predictions significantly worsened without considering that process (Figure 6c-d).

311
312 The NIT/HET ratios in trials T1 and T2 were stable at 0.1-0.15 at the beginning and middle
313 phases and then decreased to 0.05 at the end of the trials (Figure 6e-f). In contrast, the NIT/HET
314 ratios in trials T3 and T4 were relatively stable (0.04-0.05) at the beginning and middle phase,
315 and then increased to >0.05 at the end of the trials (Figure 6g-h).

316
317 Table 3 shows a list of the model parameters along with their related values and sources of
318 information. Due to high sensitivity ($S_{i,j} \geq 0.5$), the μ and K_o for all the nitrifier groups (AOB,
319 NOB1, NOB2, CMX), and μ for heterotrophs were adjusted during mathematical optimization.
320 The estimated values were further discussed in relation to literature data (see Section 4.1). The
321 extended model prediction performance for each model output ($\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$) is
322 presented in Table 4. The calibrated model exposed a high goodness-of-fit for all the outputs in
323 terms of R^2 (>0.87) and RMSE and MAE errors (2.20-3.4), while the validated model revealed
324 slightly decreased (<7%) R^2 , and slightly increased (<15%) errors (RMSE, MAE) compared to
325 the calibration period. The Janus coefficient varied in the range of 1.31-1.97, which also
326 confirmed the model validity.

327



328
329 **Figure 6.** Observed vs. predicted relative abundances of comammox bacteria (A-D), and the ratio of nitrifiers to
330 heterotrophs (E-H) for trials T1-T4

331 **Table 3.** List of the adjusted kinetic parameters during model calibration, their values and sources of information

Parameter	Unit	Bacterial group					Source
		AOB	NOB 1 <i>Nitrospira</i>	NOB 2 <i>Nitrobacter</i>	CMX	HET	
Kinetic							
μ	d ⁻¹	0.38	0.21	0.60	0.20	1.00	Optimization
K _O	mg O ₂ /L	-	-	0.45	0.30	-	Optimization
K _O	mg O ₂ /L	0.17	0.13	-	-	-	Experimental
K _O	mg O ₂ /L	-	-	-	-	0.20 ^d	Literature
K _{NH4}	mg NH ₄ /L	0.67 ^a	-	-	0.012 ^b	-	Literature
K _{NO2}	mg NO ₂ /L	-	-	0.76 ^a	6.29 ^b	0.20 ^d	Literature
K _{NO2}	mg NO ₂ /L	-	0.06	-	-	-	Experimental
K _{NO3}	mg NO ₂ /L	-	-	-	-	0.20 ^d	Literature
b	d ⁻¹	0.15 ^c	0.05 ^c	0.05 ^c	0.05 ^f	0.40 ^d	Literature
Stoichiometric							
Y	gCOD/ gN	0.15 ^c	0.05 ^c	0.05 ^c	0.15 ^c	-	Literature
Y _H	gCOD/gCOD	-	-	-	-	0.6 ^d	Literature

332 μ : Max. growth rate constant, K_O: Dissolved oxygen half-saturation constant, K_{NH4}: Ammonia half-saturation
333 constant, K_{NO2}: Nitrite half-saturation constant, Y: Yield coefficient, b: Decay rate, a: (Yu et al., 2020), b: (Koch et al
334 2019), c: (Metcalf and Eddy 2003) d: (Hiatt and Grady, 2008), e: assumed equal to AOB (Roots et al. 2019), f: equal
335 to NOB (Mehrani et al. 2021)
336

337

338 **Table 4.** Summarized information on the model efficiency during the calibration and validation periods

Variable	Calibration phase				Validation phase				
	Trials	R ²	RMSE	MAE	Trials	R ²	RMSE	MAE	J ²
NH ₄ ⁺ -N		0.90	2.30	2.42		0.87	2.58	3.11	1.25
NO ₃ ⁻ -N	T 1	0.90	2.38	2.95	T 2	0.86	2.72	3.25	1.30
NO ₂ ⁻ -N		0.86	2.47	3.48		0.81	3.44	3.67	1.93
NH ₄ ⁺ -N		-	-	-		-	-	-	-
NO ₃ ⁻ -N	T 3	0.90	2.32	2.62	T 4	0.84	3.09	3.95	1.77
NO ₂ ⁻ -N		0.88	2.56	2.69		0.80	3.58	4.32	1.95

339

340 **3.4. Contribution of accompanying processes in N conversion**

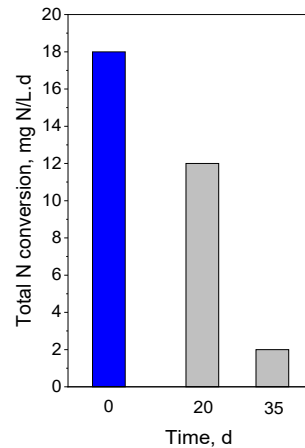
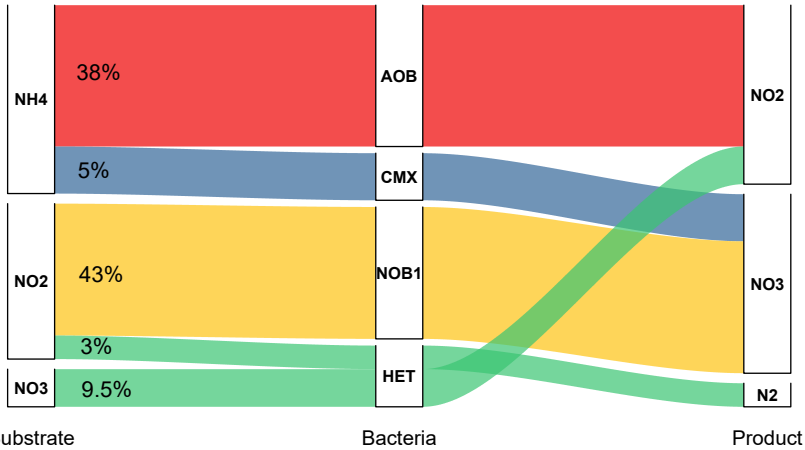
341 Figure 7 shows the N conversion pathways in trial T1 with NH₄⁺-N substrate. The relative
 342 contributions of some bacteria groups were significantly shifted during the trial. The canonical
 343 NOB1 and comammox bacteria continuously reduced their abundances - from 43 to 21% (NOB1)
 344 and from 5% to 1% (comammox). The contribution of NOB2 was negligible (<0.1%). The AOB
 345 increased their contribution from 38% at the beginning to 50% at the end of the trial. The
 346 contribution of denitrifying heterotrophs, mediating two steps of denitrification, increased from
 347 the initial 12.5% to 28% at the end of the trial.

348

349 Figure 8 shows the N conversion pathways in trial T3 with NO₂⁻-N substrate. The contribution of
 350 NOB1 was substantially decreased from 79% to 7%, while the NOB2 contribution had an
 351 increasing trend (4% to 89%) in the course of the trial. The comammox bacteria and denitrifying
 352 heterotrophs decreased their contributions, respectively, from 4% to <1% and from 16% to 3%.

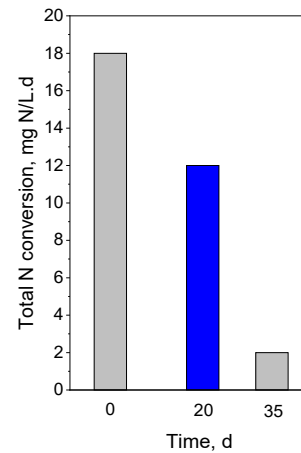
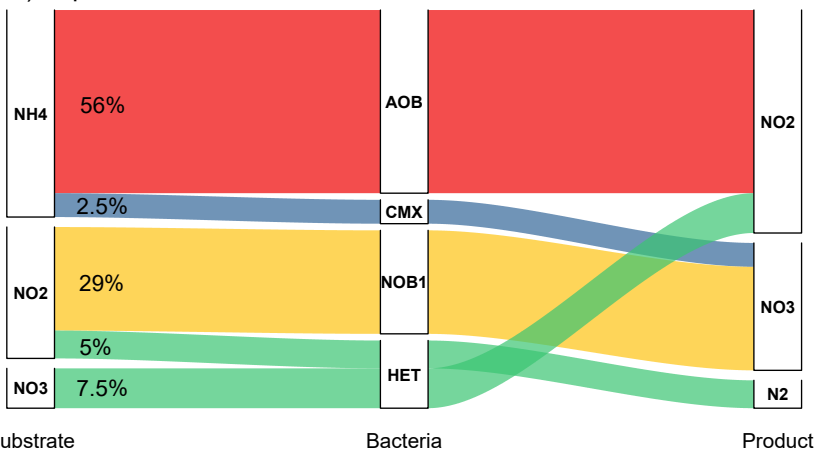
353

A) Experiment T1, T= 0d (beginning)



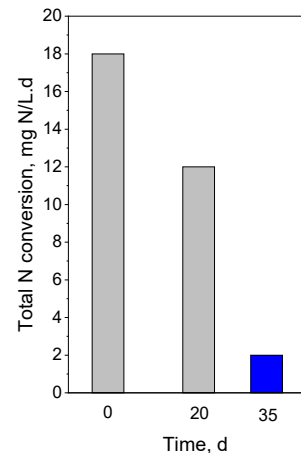
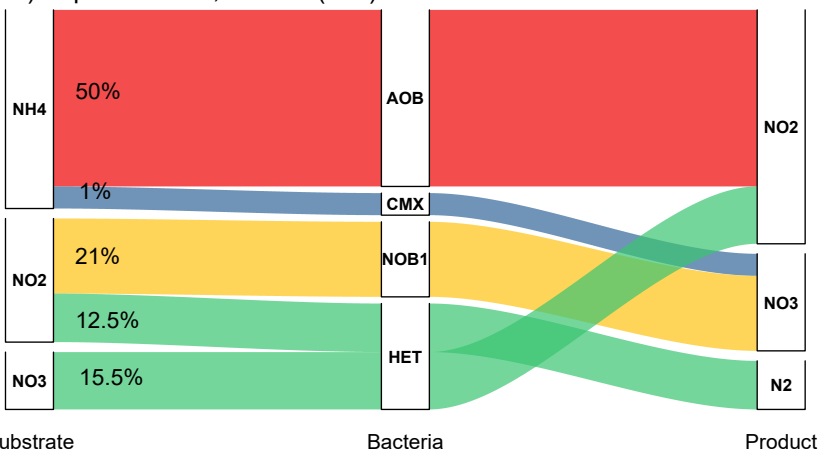
354

B) Experiment T1, T= 20d



355

C) Experiment T1, T= 35d (end)



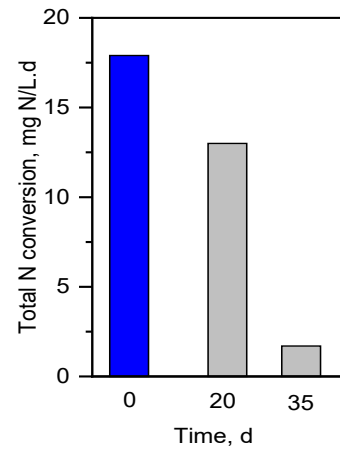
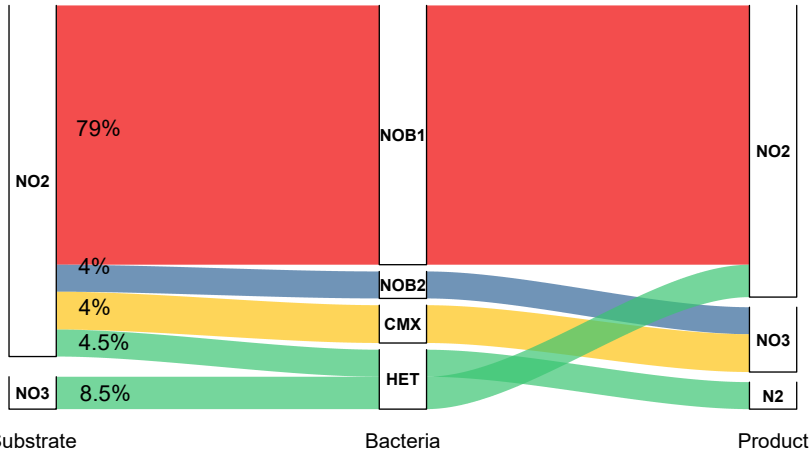
356

357 **Figure 7.** Sankey diagram showing the N conversion pathways in trial T1 (% represent the shares in the total
358 conversion rate (mg N/L.d): A) 0 d, B) 20 d, C) 35 d

359

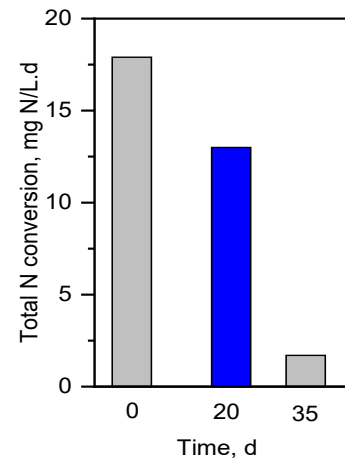
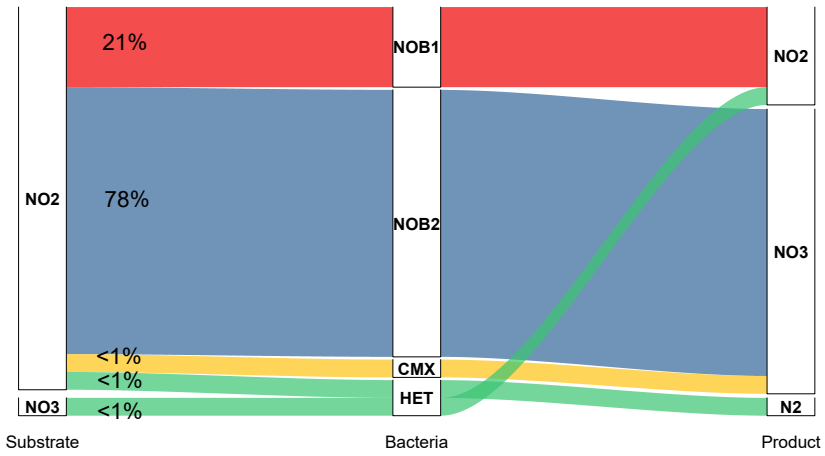
360

A) Experiment T3, T= 0d (beginning)



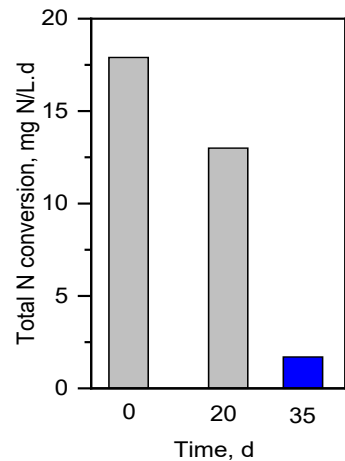
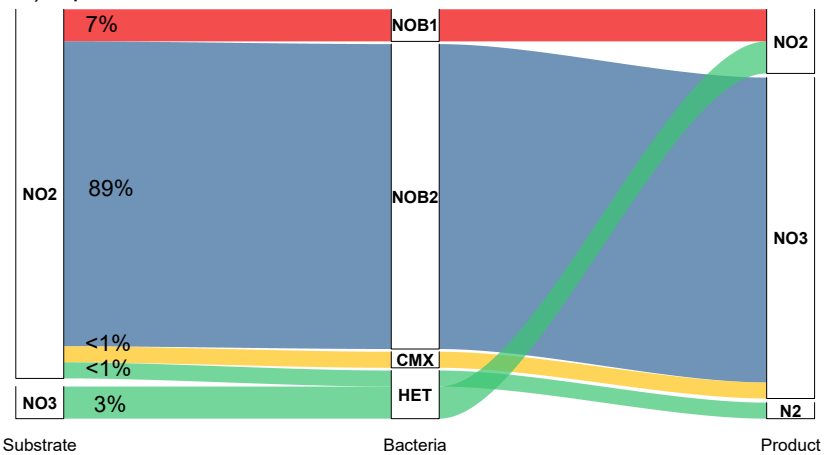
361

B) Experiment T3, T= 20d



362

C) Experiment T3, T= 35d



363

364 **Figure 8.** Conversion pathways of the N species for trial T3 (% represent the shares in the absolute total N
365 conversions (mg N/L.d): A) 0 d, B) 20 d, C) 35 d

366

367

368 **4. DISCUSSION**

369 **4.1. Factors influencing the competition between *Nitrospira* and *Nitrobacter***

370 In the present study, it has been confirmed that the NOB competition is predominantly impacted
371 by substrate (NO_2^- -N) concentrations, rather than temperature. Blackburne et al. (2007) attributed
372 the dominance of *Nitrospira* over *Nitrobacter* under low NH_4^+ -N and NO_2^- -N concentrations to
373 much lower inhibition thresholds of free ammonia (FA) and free nitrous acid (FNA). These
374 thresholds were respectively 0.04–0.08 mg NH_3 -N/L and 0.03 mg HNO_2 -N/L for *Nitrospira*, and
375 10 mg NH_3 -N/L and 0.2–0.4 mg HNO_2 -N/L for *Nitrobacter*.

376
377 It has been shown in several studies (Huang et al., 2010; Nogueira and Melo, 2006; Park et al.,
378 2017) that *Nitrobacter* was the dominating NOB at higher NO_2^- -N concentrations (> 80 mg N/L),
379 whereas *Nitrospira* thrived better under lower NO_2^- -N conditions. Moreover, Nogueira and Melo
380 (2006) observed that the dominance of *Nitrobacter* was not reverted after decreasing the NO_2^- -N
381 concentration to the original (low) level. The authors provided two possible explanations for that
382 observation, including inhibition of *Nitrospira* by high densities of *Nitrobacter* or not sufficient
383 experimental period.

384
385 The DO concentration is another important operational factor that influences *Nitrospira*
386 abundance in WWTPs (Ushiki et al., 2017; Chang et al., 2019). Park et al. (2017) attributed a
387 strong enrichment of *Nitrospira* (at the expense of *Nitrobacter*) in a DO- and NO_2^- -N -limited
388 SBR to the lower affinity constants of *Nitrospira* for both substrates (see Table S5). (Liu and
389 Wang, 2013) found that in addition to low DO concentrations (0.2-0.4 mg O_2 /L), extending the
390 SRT from 10 d to 40 d resulted in the dominance of *Nitrospira* over *Nitrobacter*.

391

392 Li et al., (2021) found that the SRT of 10 d and shorter aeration times (1.5 h) allowed to
393 completely wash out NOB from the system. Gradually prolonged aeration times (3-3.5 h),
394 applied subsequently, did not result in enriching NOB. Both *Nitrospira* and *Nitrobacter*
395 abundances remained stable, but at a low level, which resulted in maintaining long-term NO₂⁻-N
396 accumulation in a wide temperature range (18-29 C).

397
398 *Nitrospira* may be better adapted to slightly higher pH (8–8.3 vs. 7.6–8.2) and temperatures (29–
399 30 °C vs. 24–25 °C) in addition to low NO₂⁻-N and DO concentrations. Sun et al., (2022)
400 investigated the coexistence of *Nitrobacter* and *Nitrospira* at various pH (6.0-8.5). The optimum
401 pH of 7.0 was found in terms of the maximum specific growth rate (μ) and substrate affinity
402 (K_{NO_2}) for NO₂⁻-N oxidation, which also correlated with the highest absolute abundances of the
403 functional genes of *Nitrobacter* (*nxrA*) and *Nitrospira* (*nxB*).

404
405 Ushiki et al. (2017) hypothesized that *Nitrospira* had other characteristics to compete with
406 *Nitrobacter* and other NOB in nitrite-limited environments. *Nitrobacter* has a cytoplasmic nitrite
407 oxidoreductase (*nxrA*) that is responsible for the reverse transport of NO₂⁻-N and NO₃⁻-N through
408 the inner membrane. On the contrary, *Nitrospira* encodes for periplasmic *nxB*, which catalyses
409 nitratation. The periplasmic oxidation is advantageous since it generates a larger specific proton
410 motive force and avoids the transmembrane exchange of NO₂⁻-N and NO₃⁻-N (Pester et al.,
411 2014).

412
413 Table S5 shows a literature review of the kinetic and stoichiometric parameters for *Nitrospira*
414 and *Nitrobacter*. The ranges of μ for r-strategist *Nitrobacter* and K-strategist *Nitrospira* are 0.31-
415 1.28 d⁻¹ and 0.15-0.93 d⁻¹, respectively. In the present study, the estimated μ for *Nitrobacter* (0.6

416 d^{-1}) was three times higher than *Nitrospira* ($0.21 d^{-1}$). In addition, the experimentally obtained K_o
417 of $0.13 \text{ mgO}_2/\text{L}$ for NOB1 (*Nitrospira*) is slightly lower than the overall ranges in Table S6
418 ($K_{O,Nitrobacter} = 0.17\text{-}4.32 \text{ mgO}_2/\text{L}$, $K_{O,Nitrospira} = 0.33\text{-}1.35 \text{ mgO}_2/\text{L}$), while the optimized K_o of 0.45
419 mgO_2/L for NOB2 is in the lower range ($0.45\text{-}1.35 \text{ mgO}_2/\text{L}$) reported by Yu et al. (2020). It
420 should be noted, however, that in the literature (O'Shaughnessy, 2016), the K_o values for
421 *Nitrospira* and other NOB genera were as low as $0.04 d^{-1}$.

422

423 **4.2. Role of comammox-*Nitrospira* in the systems fed with different N substrates**

424 Maddela et al. (2021) noted that comammox bacteria have several competitive advantages over
425 coexisting canonical nitrifiers, including the capability of thriving at low DO levels and a high
426 substrate affinity. The high $\text{NH}_4^+\text{-N}$ affinity of pure cultured comammox bacteria (*N. inopinata*)
427 is indicated by extremely low half-saturation constants, which are 4- to 2500-fold lower than the
428 values reported for AOB. Comammox bacteria also show a lower $\text{NO}_2^-\text{-N}$ affinity than canonical
429 NOB. Moreover, the abundances of comammox bacteria (*N. inopinata*) showed significant
430 positive correlations with canonical NOB rather than AOB, which may suggest that comammox
431 bacteria more actively mediate $\text{NO}_2^-\text{-N}$ oxidation rather than $\text{NH}_4^+\text{-N}$ oxidation. The authors
432 concluded that switches between the modes of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ oxidation in comammox
433 bacteria remain unknown, and it is difficult to identify the environmental and operational factors
434 determining the preferred nitrogen source.

435

436 The simulation results of the present study, strongly support the hypothesis that comammox
437 bacteria can grow on $\text{NO}_2^-\text{-N}$. Following the discovery of comammox, it was thought that
438 comammox-*Nitrospira*, do not possess this ability (Koch et al., 2019). Based on the findings of a
439 previous investigation by Kits et al. (2017), this was explained by an extremely low $\text{NO}_2^-\text{-N}$



440 affinity. In comparison with canonical *Nitrospira*, *N. inopinata* (comammox bacteria) had a 50-
441 fold lower NO_2^- -N affinity (Kits et al., 2017). In a recent study, Shao and Wu (2021) found that
442 the NO_2^- -N affinities were variable among the comammox species. Under NH_4^+ -N limiting
443 conditions, some authors did not exclude the possibility of comammox bacteria using NO_2^- -N as
444 electron donors (Kits et al., 2017; Palomo et al., 2018; Roots et al., 2019).

445
446 There is no consensus in the literature if NO_2^- -N is released outside of the cells during the
447 comammox process. Kits et al. (2017) observed that comammox *Nitrospira* could produce NO_2^- -
448 N as an extracellular transit product during complete nitrification. Transient NO_2^- -N
449 accumulation produced by *N. inopinata* (comammox *Nitrospira*) during nitritation was reported
450 by Ren et al. (2020). In contrast, Wu et al. (2020) found that comammox *Nitrospira* consumed
451 NH_4^+ -N and produced NO_3^- -N at the ratio of nearly 1:1 without any NO_2^- -N accumulation.

452 453 **4.3. Further developments of two-NOB models describing the competition with other NOB** 454 **groups**

455 The model developed in the present study may be used to describe a competition of *Nitrospira*
456 with NOB genera other than *Nitrobacter*. In particular, *Ca. Nitrotoga* has been detected as the
457 main NOB genus, either alone or together with *Nitrospira*, in different full-scale WWTPs
458 worldwide (Lücker et al., 2015; Saunders et al., 2016; Chen et al. 2020), and many pilot-scale
459 systems (Figdore et al., 2018; Jiang et al., 2018; Persson et al., 2017; Wu et al., 2018; Zheng et
460 al., 2019b). *Ca. Nitrotoga* can oxidize NO_2^- -N at temperatures ranging from 4 to 28°C (Lantz et
461 al., 2021), its optimum temperature (22-23°C) is lower compared to other NOB genera (Zheng et
462 al., 2020; Ishii et al., 2020). Nowka et al. (2015) observed that *Ca. Nitrotoga* outcompeted
463 *Nitrospira* and *Nitrobacter* during a long-term cultivation at 5 and 10°C. Liu et al. (2021)

464 calculated a very low temperature correction factor of 1.042 in the temperature range of 4-22 °C
465 for NOB dominated by *Ca. Nitrotoga*. Speick et al. (2021) concluded that the adaptation of *Ca.*
466 *Nitrotoga* to low temperatures may be beneficial for the recovery of nitrification during cold
467 seasons.

468
469 K_O for *Ca. Nitrotoga* has been determined by Zheng et al. (2020) as 0.59 ± 0.11 mg O₂/L. This
470 value is slightly higher than K_O for *Nitrospira*, and much lower than *Nitrobacter* (see Table 5).
471 Both *Nitrospira* and *Ca. Nitrotoga* resisted at intermittent aeration in a PN/A system
472 (Gustavsson, 2020), whereas in another study, *Ca. Nitrotoga* was shown to have a lower DO
473 affinity than *Nitrospira* (Zheng et al. 2020). Qian et al. (2021) found in a partial
474 nitrification/anammox (PN/A) system that increasing the DO concentration from 0.4 to 1.8 mgO₂/L
475 led to an increased abundance of *Ca. Nitrotoga* over *Nitrospira*. On the contrary, Jiang et al.
476 (2018) found that a high DO of 2–2.5 mgO₂/L was sufficient for the washout of both *Nitrospira*
477 and *Ca. Nitrotoga* in a PN/A system.

478
479 Higher substrate concentrations may be beneficial to *Nitrotoga* (Nowka et al. 2015). Kinnunen et
480 al. (2017) discovered that at a NO₂⁻-N concentration of 1 mg N/L, *Ca. Nitrotoga* outcompeted
481 *Nitrospira* in a biofilm community, but *Nitrospira* dominated at a tenfold lower substrate
482 concentration. K_{NO_2} for *Ca. Nitrotoga* was reported in a wide range of 0.345-1.68 mg N/L (Zheng
483 et al., 2020; Kitzinger et al. 2018; Ishii et al. 2017; Wegen et al. 2019; Nowka et al. 2015). *Ca.*
484 *Nitrotoga* was also shown to be much more resistant to free ammonia (FA) and free nitrous acid
485 (FNA) exposure than *Nitrobacter* and *Nitrospira* (Li et al. 2020, Zheng et al., 2021).

486

487 The pH preference of *Ca. Nitrotoga* is neutral to slightly alkaline range as typical for nearly all
488 NOB (Speick et al., 2021). The optimum pH for *Ca. Nitrotoga* has been determined in a wide
489 range as 7.1 – 7.6 (Kitzinger et al., 2018), 7.5 (Zheng et a., 2020), 8.3 (Ishii et al., 2020).

490

491 **4.4. Interactions of nitrifiers and heterotrophs in N removal systems fed with inorganic** 492 **carbon**

493 In the present study, the strategy of progressive SRT reduction and the lack of organic carbon in
494 the feed resulted in re-arrangements within the heterotrophic bacteria subpopulation (Table S2).

495 Bacteria belonging to the order *Saprospiraceae* and genera *Caldilinea*, *Curvibacter*, *Dokdonella*,
496 *Holophaga*, *Tetrasphaera* constituted predominant components in the inoculum biomass.

497 However, during all the trials, those bacteria were systematically outcompeted by members of
498 *Acidovorax*, *Hydrogenophaga*, *Comamonas*, *Pseudomonas*, *Simplicispira*, and *Thermomonas*.

499

500 Based on the reported characteristics of the identified heterotrophs, there are three possible
501 mechanisms of the surveillance and competitiveness of those bacteria in comparison with other
502 heterotrophs under extremely short SRTs and inorganic feeding medium: (i) heterotrophic
503 denitrification with soluble microbial products (SMP) and extracellular polymeric substances
504 (EPS), generated by AOB and NOB (Nogueira et al. 2005; Sepehri and Sarrafzadeh 2019), (ii)
505 heterotrophic nitrification (Chen et al. 2012), (iii) predation of heterotrophs on autotrophic
506 bacteria (Dolinšek et al. 2013).

507

508 In particular, during the trials with $\text{NH}_4\text{-N}$ as a sole N source, a wider range and more equally
509 represented heterotrophic bacteria groups have been detected during the final stages. Similar to
510 the present study, a noticeable occurrence of the *Acidovorax* and *Pseudomonas* representatives in



511 the reactor fed with an inorganic medium was observed by Keluskar et al. (2013). The growth of
512 those heterotrophs was supported by organic compounds, such as pyruvate, excreted by AOB
513 (*Nitrosomonas*). An additional metabolic pathway enabling the survival of specific heterotrophs
514 (*Hydrogenophaga*) in the systems operated under the limited carbon availability is
515 hydrogenotrophic denitrification with the utilization of hydrogen in the absence of organic
516 compounds. Furthermore, representatives of *Rhodococcus* have been characterized as bacteria
517 which are capable of performing simultaneous heterotrophic nitrification and aerobic
518 denitrification (Chen et al. 2012).

519
520 In the present study, in the trials with NO₂-N as a sole N source, members of the *Comamonas*
521 genera outcompeted other heterotrophs, and their relative abundances exceeded 50% in both trials
522 (at 12 and 20°C). Representatives of the *Comamonas* are well known denitrifying heterotrophs,
523 which are capable of utilizing a wide range of organic compounds, such as amino acids,
524 carboxylic acids, steroids and aromatic compounds (Wu et al., 2018), thus due to wide metabolic
525 properties were capable to develop substrate dependencies with *Nitrobacter* NOB.

526
527 Predation is another factor affecting the occurrence of specific heterotrophs and their interaction
528 with the nitrifiers (Dolinšek et al. 2013). Daims et al. (2016) showed that abundances of
529 *Nitrospira* were dramatically reduced due to the predation by *B. bacteriovorus*. In the present
530 study, during the trials with NH₄-N, predator bacteria belonging to the *Bdellovibrio* genus were
531 detected in the abundances > 3%. On the other hand, lower abundances of these bacteria were
532 found during the trials with NO₂-N, when *Nitrobacter* was predominant. This finding suggests
533 that predation may indeed reflect a selective pressure on specific NOB.

534

535 **5. CONCLUSIONS**

- 536 ■ A two-step nitrification model was extended with different NOB groups (competing r
537 strategists vs. K strategists) to increase the prediction accuracy of modeling nitrifying systems
538 under diverse NO₂⁻-N availabilities in the substrate.
- 539 ■ In addition to the standard prediction parameters (N species and biomass concentrations),
540 microbiological indicators such as the relative abundance of comammox and the ratio of
541 nitrifiers to heterotrophs, are useful target variables for calibration of mechanistic models as
542 they revealed the dominant conversion pathways in the studied systems.
- 543 ■ A combination of microbiological analyses and modeling simulation results confirmed that
544 comammox bacteria can grow on NO₂⁻-N as a sole N substrate.
- 545 ■ In contrast to canonical nitrifiers, comammox played a minor role in the study system.
546 However, the impact of these bacteria needs to be further explored by modeling systems
547 containing a higher abundance of *Nitrospira*.
- 548 ■ Even though comammox played a minor role in the studied system compared to the canonical
549 nitrifiers, the impact of these bacteria should further be explored by modeling systems
550 containing a higher abundances of *Nitrospira*.

551

552 **ACKNOWLEDGMENTS**

553 This study was supported by the Polish National Science Center under project no. UMO-
554 2017/27/B/NZ9/01039.

555

556

557

558 **REFERENCES**

- 559 Al-Hazmi HE, Lu X, Majtacz J, Kowal P, Xie L, Makinia J. Optimization of the Aeration Strategies
560 in a Deammonification Sequencing Batch Reactor for Efficient Nitrogen Removal and
561 Mitigation of N₂O Production. *Environmental Science & Technology* 2021; 55: 1218-1230.
- 562 Blackburne R, Vadivelu VM, Yuan Z, Keller J. Kinetic characterisation of an enriched Nitrospira
563 culture with comparison to Nitrobacter. *Water Research* 2007; 41: 3033-3042.
- 564 Cao J, Zhang T, Wu Y, Sun Y, Zhang Y, Huang B, et al. Correlations of nitrogen removal and core
565 functional genera in full-scale wastewater treatment plants: Influences of different
566 treatment processes and influent characteristics. *Bioresource Technology* 2020; 297:
567 122455.
- 568 Cao Y, van Loosdrecht MCM, Daigger GT. Mainstream partial nitrification–anammox in municipal
569 wastewater treatment: status, bottlenecks, and further studies. *Applied Microbiology and
570 Biotechnology* 2017; 101: 1365-1383.
- 571 Chang M, Wang Y, Pan Y, Zhang K, Lyu L, Wang M, et al. Nitrogen removal from wastewater
572 via simultaneous nitrification and denitrification using a biological folded non-aerated filter.
573 *Bioresource Technology* 2019; 289: 121696.
- 574 Chen P, Li J, Li QX, Wang Y, Li S, Ren T, et al. Simultaneous heterotrophic nitrification and
575 aerobic denitrification by bacterium *Rhodococcus* sp. CPZ24. *Bioresource Technology*
576 2012; 116: 266-270.
- 577 Chen H, Wang M, Chang S. Disentangling Community Structure of Ecological System in
578 Activated Sludge: Core Communities, Functionality, and Functional Redundancy.
579 *Microbial Ecology* 2020; 80: 296-308.
- 580 Daims H, Lücker S, Wagner M. A New Perspective on Microbes Formerly Known as Nitrite-
581 Oxidizing Bacteria. *Trends in Microbiology* 2016; 24: 699-712.

- 582 Daims H, Lebedeva EV, Pjevac P, Han P, Herbold C, Albertsen M, et al. Complete nitrification by
583 Nitrospira bacteria. *Nature* 2015; 528: 504.
- 584 Dolinšek J, Lagkouvardos I, Wanek W, Wagner M, Daims H. Interactions of Nitrifying Bacteria
585 and Heterotrophs: Identification of a Micavibrio-Like Putative Predator of Nitrospira spp.
586 *Applied and Environmental Microbiology* 2013; 79: 2027-2037.
- 587 Duan H, Ye L, Wang Q, Zheng M, Lu X, Wang Z, et al. Nitrite oxidizing bacteria (NOB) contained
588 in influent deteriorate mainstream NOB suppression by sidestream inactivation. *Water*
589 *Research* 2019; 162: 331-338.
- 590 Feng S, Tan CH, Constancias F, Kohli GS, Cohen Y, Rice SA. Predation by *Bdellovibrio*
591 bacteriovorus significantly reduces viability and alters the microbial community
592 composition of activated sludge flocs and granules. *FEMS Microbiology Ecology* 2017; 93:
593 20-28.
- 594 Figdore BA, Stensel HD, Winkler M-KH. Comparison of different aerobic granular sludge types
595 for activated sludge nitrification bioaugmentation potential. *Bioresource Technology* 2018;
596 251: 189-196.
- 597 Gustavsson DJI, Suarez C, Wilén B-M, Hermansson M, Persson F. Long-term stability of partial
598 nitritation-anammox for treatment of municipal wastewater in a moving bed biofilm reactor
599 pilot system. *Science of The Total Environment* 2020; 714: 136342.
- 600 Hauduc H, Neumann MB, Muschalla D, Gamerith V, Gillot S, Vanrolleghem PA. Efficiency
601 criteria for environmental model quality assessment: A review and its application to
602 wastewater treatment. *Environmental Modelling & Software* 2015; 68: 196-204.
- 603 Henze MG, W., Mino, T., van Loosdrecht, M.C.M. Activated sludge models ASM1, ASM2,
604 ASM2d and ASM3. London, UK: IWA Publishing, 2000.



- 605 Hiatt WC, Grady CPL. An Updated Process Model for Carbon Oxidation, Nitrification, and
606 Denitrification. *Water Environment Research* 2008; 80: 2145-2156.
- 607 Huang Z, Gedalanga PB, Asvapathanagul P, Olson BH. Influence of physicochemical and
608 operational parameters on *Nitrobacter* and *Nitrospira* communities in an aerobic activated
609 sludge bioreactor. *Water Research* 2010; 44: 4351-4358.
- 610 Ishii K, Fujitani H, Sekiguchi Y, Tsuneda S. Physiological and genomic characterization of a new
611 ‘*Candidatus Nitrotoga*’ isolate. *Environmental Microbiology* 2020; 22: 2365-2382.
- 612 Jiang H, Liu G-h, Ma Y, Xu X, Chen J, Yang Y, et al. A pilot-scale study on start-up and stable
613 operation of mainstream partial nitrification-anammox biofilter process based on online
614 pH-DO linkage control. *Chemical Engineering Journal* 2018; 350: 1035-1042.
- 615 Keene NA, Reusser SR, Scarborough MJ, Grooms AL, Seib M, Santo Domingo J, et al. Pilot plant
616 demonstration of stable and efficient high rate biological nutrient removal with low
617 dissolved oxygen conditions. *Water Research* 2017; 121: 72-85.
- 618 Keluskar R, Nerurkar A, Desai A. Mutualism between autotrophic ammonia-oxidizing bacteria
619 (AOB) and heterotrophs present in an ammonia-oxidizing colony. *Archives of*
620 *Microbiology* 2013; 195: 737-747.
- 621 Kent TR, Sun Y, An Z, Bott CB, Wang Z-W. Mechanistic understanding of the NOB suppression
622 by free ammonia inhibition in continuous flow aerobic granulation bioreactors.
623 *Environment International* 2019; 131: 105005.
- 624 Kinnunen M, Gülay A, Albrechtsen H-J, Dechesne A, Smets BF. *Nitrotoga* is selected over
625 *Nitrospira* in newly assembled biofilm communities from a tap water source community at
626 increased nitrite loading. *Environmental Microbiology* 2017; 19: 2785-2793.
- 627 Kits KD, Sedlacek CJ, Lebedeva EV, Han P, Bulaev A, Pjevac P, et al. Kinetic analysis of a
628 complete nitrifier reveals an oligotrophic lifestyle. *Nature* 2017; 549: 269-272.

- 629 Koch H, van Kessel MAHJ, Lücker S. Complete nitrification: insights into the ecophysiology of
630 comammox *Nitrospira*. *Applied Microbiology and Biotechnology* 2019; 103: 177-189.
- 631 Laanbroek HJ, Bodelier PLE, Gerards S. Oxygen consumption kinetics of *Nitrosomonas europaea*
632 and *Nitrobacter hamburgensis* grown in mixed continuous cultures at different oxygen
633 concentrations. *Archives of Microbiology* 1994; 161: 156-162.
- 634 Lantz MA, Boddicker AM, Kain MP, Berg OMC, Wham CD, Mosier AC. Physiology of the
635 Nitrite-Oxidizing Bacterium *Candidatus Nitrotoga* sp. CP45 Enriched From a Colorado
636 River. *Frontiers in microbiology* 2021; 12: 709371-709371.
- 637 Lawson CE, Lücker S. Complete ammonia oxidation: an important control on nitrification in
638 engineered ecosystems? *Current Opinion in Biotechnology* 2018; 50: 158-165.
- 639 Li S, Duan H, Zhang Y, Huang X, Yuan Z, Liu Y, et al. Adaptation of nitrifying community in
640 activated sludge to free ammonia inhibition and inactivation. *Science of The Total*
641 *Environment* 2020; 728: 138713.
- 642 Li S, Li J, Yang S, Zhang Q, Li X, Zhang L, et al. Rapid achieving partial nitrification in domestic
643 wastewater: Controlling aeration time to selectively enrich ammonium oxidizing bacteria
644 (AOB) after simultaneously eliminating AOB and nitrite oxidizing bacteria (NOB).
645 *Bioresource Technology* 2021; 328: 124810.
- 646 Liu G, Wang J. Long-Term Low DO Enriches and Shifts Nitrifier Community in Activated Sludge.
647 *Environmental Science & Technology* 2013; 47: 5109-5117.
- 648 Liu H, Zhu L, Tian X, Yin Y. Seasonal variation of bacterial community in biological aerated filter
649 for ammonia removal in drinking water treatment. *Water Research* 2017; 123: 668-677.
- 650 Liu X, Huang M, Bao S, Tang W, Fang T. Nitrate removal from low carbon-to-nitrogen ratio
651 wastewater by combining iron-based chemical reduction and autotrophic denitrification.
652 *Bioresource Technology* 2020; 301: 122731.

- 653 Liu Y, Li S, Ni G, Duan H, Huang X, Yuan Z, et al. Temperature Variations Shape Niche
654 Occupation of Nitrotoga-like Bacteria in Activated Sludge. ACS ES&T Water 2021; 1:
655 167-174.
- 656 Lückner S, Schwarz J, Gruber-Dorninger C, Spieck E, Wagner M, Daims H. Nitrotoga-like bacteria
657 are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants.
658 The ISME Journal 2015; 9: 708-720.
- 659 Ma Y, Domingo-Félez C, Plósz BG, Smets BF. Intermittent Aeration Suppresses Nitrite-Oxidizing
660 Bacteria in Membrane-Aerated Biofilms: A Model-Based Explanation. Environmental
661 Science & Technology 2017; 51: 6146-6155.
- 662 Maddela NR, Gan Z, Meng Y, Fan F, Meng F. Occurrence and Roles of Comammox Bacteria in
663 Water and Wastewater Treatment Systems: A Critical Review. Engineering 2021; 0-
- 664 Mehrani M-J, Lu X, Sobotka D, Kowal P, Makinia J. Incorporation of the complete ammonia
665 oxidation (comammox) process for nitrification modeling in activated sludge systems.
666 Journal of Environmental Management 2021; 297: 113223.
- 667 Mehrani M-J, Sobotka D, Kowal P, Ciesielski S, Makinia J. The occurrence and role of Nitrospira
668 in nitrogen removal systems. Bioresource Technology 2020; 303: 122936.
- 669 Mehrani M-J, Sobotka D, Kowal P, Guo J, Makinia J. New insights into modeling two-step
670 nitrification in activated sludge systems – the effects of initial biomass concentrations,
671 comammox and heterotrophic activities. Science of the Total Environment 2022, 157628.
- 672 Metcalf and Eddy, Inc., Wastewater Engineering: Treatment and Resource Recovery, 5th Edition.
673 McGraw-Hill , New York, United States, 2014.
- 674 Metcalf and Eddy, Inc., Wastewater Engineering : Treatment and Reuse, 4th Edition. McGraw-
675 Hill, New York, United States, 2003.

- 676 Metcalf and Eddy, Inc., Wastewater Engineering: Treatment, Disposal, and Reuse, 3rd Edition.
677 McGraw-Hill, New York, United States, 1990.
- 678 Nogueira R, Melo LF. Competition between *Nitrospira* spp. and *Nitrobacter* spp. in nitrite-
679 oxidizing bioreactors. *Biotechnology and Bioengineering* 2006; 95: 169-175.
- 680 Nogueira R, Elenter D, Brito A, Melo LF, Wagner M, Morgenroth E. Evaluating heterotrophic
681 growth in a nitrifying biofilm reactor using fluorescence in situ hybridization and
682 mathematical modeling. *Water Science and Technology* 2005; 52: 135-141.
- 683 Nowka B, Daims H, Spieck E. Comparison of Oxidation Kinetics of Nitrite-Oxidizing Bacteria:
684 Nitrite Availability as a Key Factor in Niche Differentiation. *Applied and Environmental*
685 *Microbiology* 2015; 81: 745.
- 686 O'Shaughnessy M. Mainstream Deammonification (WERF Report INFR6R11). Water
687 Environment Research Foundation, 2016, Alexandria, VA (USA).
- 688 Palomo A, Pedersen AG, Fowler SJ, Dechesne A, Sicheritz-Pontén T, Smets BF. Comparative
689 genomics sheds light on niche differentiation and the evolutionary history of comammox
690 *Nitrospira*. *Isme j* 2018; 12: 1779-1793.
- 691 Park M-R, Park H, Chandran K. Molecular and Kinetic Characterization of Planktonic *Nitrospira*
692 spp. Selectively Enriched from Activated Sludge. *Environmental Science & Technology*
693 2017; 51: 2720-2728.
- 694 Pérez J, Lotti T, Kleerebezem R, Picioreanu C, van Loosdrecht MCM. Outcompeting nitrite-
695 oxidizing bacteria in single-stage nitrogen removal in sewage treatment plants: A model-
696 based study. *Water Research* 2014; 66: 208-218.
- 697 Persson F, Suarez C, Hermansson M, Plaza E, Sultana R, Wilén B-M. Community structure of
698 partial nitrification-anammox biofilms at decreasing substrate concentrations and low
699 temperature. *Microbial Biotechnology* 2017; 10: 761-772.

- 700 Qian F, Huang Z, Liu Y, Grace OOw, Wang J, Shi G. Conversion of full nitrification to partial
701 nitrification/anammox in a continuous granular reactor for low-strength ammonium
702 wastewater treatment at 20 °C. *Biodegradation* 2021; 32: 87-98.
- 703 Regmi P, Miller MW, Holgate B, Bunce R, Park H, Chandran K, et al. Control of aeration, aerobic
704 SRT and COD input for mainstream nitrification/denitrification. *Water Research* 2014; 57:
705 162-171.
- 706 Roots P, Wang Y, Rosenthal AF, Griffin JS, Sabba F, Petrovich M, et al. Comammox *Nitrospira*
707 are the dominant ammonia oxidizers in a mainstream low dissolved oxygen nitrification
708 reactor. *Water Research* 2019; 157: 396-405.
- 709 Sakoula D, Koch H, Frank J, Jetten MSM, van Kessel MAHJ, Lücker S. Enrichment and
710 physiological characterization of a novel comammox *Nitrospira* indicates ammonium
711 inhibition of complete nitrification. *The ISME Journal* 2021; 15: 1010-1024.
- 712 Saunders AM, Albertsen M, Vollertsen J, Nielsen PH. The activated sludge ecosystem contains a
713 core community of abundant organisms. *The ISME Journal* 2016; 10: 11-20.
- 714 Sepehri A, Sarrafzadeh M-H. Activity enhancement of ammonia-oxidizing bacteria and nitrite-
715 oxidizing bacteria in activated sludge process: metabolite reduction and CO₂ mitigation
716 intensification process. *Applied Water Science* 2019; 9: 131.
- 717 Shao Y-H, Wu J-H. Comammox *Nitrospira* Species Dominate in an Efficient Partial Nitrification–
718 Anammox Bioreactor for Treating Ammonium at Low Loadings. *Environmental Science*
719 & Technology 2021; 55: 2087-2098.
- 720 Spieck E, Wegen S, Keuter S. Relevance of *Candidatus Nitrotoga* for nitrite oxidation in technical
721 nitrogen removal systems. *Applied Microbiology and Biotechnology* 2021; 105: 7123-7139.

- 722 Sun H, Zhang H, Zhang F, Yang H, Lu J, Ge S, et al. Response of substrate kinetics and biological
723 mechanisms to various pH constrains for cultured Nitrobacter and Nitrospira in nitrifying
724 bioreactor. *Journal of Environmental Management* 2022; 307: 114499.
- 725 Ushiki N, Jinno M, Fujitani H, Suenaga T, Terada A, Tsuneda S. Nitrite oxidation kinetics of two
726 Nitrospira strains: The quest for competition and ecological niche differentiation. *Journal*
727 *of Bioscience and Bioengineering* 2017; 123: 581-589.
- 728 Vadivelu VM, Yuan Z, Fux C, Keller J. Stoichiometric and kinetic characterisation of Nitrobacter
729 in mixed culture by decoupling the growth and energy generation processes. *Biotechnology*
730 *and Bioengineering* 2006; 94: 1176-1188.
- 731 van Kessel MAHJ, Speth DR, Albertsen M, Nielsen PH, Op den Camp HJM, Kartal B, et al.
732 Complete nitrification by a single microorganism. *Nature* 2015; 528: 555.
- 733 Wegen S, Nowka B, Spieck E. Low Temperature and Neutral pH Define "Candidatus Nitrotoga
734 sp." as a Competitive Nitrite Oxidizer in Coculture with Nitrospira defluvii. *Appl Environ*
735 *Microbiol* 2019; 85.
- 736 Winkler MKH, Boets P, Hahne B, Goethals P, Volcke EIP. Effect of the dilution rate on microbial
737 competition: r-strategist can win over k-strategist at low substrate concentration. *PLOS*
738 *ONE* 2017; 12: e0172785.
- 739 Wu L, Ning D, Zhang B, Li Y, Zhang P, Shan X, et al. Global diversity and biogeography of
740 bacterial communities in wastewater treatment plants. *Nature Microbiology* 2019; 4: 1183-
741 1195.
- 742 Wu L, Li Z, Zhao C, Liang D, Peng Y. A novel partial-denitrification strategy for post-anammox
743 to effectively remove nitrogen from landfill leachate. *Science of The Total Environment*
744 2018; 633: 745-751.

- 745 Yin Q, Sun Y, Li B, Feng Z, Wu G, The r/K selection theory and its application in biological
746 wastewater treatment processes. *Science of the Total Environment* 2022; 824: 153836
- 747 Yu L, Chen S, Chen W, Wu J. Experimental investigation and mathematical modeling of the
748 competition among the fast-growing “r-strategists” and the slow-growing “K-strategists”
749 ammonium-oxidizing bacteria and nitrite-oxidizing bacteria in nitrification. *Science of The*
750 *Total Environment* 2020; 702: 135049.
- 751 Zaborowska E, Lu X, Małkonia J. Strategies for mitigating nitrous oxide production and decreasing
752 the carbon footprint of a full-scale combined nitrogen and phosphorus removal activated
753 sludge system. *Water Research* 2019; 162: 53-63.
- 754 Zheng M, Li S, Ni G, Xia J, Hu S, Yuan Z, et al. Critical Factors Facilitating Candidatus Nitrotoğa
755 To Be Prevalent Nitrite-Oxidizing Bacteria in Activated Sludge. *Environmental Science &*
756 *Technology* 2020; 54: 15414-15423.
- 757 Zheng M, Li S, Dong Q, Huang X, Liu Y. Effect of blending landfill leachate with activated sludge
758 on the domestic wastewater treatment process. *Environmental Science: Water Research &*
759 *Technology* 2019a; 5: 268-276.
- 760 Zheng Z, Huang S, Bian W, Liang D, Wang X, Zhang K, et al. Enhanced nitrogen removal of the
761 simultaneous partial nitrification, anammox and denitrification (SNAD) biofilm reactor for
762 treating mainstream wastewater under low dissolved oxygen (DO) concentration.
763 *Bioresource Technology* 2019b; 283: 213-220.
- 764 Zhu A, Guo J, Ni B-J, Wang S, Yang Q, Peng Y. A Novel Protocol for Model Calibration in
765 Biological Wastewater Treatment. *Scientific Reports* 2015; 5: 8493.
- 766
- 767



768

Supplementary Information

769 *The coexistence and competition of canonical and comammox nitrite oxidizing*
770 *bacteria in a nitrifying activated sludge system – experimental observations and*
771 *simulation studies*

772

773 **Mohamad-Javad Mehrani¹, Przemyslaw Kowal¹, Dominika Sobotka¹, Martyna Godzieba², Sławomir**
774 **Ciesielski², Jianhua Guo³, Jacek Makinia^{1*}**

775 ¹ Faculty of Civil and Environmental Engineering, Gdansk University of Technology, Narutowicza Street 11/12, 80-233 Gdansk,
776 Poland

777 ² Department of Environmental Biotechnology, Department of Environmental Biotechnology, University of Warmia and Mazury
778 in Olsztyn, Sloneczna 45G, 10-719 Olsztyn, Poland

779 ³Australian Centre for Water and Environmental Biotechnology (ACWEB, formerly AWMC), The University of Queensland, St.
780 Lucia, Queensland 4072, Australia

781 *Correspondence: Jacek Makinia (Email: jmakinia@pg.edu.pl, Tel: +48 58 347 19 54, Address: Gdansk University of
782 Technology, ul. Narutowicza 11/12, 80-233, Gdansk, Poland)

783

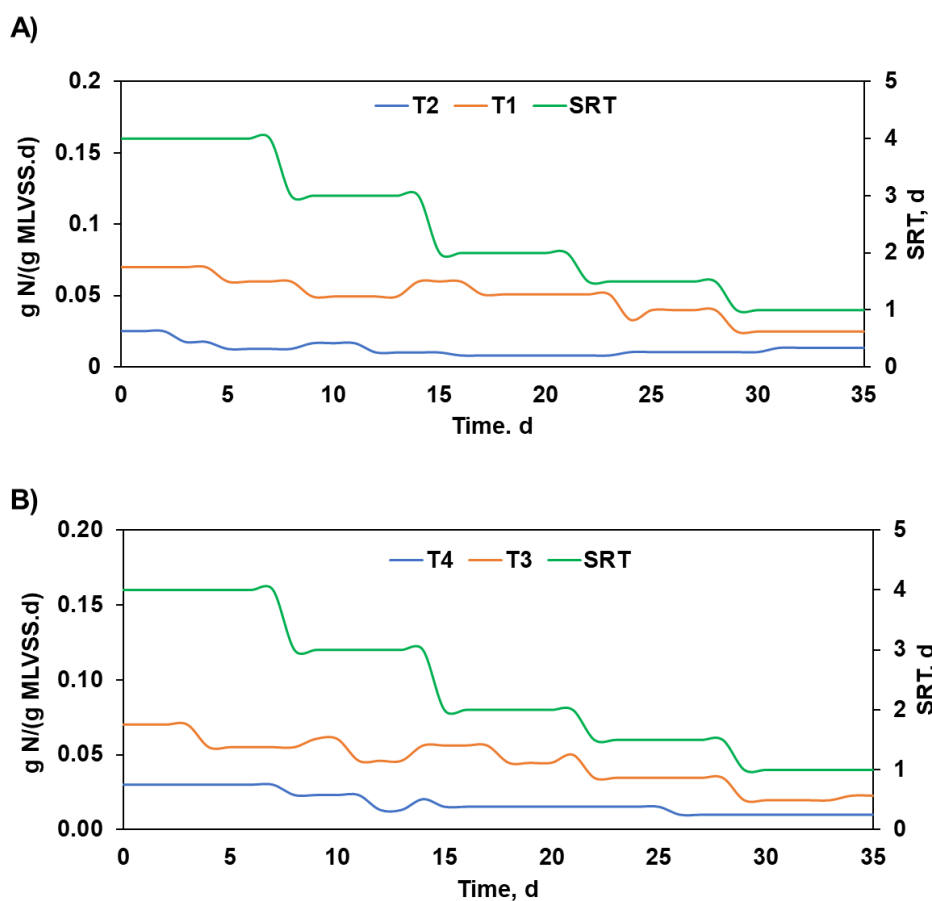
784 **S1. Synthetic medium used for the long-term experiments and Influent substrate**

785 **concentrations**

786 **Table S1.** Characteristics of the synthetic medium (NH₄-N vs. NO₂-N substrate) used for the long-term washout
787 experiments

Substrate	Main elements		Tracer solution*	
	Compound	Concentration mg/L	Compound	Concentration g/L
NH₄-N	NH ₄ CL	762	ZnSO ₄ ·7H ₂ O	0.43
	KHCO ₃	1512	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.22
	CaCl ₂	141	CuSO ₄ ·5H ₂ O	0.25
	KH ₂ PO ₄	50	MnCl ₂ ·4H ₂ O	0.99
	MgSO ₄	58.6	CoCl ₂ ·6H ₂ O	0.24
	FeSO ₄ ·7H ₂ O	9.1	NaWO ₄ ·2H ₂ O	0.005
	EDTA	6.3	EDTA	15
	Tracer solution*	1	NiCl ₂ ·6H ₂ O	0.2
			NaSeO ₄ ·10H ₂ O	0.2
			H ₃ BO ₃	0.014
NO₂-N	NO ₂ ⁻	172 - 1470	ZnSO ₄ ·7H ₂ O	0.43
	KHCO ₃	1512	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.22
	CaCl ₂	141	CuSO ₄ ·5H ₂ O	0.25
	KH ₂ PO ₄	50	MnCl ₂ ·4H ₂ O	0.99
	MgSO ₄	58.6	CoCl ₂ ·6H ₂ O	0.24
	FeSO ₄ ·7H ₂ O	9.1	NaWO ₄ ·2H ₂ O	0.005
	EDTA	6.3	EDTA	15
	Tracer solution*	1	NiCl ₂ ·6H ₂ O	0.2
			NaSeO ₄ ·10H ₂ O	0.2
			H ₃ BO ₃	0.014

788



790
 791 **Figure S1-** SRT and influent N loading rate for A) NH₄⁺-N substrate experiments (T1, T2), and B) NO₂⁻-N substrate
 792 experiments (T3, T4)

793

794 S2. Microbiological analytical methods

795 S.2.1. DNA extraction

796 Biomass samples for microbial analysis were collected from the SBRs three times: at the beginning
 797 (inoculum), during the middle phase (25th day), and at the of the experiment (35th day). The

798 biomass samples from the initial experimental stages were transferred to the 50 ml Falcon type
 799 tubes for sedimentation and thickening. The biomass samples withdrawn during the terminal stages,

800 were separated from the outflows from the experimental SBR by filtration trough through 0.22 μm
 801 pore size filters. Each sample was collected in duplicate. Both types of the samples (thickened

802 sludge and biomass deposited on filters) were stored at -25°C prior to DNA extraction. The

803 extraction of the DNA from the biomass samples (100 mg of the thickened sludge or filter slices
804 with deposited biomass) was carried out using the FastDNA™ SPIN KIT (MP Biomedicals, USA)
805 following the manufacturer's manual. The DNA acquired from purification was subsequently used
806 for the Illumina Next Generation Sequencing protocol.

807

808 *S.2.2. High-throughput 16S rDNA sequencing*

809 High-throughput Illumina sequencing targeting the V3-V4 region of the 16S rRNA gene was
810 performed with S-d-Bact-0341-b-S-17 and S-d-Bact-0785-a-A-21 primers (Klindworth et al., 2012)
811 and NEBNext®High-Fidelity 2X PCR Master Mix (Bio Labs inc., USA) following the
812 manufacturer's manual. The sequencing reactions were carried out with MiSeq sequencer (Illumina,
813 USA) by applying the paired-end technology, 2×250 nt with MiSeq Reagent Kit V2 following the
814 manufacturer's protocols. The obtained raw DNA sequence reads were subjected to quality control
815 (QC) using the tools available at Usegalaxy server (usegalaxy.org). Paired-end reads were initially
816 combined with the fastq-join algorithm. The reads were subsequently trimmed and filtered in terms
817 of the length (≥ 400 bp) and quality (Phred score ≥ 20) with Filter FASTQ algorithm. The reads
818 quality at each QC step was validated with FastQC software
819 (www.bioinformatics.babraham.ac.uk/projects/fastqc).

820

821 Chimera presence was verified with a chimera check on-line tool powered by UCHIME
822 (<http://pyro.cme.msu.edu>). The classification of the reads on each taxonomical level was carried
823 out with the SILVA server (www.arb-silva.de) using the database release version 132 at the
824 similarity level of 90% and operational taxonomic units (OTUs) clustering at 97%. The output data
825 from the Usegalaxy server were saved into FASTA format and uploaded to the MetaGenome Rapid

826 Annotation Subsystems Technology (MG-RAST) (Meyer et al., 2008) to enable public access to
827 the files under the accession numbers ranging from mgm4899014.3 to mgm4899025.3.

828

829 ***S.2.3 Absolute quantification of the Comammox bacteria by qPCR***

830 Absolute quantification of the comammox amoA genes was performed with primers sets comaA-
831 244F & comaA-659R and comaB-244F & comaB-659R in order detect and enumerate comammox
832 *Nitrospira* clades A and B, respectively, in accordance with the protocol proposed by (Pjevac et
833 al., 2017). The real-time PCR reactions were performed on ABI 7500 real-time PCR thermocycler
834 (Applied Biosystems) in MicroAmp™ Optical 96-well reaction plates (Applied Biosystems).
835 Each sample was analysed in triplicate.

836

837 **Table S2.** Composition of the overall bacterial community and dynamics of the most abundant HET bacteria during
 838 particular experimental trials. The relative abundances are presented in the form of the heatmap in accordance to the
 839 presented pattern presented pattern

Experimental series (day of the experiment)	NO ₂ -N only; 12°C			NO ₂ -N only; 20°C			NH ₄ -N only; 12°C			NH ₄ -N only; 20°C		
	T1			T2			T3			T4		
	1	15	28	1	14	30	1	21	35	1	21	35
BLAST assignment (at >85% similarity)												
uncultured member of Saprospiraceae (HET)	7.1%	3.7%	0.1%	7.6%	3.4%	0.0%	4.16%	1.52%	0.43%	7.26%	0.08%	0.69%
<i>Curvibacter</i> (DEN)	7.5%	1.9%	0.1%	3.7%	0.9%	0.0%	0.0%	0.2%	0.0%	0.2%	0.2%	0.1%
<i>Tetrasphaera</i> (DPAO)	3.5%	2.8%	0.0%	4.8%	2.1%	0.0%	3.7%	0.6%	0.1%	3.0%	0.0%	0.2%
<i>Candidatus</i> Saccharibacteria (DEN)	1.8%	1.3%	0.0%	1.0%	0.5%	0.1%	5.5%	1.8%	0.5%	4.5%	1.3%	0.3%
<i>Dokdonella</i> (DEN)	4.5%	3.6%	0.5%	1.9%	3.3%	0.1%	1.5%	1.3%	1.0%	2.3%	0.7%	4.9%
<i>Gemmatimonas</i> (DPAO)	1.2%	0.9%	0.1%	1.3%	1.4%	0.0%	1.5%	1.1%	0.3%	1.8%	0.0%	1.3%
<i>Holophaga</i> (HET)	2.2%	2.0%	0.1%	0.9%	1.0%	0.0%	2.4%	0.8%	0.2%	3.1%	0.0%	0.5%
<i>Thermomonas</i> (DEN)	4.3%	0.9%	1.0%	3.5%	2.5%	0.1%	0.3%	2.2%	2.7%	0.8%	3.7%	3.1%
<i>Candidatus</i> Microthrix (DPAO)	2.4%	0.8%	0.1%	3.0%	1.8%	0.0%	2.5%	0.5%	0.1%	1.4%	0.0%	0.1%
<i>Acidovorax</i> (DEN)	4.7%	7.9%	2.7%	8.0%	16.2%	1.1%	1.6%	11.3%	12.6%	1.4%	2.7%	3.2%
<i>Terrimonas</i> (HET)	2.1%	1.6%	0.2%	2.4%	1.5%	0.1%	1.0%	0.7%	0.6%	0.9%	0.2%	1.1%
<i>Caldilinea</i> (HET/DEN)	2.4%	2.5%	0.1%	1.7%	2.5%	0.1%	5.8%	1.6%	0.4%	4.8%	0.2%	1.0%
<i>Pelomonas</i> (DEN)	1.0%	6.8%	1.6%	0.9%	16.0%	0.9%	0.8%	1.9%	1.7%	0.5%	2.0%	1.1%
<i>Hyphomicrobium</i> (DEN)	1.6%	0.5%	0.0%	0.5%	0.4%	0.0%	1.5%	0.4%	0.2%	0.8%	0.2%	0.1%
<i>Candidatus</i> Competibacter (GAO)	0.3%	0.6%	0.0%	0.2%	0.2%	0.0%	1.9%	0.8%	0.1%	1.6%	0.0%	0.1%
<i>Asprobacter</i> (HET)	1.3%	0.6%	0.2%	1.0%	0.7%	0.1%	0.2%	0.1%	0.3%	0.2%	0.3%	0.1%
<i>Ferruginibacter</i> (HET)	1.0%	0.7%	0.0%	1.2%	1.0%	0.0%	0.6%	0.3%	0.3%	0.5%	0.2%	1.3%
<i>Dechloromonas</i> (DPAO)	0.3%	0.2%	0.0%	0.8%	0.3%	0.0%	0.2%	0.5%	0.1%	0.0%	0.0%	0.1%
<i>Denitratisoma</i> (DEN)	0.2%	0.1%	0.0%	0.1%	0.1%	0.0%	0.2%	0.1%	0.1%	0.1%	0.0%	0.2%
<i>Thauera</i> (DEN)	0.7%	0.2%	0.2%	1.2%	0.2%	0.0%	2.5%	0.2%	0.0%	1.7%	0.0%	1.6%
<i>Hydrogenophaga</i> (DEN)	0.1%	0.4%	3.6%	0.4%	4.2%	0.7%	0.0%	5.0%	7.6%	0.0%	5.1%	5.1%
<i>Zoogloea</i> (DEN and NIT)	0.0%	0.0%	0.2%	0.2%	0.0%	0.0%	0.0%	0.4%	1.2%	0.1%	0.2%	0.2%
<i>Mycolicibacter</i> (HET)	0.2%	0.2%	4.6%	0.1%	0.1%	3.2%	0.6%	0.2%	0.3%	0.4%	0.4%	0.1%
<i>Comamonas</i> (HET/DEN???)	0.4%	10.9%	48.6%	0.5%	4.1%	77.7%	0.0%	11.2%	6.5%	0.1%	16.7%	6.9%
<i>Legionella</i> (HET)	0.1%	0.1%	3.9%	0.3%	0.2%	0.5%	0.1%	0.0%	0.1%	0.1%	0.6%	0.0%
<i>Simplicispira</i> (DEN)	0.0%	3.7%	4.1%	0.1%	1.0%	0.0%	0.0%	5.0%	4.9%	0.0%	1.5%	2.5%
<i>Pseudoxanthomonas</i> (DEN/new DPAO???)	0.0%	0.0%	0.7%	0.0%	0.1%	0.2%	0.0%	0.9%	4.9%	0.0%	4.0%	0.5%
<i>Rhodoferax</i> (DEN)	7.5%	1.9%	0.1%	3.7%	0.9%	0.0%	0.0%	0.2%	0.0%	0.2%	0.2%	0.1%
<i>Rhodococcus</i> (HET DEN and NIT)	0.0%	0.1%	0.3%	0.0%	0.1%	0.4%	0.0%	0.6%	4.5%	0.0%	13.7%	8.7%
<i>Rhodobacter</i> (DEN)	0.7%	1.1%	0.6%	1.1%	1.0%	0.1%	1.2%	0.5%	0.6%	0.8%	0.1%	0.1%
<i>Sphingopyxis</i> (HET)	0.0%	0.0%	0.3%	0.0%	0.1%	0.2%	0.0%	1.3%	1.4%	0.0%	0.8%	0.6%
<i>Pseudomonas</i> (DEN)	0.0%	1.7%	0.4%	0.2%	2.6%	0.0%	0.0%	0.6%	2.7%	0.0%	14.9%	3.6%
<i>Brevundimonas</i> (DEN)	0.3%	0.2%	0.4%	0.1%	0.9%	0.1%	0.1%	0.6%	1.4%	0.0%	0.6%	1.9%
<i>Denitratimonas</i> (DEN)	1.1%	0.6%	0.2%	2.4%	1.0%	0.0%	0.0%	0.1%	0.2%	0.1%	0.5%	0.9%
<i>Chryseolinea</i> related (HET)	0.8%	0.8%	0.0%	1.0%	1.2%	0.0%	0.8%	2.4%	3.7%	0.5%	0.2%	0.3%
<i>Simplicispira</i> (DEN)	0.0%	3.7%	4.1%	0.1%	1.0%	0.0%	0.0%	5.0%	4.9%	0.0%	1.5%	2.5%
<i>Bdellovibrio</i> (DEN predator)	0.4%	0.2%	0.2%	0.4%	0.5%	0.0%	0.3%	0.5%	0.3%	0.3%	0.4%	3.3%
<i>Ferruginibacter</i> (DEN)	1.0%	0.7%	0.0%	1.2%	1.0%	0.0%	0.6%	0.3%	0.3%	0.5%	0.2%	1.3%
<i>Glutamicibacter</i> (HET)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.5%	0.2%	1.3%

<i>Flavobacterium (DEN)</i>	0.2%	0.2%	0.1%	0.5%	0.3%	0.1%	0.2%	0.4%	2.3%	0.3%	0.3%	3.1%
<i>TOTAL:</i>	62.9%	66.3%	79.6%	58.0%	76.3%	86.3%	41.8%	63.3%	69.5%	40.7%	63.9%	73.7%

840

841 **S3. Initial biomass concentrations for simulation of the long-term experiments**842 **Table S3.** Initial biomass concentrations for simulation of the long-term experiments

Parameters	Units	Initial biomass concentration			
		T1	T2	T3	T4
X_{AOB}	mg COD/L	36.4	34.3	0	0
X_{NOB1}	mg COD/L	13	12.5	11.5	11
X_{NOB2}	mg COD/L	0.13	0.12	0.11	0.11
X_{CMX}	mg COD/L	4.8	6.0	4.5	4.0
X_{HET}	mg COD/L	380	405	385	390

843

844

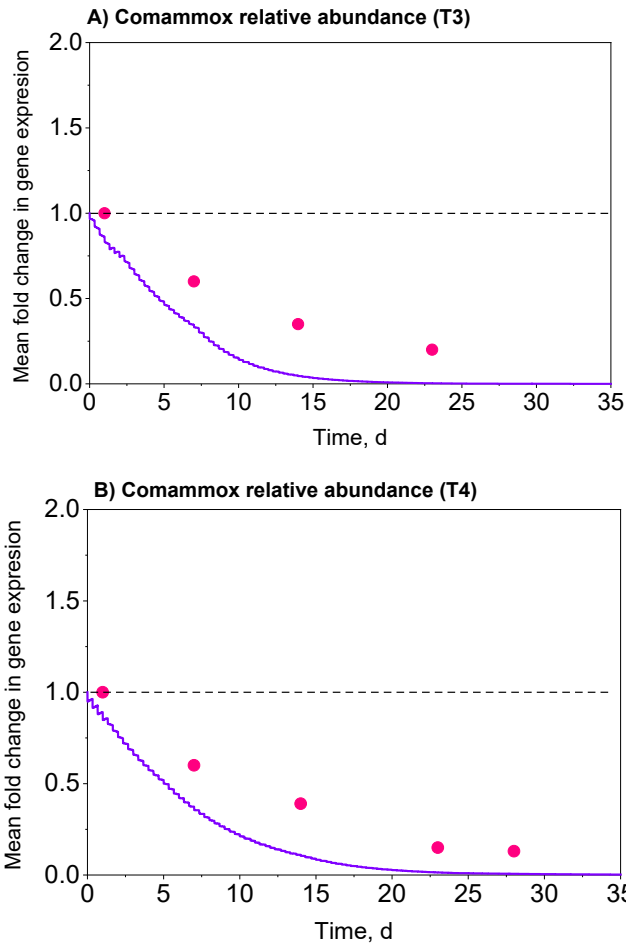
845 **S4. Review on stoichiometric and kinetic parameters**

846 **Table S4.** Review of recent literature data on the stoichiometric and kinetic parameters related to the specific groups
 847 of canonical AOB (r-AOB vs. K-AOB), canonical NOB (r-NOB vs. K-NOB), and comammox bacteria

Parameters (Unit)	Canonical r-AOB (<i>Nitrosomonas</i>)	Canonical K-AOB (<i>Nitrospira</i>)	Canonical r-NOB (<i>Nitrospira</i>)	Canonical K-NOB (<i>Nitrobacter</i>)	Comammox (<i>Nitrospira</i>)	References
μ_{max} (d ⁻¹)		0.24		0.18		(Liu and Wang 2014)
		0.2-0.9				(Metcalf et al., 2003)
	3.03	1.01	0.93	0.31		(Yu et al., 2020)
b (d ⁻¹)	0.54-2.1	0.7-0.9				(Zhang et al., 2019)
			0.39-1.28		0.69	(Park et al., 2017)
K _{NH4} (mg N/L)	0.94-0.99		2.25-2.51			(Liu et al., 2017)
	0.09-0.27		0.09-0.27			(Metcalf et al., 2003)
K _{NO2} (mg N/L)	0.05-0.15		0.05-0.15			(Henze 2000)
	0.675	0.225				(Yu et al., 2020)
			0.765	0.255		(Sakoula et al., 2021)
K _O (mg O ₂ /L)					0.0005	(Koch et al., 2019)
					0.012	(Kits et al., 2017)
Y (mg COD/mg N)	0.076-0.612				0.009	(Yu et al., 2020)
					0.175	(Sakoula et al., 2021)
Y (mg COD/mg N)				0.084-0.378		(Koch et al., 2019)
			0.3-7.6	0.1-1.1		(Park et al., 2017)
Y (mg COD/mg N)	-	-	0.69-7.62	0.126-0.378	5.21	(Nowka et al., 2015)
	0.42	0.14	1.35	0.45		(Yu et al., 2020)
Y (mg COD/mg N)	0.15-1.15		0.13-1.10			(Zhang et al., 2019)
			0.17-4.32		0.33	(Park et al., 2017)
Y (mg COD/mg N)	0.36		0.54			(Liu et al., 2017)
		0.18		0.08		(Jubany et al., 2008)
Y (mg COD/mg N)			0.07-0.14			(Park et al., 2017)
					0.2-0.38	(Kits et al., 2017)
	0.15-0.24					(Eldyasti et al., 2012)

848 μ : maximum specific growth rate, K: half-saturation coefficient, b: decay coefficient, Y: growth yield coefficient

849



851
 852 **Figure S2-** Observed vs. predicted relative abundances of comammox bacteria in trial T3 (comammox can growth
 853 only on $\text{NH}_4\text{-N}$)
 854
 855

856 **Table S5.** Kinetic and stoichiometric parameters for canonical *Nitrospira*, comammox *Nitrospira*, and *Nitrobacter*
 857 reported in the literature

Bacteria	μ_{\max} (d ⁻¹)	b (d ⁻¹)	Y (g VSS/g N or g COD/g N)	K _{NH4} (mg N/L)	K _{NO2} (mg N/L)	K _O (mg O ₂ /L)	Remarks	Reference
<i>Nitrospira</i>								
	0.31	0.02	-		0.25	0.45	T= 17-22 °C, pH=7.5–8.2, Enriched culture	(Yu et al., 2020)
	0.69 ± 0.10	-	0.14 ± 0.02 (g VSS/g N)	-	0.52 ± 0.14	0.33 ± 0.04	T=22 °C, pH=7.5±0.1 Enriched culture, lineage I	(Park et al. 2017)
			0.12 ± 0.04 (g VSS/g N)	-	0.9-1.1	0.54	T=22 °C, pH=7.5 Enriched culture	(Blackburne et al. 2007)
	0.45-0.52	-	-	-	0.13-0.39	-	T=28–37 °C, pH=7.4-8.6 Pure culture, <i>Nitrospira defluvii</i> , and <i>Moscoviensis</i> ,	(Nowka et al. 2015)
	-		-	-	0.08 (Lineage I) 0.14 (Lineage II)	-	T=29°C, Pure culture, <i>Nitrospira</i> lineage I and II	(Ushiki et al. 2017)
	0.45	-	-	-	-	-	T=N/a, Enriched culture	(Lawson and Lücker 2018)
	-	-	-	-	5.21	-	T=37 °C, Pure culture, <i>clade 1</i> , <i>lineage II</i>	(Kits et al. 2017)

0.2	0.04	0.05 (g COD/g N)	0.06	0.13	T=12-20 °C, Mixed culture	This study
-----	------	---------------------	------	------	------------------------------	------------

858

Comammox <i>-Nitrospira</i>	0.148	-	-	0.009	-	-	T=N/a, Enriched culture Clade A	(Lawson and Lücker, 2018)
	-	-	-	0.012	6.29	-	T= NA, pH= NA Pure culture, <i>lineage II</i>	(Koch et al. 2019)
	-	-	-	0.0005	0.175	-	-	(Sakoula et al., 2021)
	0.2	0.04	0.15 (g COD/g N)	-	-	-	T=12-20 °C, Mixed culture Clade A	This study
<i>Nitrobacter</i>	0.93	0.05	-	-	0.76	1.35	T= 17-22 °C, pH=7.5–8.2, Enriched culture	(Yu et al., 2020)
	-	-	0.08 (g VSS/g N)	-	1.2–1.3	0.43	T=22 °C, Enriched culture,	(Blackburne et al., 2007)
	0.39–1.28	-	-	-	0.69–7.6	-	T= 28–37 °C pH=7.4-8.6, Pure culture	(Nowka et al., 2015)
	-	-	-	-	-	0.17–4.32	T=25 °C, pH=7.3-8.2, Pure culture	(Laanbroek et al., 1994)
	0.48	-	0.07–0.1 (g VSS/g N)	-	1.49	-	T=22 °C, pH=7.3, Mixed culture	(Vadivelu et al., 2006)
	0.6	0.08	0.05 (g COD/g N)	-	0.06	0.45	-	This study
	-	-	-	-	-	-	-	-

859 μ_{\max} : Maximum growth rate, Y: Yield coefficient, Ko: DO affinity constant, K_{NH_4} : Ammonia affinity constant, K_{NO_2} :

860 Nitrite affinity constant,

861

862



863 **References**

- 864 Blackburne R, Vadivelu VM, Yuan Z, Keller J. Kinetic characterisation of an enriched *Nitrospira*
865 culture with comparison to *Nitrobacter*. *Water Research* 2007; 41: 3033-3042.
866
- 867 Eldyasti, A., Nakhla, G. and Zhu, J., 2012. Development of a calibration protocol and
868 identification of the most sensitive parameters for the particulate biofilm models used in
869 biological wastewater treatment. *Bioresource Technology* 111, 111-121.
870 10.1016/j.biortech.2012.02.021
871
- 872 Henze, M.G., W., Mino, T., van Loosdrecht, M.C.M. (2000) *Activated sludge models ASM1,*
873 *ASM2, ASM2d and ASM3*, IWA Publishing, London, UK.
874
- 875 Jubany, I., Carrera, J., Lafuente, J. and Baeza, J.A., 2008. Start-up of a nitrification system with
876 automatic control to treat highly concentrated ammonium wastewater: Experimental results
877 and modeling. *Chemical Engineering Journal* 144(3), 407-419. 10.1016/j.cej.2008.02.010
878
- 879 Kits, K.D., Sedlacek, C.J., Lebedeva, E.V., Han, P., Bulaev, A., Pjevac, P., Daebeler, A.,
880 Romano, S., Albertsen, M., Stein, L.Y., Daims, H. and Wagner, M., 2017. Kinetic analysis
881 of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* 549(7671), 269-272.
882 10.1038/nature23679
883
- 884 Koch, H., van Kessel, M.A.H.J. and Lücker, S., 2019. Complete nitrification: insights into the
885 ecophysiology of comammox *Nitrospira*. *Applied Microbiology and Biotechnology* 103(1),
886 177-189. 10.1007/s00253-018-9486-3
887
- 888 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F.O.,
889 2012. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
890 generation sequencing-based diversity studies. *Nucleic Acids Research* 41(1), e1-e1.
891 10.1093/nar/gks808
892
- 893 Liu, G. and Wang, J., 2014. Role of Solids Retention Time on Complete Nitrification:
894 Mechanistic Understanding and Modeling. *Journal of Environmental Engineering* 140(1),
895 48-56. 10.1061/(ASCE)EE.1943-7870.0000779
896
- 897 Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., Paczian, T.,
898 Rodriguez, A., Stevens, R., Wilke, A., Wilkening, J. and Edwards, R.A., 2008. The
899 metagenomics RAST server – a public resource for the automatic phylogenetic and
900 functional analysis of metagenomes. *BMC Bioinformatics* 9(1), 386-399. 10.1186/1471-
901 2105-9-386
902
- 903 Laanbroek HJ, Bodelier PLE, Gerards S. Oxygen consumption kinetics of *Nitrosomonas*
904 *europaea* and *Nitrobacter hamburgensis* grown in mixed continuous cultures at different
905 oxygen concentrations. *Archives of Microbiology* 1994; 161: 156-162.
906
- 907 Lawson CE, Lücker S. Complete ammonia oxidation: an important control on nitrification in
908 engineered ecosystems? *Current Opinion in Biotechnology* 2018; 50: 158-165.

- 909
910 Liu, J., Wu, Y., Wu, C., Muylaert, K., Vyverman, W., Yu, H.-Q., Muñoz, R. and Rittmann, B.,
911 2017. Advanced nutrient removal from surface water by a consortium of attached
912 microalgae and bacteria: A review. *Bioresource Technology* 241, 1127-1137.
913 10.1016/j.biortech.2017.06.054
914
915 Metcalf and Eddy, Inc. 2003. *Wastewater engineering : treatment and reuse*, Fourth edition /
916 revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel. Boston :
917 McGraw-Hill, 2003.
918
919 Nowka, B., Daims, H. and Spieck, E., 2015. Comparison of Oxidation Kinetics of Nitrite-
920 Oxidizing Bacteria: Nitrite Availability as a Key Factor in Niche Differentiation. *Applied*
921 *and Environmental Microbiology* 81(2), 745. 10.1128/AEM.02734-14
922
923 Park, M.-R., Park, H. and Chandran, K., 2017. Molecular and Kinetic Characterization of
924 Planktonic *Nitrospira* spp. Selectively Enriched from Activated Sludge. *Environmental*
925 *Science & Technology* 51(5), 2720-2728. 10.1021/acs.est.6b05184
926
927 Pjevac, P., Schaubberger, C., Poghosyan, L., Herbold, C.W., van Kessel, M.A.H.J., Daebeler, A.,
928 Steinberger, M., Jetten, M.S.M., Lücker, S., Wagner, M. and Daims, H., 2017. AmoA-
929 Targeted Polymerase Chain Reaction Primers for the Specific Detection and Quantification
930 of Comammox *Nitrospira* in the Environment. *Frontiers in Microbiology* 8(1508).
931 10.3389/fmicb.2017.01508
932
933 Sakoula, D., Koch, H., Frank, J., Jetten, M.S.M., van Kessel, M.A.H.J. and Lücker, S., 2021.
934 Enrichment and physiological characterization of a novel comammox *Nitrospira* indicates
935 ammonium inhibition of complete nitrification. *The ISME Journal* 15(4), 1010-1024.
936 10.1038/s41396-020-00827-4
937
938 Vadivelu VM, Yuan Z, Fux C, Keller J. 2006. Stoichiometric and kinetic characterisation of
939 *Nitrobacter* in mixed culture by decoupling the growth and energy generation processes.
940 *Biotechnology and Bioengineering* 94: 1176-1188.
941
942 Yu, L., Chen, S., Chen, W. and Wu, J., 2020. Experimental investigation and mathematical
943 modeling of the competition among the fast-growing “r-strategists” and the slow-growing
944 “K-strategists” ammonium-oxidizing bacteria and nitrite-oxidizing bacteria in nitrification.
945 *Science of The Total Environment* 702, 135049. 10.1016/j.scitotenv.2019.135049
946
947 Zhang, D., Su, H., Antwi, P., Xiao, L., Liu, Z. and Li, J., 2019. High-rate partial-nitrification and
948 efficient nitrifying bacteria enrichment/out-selection via pH-DO controls: Efficiency,
949 kinetics, and microbial community dynamics. *Science of The Total Environment* 692, 741-
950 755. 10.1016/j.scitotenv.2019.07.308
951
952

953

