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

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Abstract

The second step of nitrification can be mediated by nitrite oxidizing bacteria (NOB), i.e. *Nitrospira* and *Nitrobacter*, with different characteristics in terms of the r/K theory. In this study, an activated sludge model was developed to account for competition between two groups of canonical NOB and comammox bacteria. Heterotrophic denitrification on soluble microbial products was also incorporated into the model. Four 5-week washout trials were carried out at dissolved oxygen-limited conditions for different temperatures (12°C vs. 20°C) and main substrates (NH_4^+-N vs. NO_2^--N). Due to the aggressive reduction of solids retention time (from 4 to 1 d), the biomass concentrations were continuously decreased and stabilized after two weeks at a level below 400 mg/L. The collected experimental data (N species, biomass concentrations, and microbiological analyses) were used for model calibration and validation. In addition to the standard predictions (N species and biomass), the newly developed model also accurately predicted two microbiological indicators, including the relative abundance of comammox bacteria as well

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

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

List of Abbreviations

Abbreviation	**Complete name**
AOB	Ammonia oxidizing bacteria
ASM1	Activated Sludge Model No. 1
CMX	Comammox bacteria
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
HET	Heterotrophic bacteria
J^2	Janus coefficient
N	Nitrogen
NIT	Nitrifying bacteria
NOB	Nitrite oxidizing bacteria
MAE	Mean absolute error
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
R^2	Determination coefficient
RMSE	Root mean square error
SBR	Sequencing batch reactors
SMP	Soluble microbial products
SRT	Solids retention time
WAS	Waste activated sludge
WWTP	Wastewater treatment plant

1. INTRODUCTION

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

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

These two genera (*Nitrobacter* and *Nitrospira*) reveal different characteristics in terms of the r/K theory. *Nitrobacter* represents the r-strategists which grow faster at high concentrations of the substrates (NO_2^--N and dissolved oxygen (DO)), whereas *Nitrospira* is the K-strategist with high substrate affinity at low concentration (Yu et al., 2020). Due to these differences, a more complex suppression strategy would be required for NOB. In general, the controlled solids retention time

(SRT), combined with DO-limited conditions and high residual ammonia, have been recommended (Regmi et al., 2014). However, low DO conditions (<1.0 mg O_2/L) can be inefficient with respect to the suppression of K-strategist *Nitrospira* (Cao et al., 2017).

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

The dominance of specific groups of nitrifying bacteria results from selective pressures of the operational conditions in the bioreactor, including substrate (NH_4^+-N or NO_2^-N) concentration, DO concentration, and temperature (Metcalf and Eddy, 2014). An efficient approach to the investigation of the complex nitrifier competition would be a combination of dedicated physical experiments with advanced microbiological analyses and mathematical modeling. Cao et al. (2017) noted that the nitrification models should accommodate appropriately the competition between AOB and NOB to understand factors influencing the competition between autotrophic N-converting organisms. Two-step nitrification models have been continuously developed for almost 60 years as summarized by (Mehrani et al., 2022). Such models were used for the development of suppression strategies for NOB considered collectively as one group (Duan et al., 2019; Kent et al., 2019; Ma et al., 2017; Pérez et al., 2014). Very recent theoretical advances

include the examination of the competition for different r/K strategist groups of AOB and NOB (Yu et al., 2020; Yin et al., 2022) and the incorporation of comammox in the traditional two-step model (Mehrani et al., 2021). However, no models have been applied in practice to investigate the competition of different NOB groups under different substrate (limited vs. unlimited) conditions.

The purpose of this study was to develop and validate a model describing the co-existence and competition between two groups of canonical NOB (*Nitrospira* and *Nitrobacter*) and comammox NOB, revealing different r/K characteristics, in response to decreasing SRTs under different substrate limitation conditions. The experimental data were collected during four long-term washout experiments carried out at two temperatures (12°C vs. 20°C) with different substrates (NH_4^+-N vs. $NO2^-$- N). The effect of the substrate was also investigated in terms of the behavior of comammox bacteria. Overall, it was hypothesized that the newly developed model would provide a better explanation of the competition between the different NOB groups. Such a model can be a diagnosis and optimization tool for practical applications of the novel shortcut N removal processes under different NO_2^--N availabilities.

2. MATERIAL AND METHODS

2.1. Laboratory experiments and data collection for modeling

2.1.1. Long-term washout experiments with different nitrogen substrates

Four long-term washout trials were carried out under various laboratory conditions concerning the nitrogen substrate and temperature (Table 1). For each experiment, new inoculum biomass samples were obtained from a large municipal wastewater treatment plant (WWTP) in Swarzewo (180 000 PE), located in northern Poland. The biological part of that plant, performing biological

nutrient removal, consists of six parallel sequencing batch reactors (SBRs). The effluent standards have been set following the European Union Urban Wastewater Directive (91/21/EEC), i.e., total N (TN) = 10 mg N/L and total P (TP) = 1 mg P/L.

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

In each experiment, the reactor was operated at three cycles per day (8 hours each), including three phases: feeding (15 minutes), reaction (450 minutes), and decantation (15 minutes). The latter phase was carried out while mixing, so the solids retention time (SRT) became equal to the hydraulic retention time. The amount of waste activated sludge (WAS), removed during the decantation phase, was progressively increasing. This resulted in a gradually decreasing SRT from the initial 4 d to 1 d at the end of the trial. With this operational strategy, highly dynamic conditions were obtained to provide suitable data for model calibration/validation.

For all the experiments, the continuous aeration 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 dosing NaOH (2M solution). The temperature setpoints were kept close to the actual process conditions, i.e., 12^{o}C (winter) and 20^{o}C (summer). For a better understanding of the competition between the NOB groups, the reactor was fed with either NH_4^+-N or NO_2^--N as the sole inorganic N substrate. The influent N loadings and detailed feed composition are shown in the SI (Figure S1 and Table S1, respectively).

Mixed liquor samples were collected 3 times per week, then filtered and analyzed for different N forms, including NH_4^+-N, NO_3^--N, and NO_2^--N, at the initial and the end of the reaction phase. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were analyzed at the initial of the reaction phase. For microbiological analyses, biomass samples were collected from the reactor in duplicate three times: at the beginning (0 d), in the middle phase (20 d), and at the end of each trial (35 d). The samples from the initial and middle phases were transferred to 50 mL Falcon-type tubes for sedimentation and thickening. The terminal samples, due to the dilution of mixed liquor, were obtained by filtering 5 L of the effluent through 0.22 μm pore size filters. The biomass samples were stored at -25°C prior to DNA extraction.

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

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	NH_4^+-N	2140/1500
T2	12	7.5±0.2	0.6±0.1	NH_4^+-N	1890/1450
T3	20	7.5±0.2	0.6±0.1	NO_2^--N	1970/1520
T4	12	7.5±0.2	0.6±0.1	NO_2^--N	2020/1440

2.1.2. Chemical and microbiological analytical methods

The analytical methods used by Dr. Lange and Shimadzu were based on the APHA Standard Methods (2002). Cuvette tests in a Xion 500 spectrophotometer were used to determine NH_4^+-N, NO_3^--N, and NO_2^--N concentrations (Hach Lange, Germany). Before the analysis, mixed liquor samples were filtered under vacuum pressure using a 1.2 m pore size nitrocellulose filter MFV-3

(Millipore, USA). The gravimetric technique was used to determine the MLSS and MLVSS concentrations in line with the APHA Standard Methods (2002).

The DNA extraction was performed followed by FastDNA™ SPIN KIT (MP Biomedicals, USA) based on the manufacturer's manual. Genomic DNA extracted from the duplicated samples was pooled together. Following purification, the DNA was utilized for the Illumina Next Generation Sequencing procedure. Similar to our previous studies (Al-Hazmi et al., 2021), high-throughput Illumina sequencing of the V3-V4 regions of the 16S rRNA gene was carried out, processed and analyzed for the DNA sequencing data. The classification of the reads on each taxonomical level was carried out with the SILVA server (*www.arb-silva.de*) using the database release version 132 at the similarity level of 90% and operational taxonomic units (OTUs) clustering at 97%. The contribution of AOB and NOB within the total bacterial community was considered as a sum of the relative abundances of the 16S rRNA gene amplicons assigned to the specified functional group according to Biological Nitrogen Removal Database (http://bnrdb.genome-mining.cn). In addition, 40 of the most abundant genera of heterotrophs in the initial and final samples were determined for each test, with a special focus on the potential denitrifiers.

The comammox *Nitrospira* subpopulation changes were monitored by the quantitative Polymerase Chain Reaction (qPCR), as outlined previously by Kowal et. al (2022). The qPCR curves with the critical thresholds (CT) from the detection of specified genes and negative control were presented in SI (Figure S2). The relative quantification (RQ) method of Livak and Schmittgen (2001) was adapted to analyze trends in the comammox *Nitrospira* abundance:

$$\mathrm{RQ} = 2^{-\Delta\Delta\mathrm{Ct}} \quad (1)$$

where: $\Delta\Delta Ct = (Ct_{marker\ gene}-Ct_{16SrRNA(reference\ gene)})_{Time\ x} -(Ct_{marker\ gene}-Ct_{16SrRNA(reference\ gene)})_{Time\ 0,}$ Ct - cycle threshold. The reaction performances were assumed at 100%.

To detect the amoA gene specific to comammox *Nitrospira*, the protocol proposed by Pjevac et al. (2017) was applied. An amplicon of a partial 16S rRNA gene of the total bacterial population, amplified using primers 341F & 515R (Wang and Qian, 2009) was used as a reference gene.

2.2. Organization of the modeling study

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

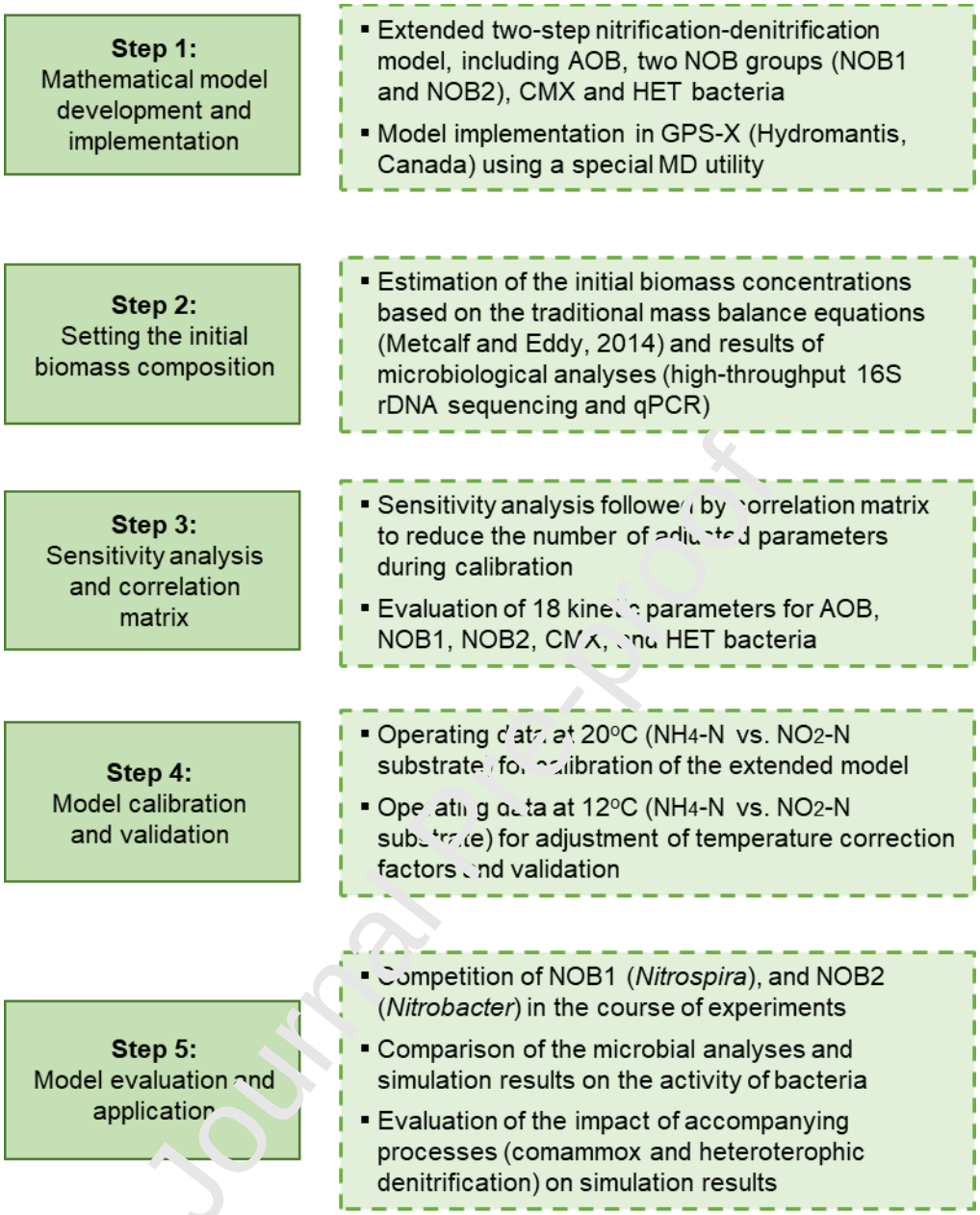

Figure 1. Flowchart diagram of the simulation procedure and model application for the competition of two NOB groups and interactions with comammox and heterotrophic denitrification

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) (Mehrani et al., 2022). In the present study, NOB were divided into two subgroups depending on their different characteristics in terms of the r/K theory, i.e., *Nitrospira* as K-strategists vs. *Nitrobacter* as r-strategists (Figure 2). Their behavior and competition were assessed during the washout experiments under different substrate availabilities (Table 2). It was assumed that the substrate affinity constant is higher for *Nitrobacter* than *Nitrospira* ($K_{NO2,NB} > K_{NO2,NS}$) allowing the latter to prevail under NO_2^--N limited conditions (NH_4^+-N feeding). On the other hand, *Nitrobacter* can outcompete *Nitrospira* under NO_2^--N feeding due to a higher maximum specific growth rate ($\mu_{max,NB} > \mu_{max,NS}$).

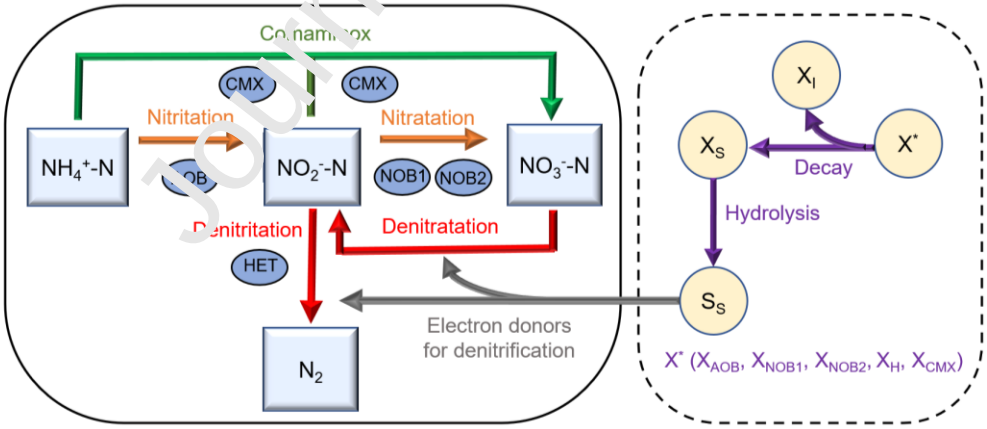

Figure 2. A concept of the extended nitrification model considering two NOB groups and interactions with comammox and heterotrophic denitrification

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

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

NOB substrate availability	*Nitrospira* (K-strategist)	*Nitrobacter* (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)	↓ $\mu_{max,NS}\frac{S_{NO2}}{S_{NO2}+K_{NO2,NS}}\frac{S_O}{S_O+K_{O,NS}}$	↑ $\mu_{max,NB}\frac{S_{NO2}}{S_{NO2}+K_{NO2,NB}}\frac{S_O}{S_O+K_{O,NB}}$

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

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

The initial values of kinetic and stoichiometric parameters were adopted from the literature (Koch et al., 2019; Yu et al., 2020; Metcalf and Eddy 2003; Hiatt and Grady, 2008; Roots et al. 2019). Subsequently, the kinetic parameters were subjected to sensitivity analysis (see Section 2.3). Three kinetic parameters, including K_O for AOB and NOB1 together with K_{NO2} for NOB1, were

experimentally determined in batch tests (Mehrani et al., 2022). These parameters were not further adjusted during model calibration.

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 (NH_4^+-N substrate) and trial T3 (NO_2^-N substrate). For trial T1, the different microbial groups (AOB, NOB1, CMX, and HET) were subjected to the sensitivity analysis concerning NH_4^+-N, NO_3^-N, and NO_2^-N behavior. In trial T3, AOB were not considered due to the lack of substrate (NH_4^+-N) for these bacteria. Instead, NOB2 were included concerning NO_3^-N and NO_2^-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 (NH_4^+-N vs. NO_2^--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).

2.5. Model evaluation and application

After model validation, the competition between two NOB groups (*Nitrospira* and *Nitrobacter*) under different substrate conditions (NH_4^+-N and NO_2^--N) was investigated by comparing the predicted process rates (mg N/L·d) in trials T1 and T3. Sankey diagrams were developed based on the daily average rates at different phases of the experiment, i.e. 0 d (beginning), 20 d, and 35 d (end). The diagrams were used to identify the dominant N conversion pathways and assess the effect of the accompanying processes (comammox, heterotrophic denitrification).

3. RESULTS

3.1. Bacterial community structure during the washout experiments

The strategy of progressive SRT reduction and the lack of organic carbon in the feed resulted in re-arrangements within the heterotrophic bacteria subpopulation (Table S2). Bacteria belonging to the order *Saprospiraceae* and genera *Caldilinea, Curvibacter, Dokdonella, Holophaga, Tetrasphaera* constituted predominant components in the inoculum biomass. However, during all the trials, those bacteria were systematically outcompeted by members of *Acidovorax, Hydrogenophaga, Comamonas, Pseudomonas, Simplicispira,* and *Thermomonas*.

The inoculum biomass samples revealed a consistent AOB abundance (1.31 ± 0.1%) within the total bacterial community. *Nitrosomonas* bacteria were the only representatives of AOB detected

in all the analyzed biomass samples. In the course of the experiment, regardless of the nitrogen source and temperature, *Nitrosomonas* tended to stabilize their abundance in the community until the SRT <2.0 d.

The initial NOB population was more diverse and reflected different abundances of the specific representatives, with the predominance of *Nitrospira* (0.84 ± 0.44%) and a minor contribution of *Nitrobacter* (0.05 ± 0.04%). The washout effect of the specific NOB was influenced by the nitrogen substrate and process temperature. In the later phases of the trials with NH_4^+-N, NOB were represented exclusively by *Nitrospira*, with their systematic washout from the systems, less effective under moderate temperature conditions (20 °C). During the tests with NO_2^--N, NOB increased their share in the total bacterial community. For both temperatures, when the SRT ≥ 2.0 d, the balanced share of *Nitrospira* and *Nitrobacter* was observed at the abundance of approximately 1.0% for each taxon. With the reduction of SRT <2.0 d, *Nitrospira* was gradually washed out and outcompeted by *Nitrobacter*, which became not only the most abundant NOB, but also one the most abundant component of the overall bacterial community (5.8% and 4.5% under temperatures 20°C and 12°C respectively). In addition, in the trial under the low temperature, representatives of *Ca*. Nitrotoga were detected and reached a noticeable abundance of 1.22%. The shifts within the nitrifiers population are summarized in Figure S3.

The qPCR analysis enabled to detect comammox *Nitrospira* in the biomass and monitor the washout process efficiency (see section 3.3 for details). In all the trials, the RQ of the comammox specified amoA gene revealed a systematic washout of comammox bacteria. The washout was more efficient in the experiments with NO_2^--N substrate, which may suggest that NH_4^+-N was the preferable substrate for comammox *Nitrospira* in the studied system.

3.2. Initial biomass composition simulation of the long-term experiments

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

3.3. Sensitivity analysis and correlation matrix

Figure 3a,c shows the sensitivity coefficients, derived for all 13 kinetic coefficients (μ, K, b), which were obtained based on the results from trial T1 (NH_4^+-N substrate) and trial T3 (NO_2^-N substrate). For trial T1, the different microbial groups (AOB, NOB1, CMX, and HET) were subjected to the sensitivity analysis for the behavior of NH_4^+-N, NO_3^-N, and NO_2^-N. For trial T3, NOB2 were considered instead of AOB, and the sensitivity analysis was performed based on the behavior of NO_3^-N and NO_2^-N.

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

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

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

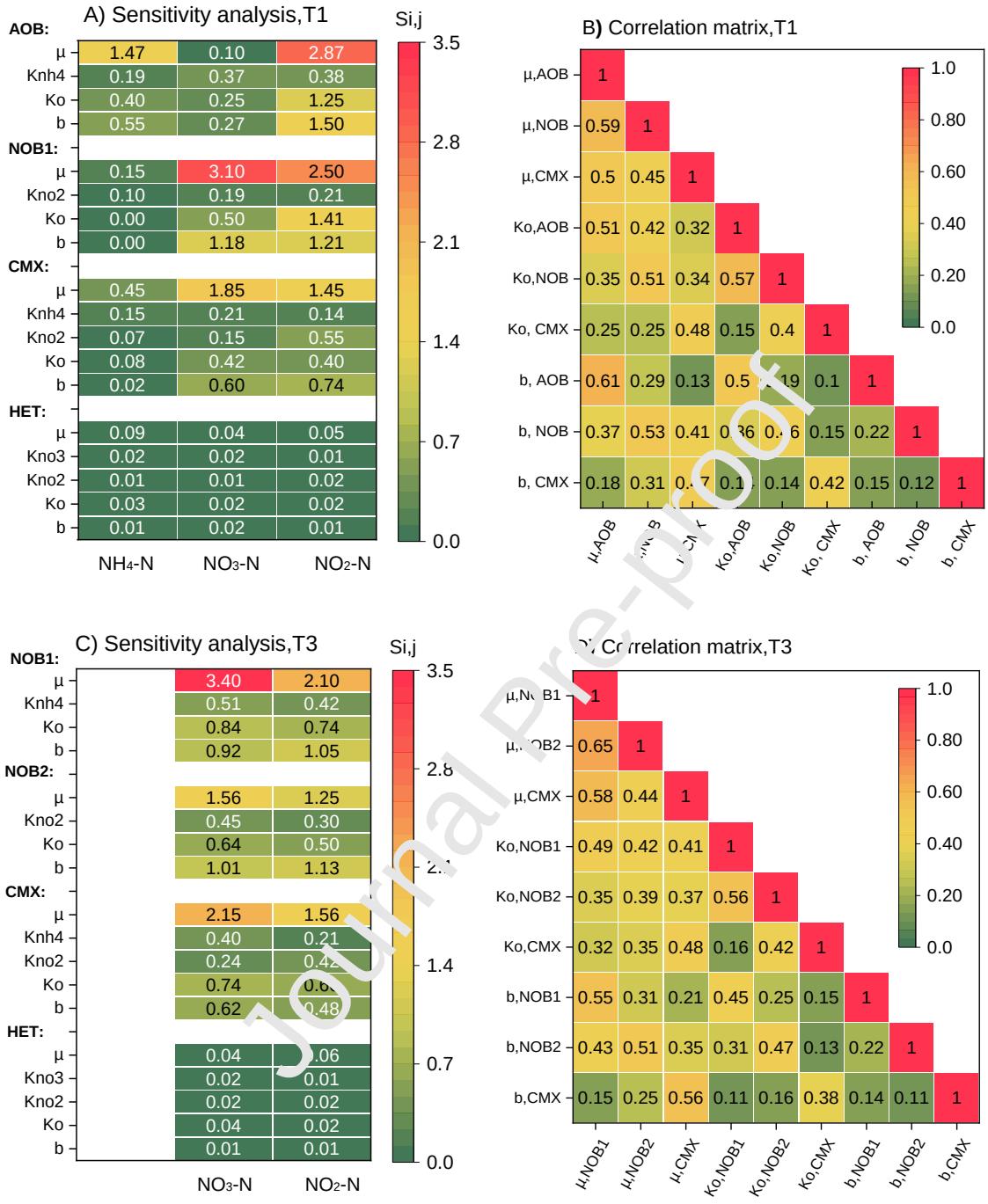

Figure 3. Sensitivity coefficients, $S_{i,j}$, and correlation matrix for the most sensitive kinetic parameters (μ, K, b) in trial T1 (A-B) and trial T3 (C-D) (the effect of NOB2 in trial T1 was not considered due to very low sensitivity ($Si,j \leq 0.01$))

3.4. Model calibration and validation

The experimental observations and predicted behaviors of N species (NH_4^+-N, NO_3^-N, and NO_2^-N), and biomass concentrations for the calibrated model (trials T1 and T3) are shown in Figure 4. Similar data for the validated model (trials T2 and T4) are shown in Figure 5. In all the trials, the biomass concentrations (MLSS, MLVSS) were continuously decreased and apparently stabilized after two weeks at below 400 mg/L. Trends of the individual biomass components are shown in the SI (Figure S4). Moreover, regardless of the substrate (NH_4^+-N vs. NO_2^--N), the behavior of NO_3^-N was generally similar with a peak concentration occurring after one week.

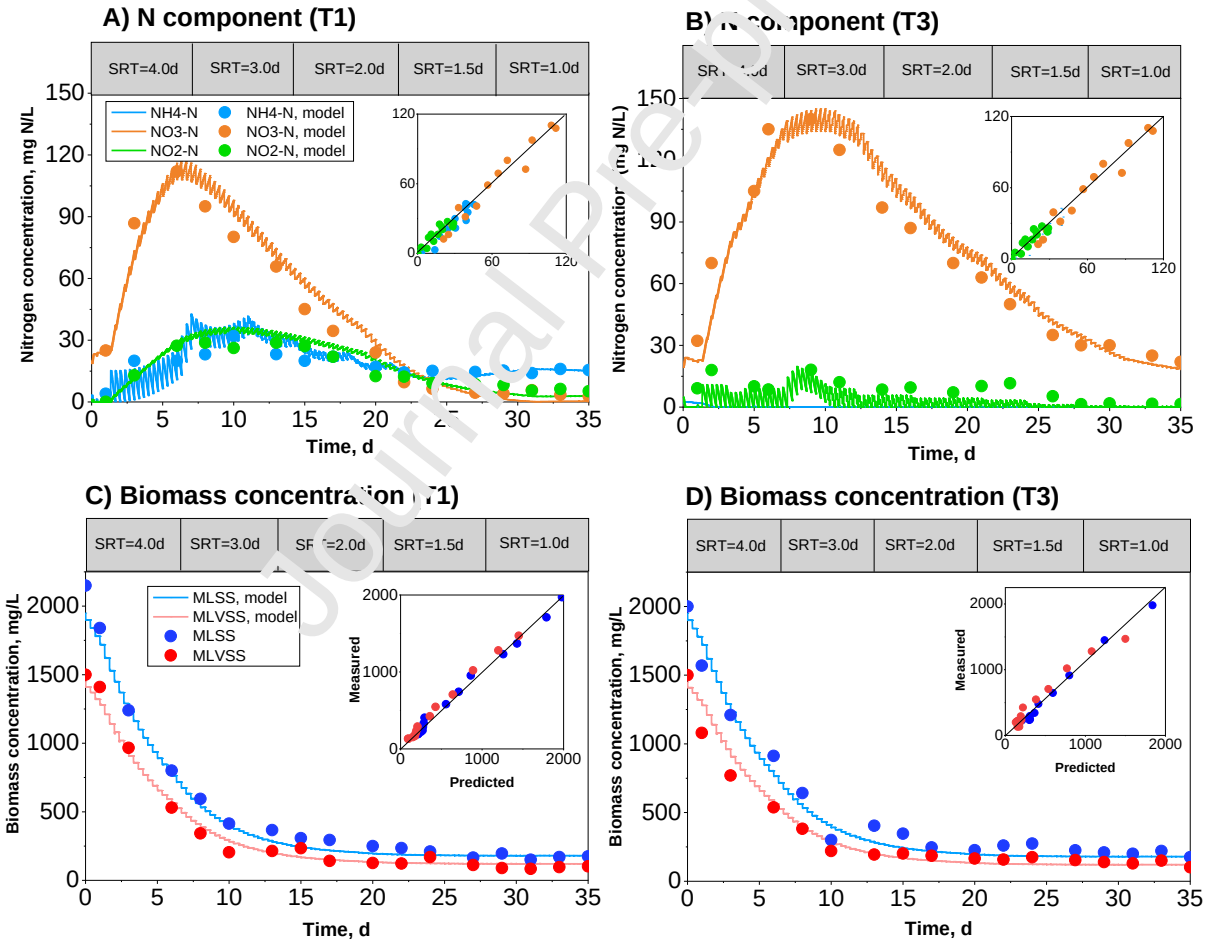

Figure 4. Observed data vs. model predictions for the calibrated model: A) N components in trial T1, B) N components in trial T3, C) Biomass concentrations in trial T1, D) Biomass concentrations in trial T3

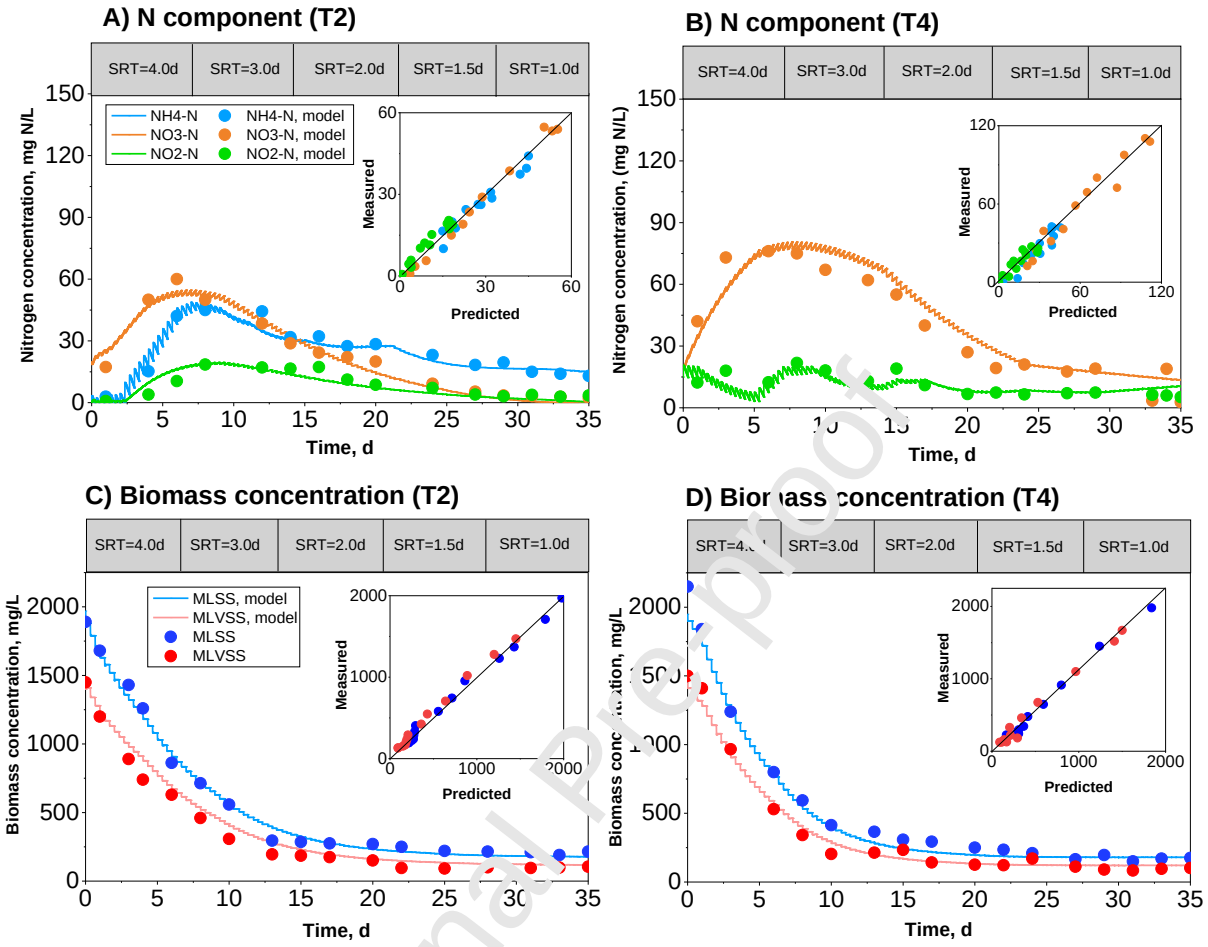

Figure 5. Observed data vs. model predictions for the validated model: A) N components in T2, B) N components in T4, C) Biomass concentrations in T2, D) Biomass concentrations in T4

In addition to the standard predictions (N species and biomass), the newly developed model also predicted two microbiological indicators, including the relative abundance of comammox bacteria and nitrifiers (NIT) to heterotrophs (HET) ratio (Figure 6). The relative abundance of comammox bacteria in the trials with NH_4^+-N substrate (T1 and T2) decreased and stabilized at 0.4-0.5 (Figure 6a-b), while in the trials with NO_2^--N substrate (T3 and T4), the abundance sharply decreased in the first week and stabilized at 0.1-0.2 at the end of trials (Figure 6c-d). The

results with NO_2^-N substrate strongly suggest that comammox bacteria can grow on NO_2^-N, as the model predictions significantly worsened without considering that process (Figure S5).

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

Table 3 shows a list of the model parameters along with their related values and sources of information. Due to high sensitivity ($S_{i,j} \geq 0.5$), the μ and K_O for all the nitrifier groups (AOB, NOB1, NOB2, CMX), and μ for heterotrophs were adjusted during mathematical optimization. The estimated values were further discussed in relation to literature data (see Section 4.1). The extended model prediction performance for each model output (NH_4^+-N, NO_3^-N, NO_2^-N) is presented in Table 4. The calibrated model exposed a high goodness-of-fit for all the outputs in terms of R^2 (>0.87) and RMSE and MAE errors (2.20-3.4), while the validated model revealed slightly decreased (<7%) R^2 and slightly increased (<15%) errors (RMSE, MAE) compared to the calibration period. The Janus coefficient varied in the range of 1.31-1.97, which also confirmed the model validity.

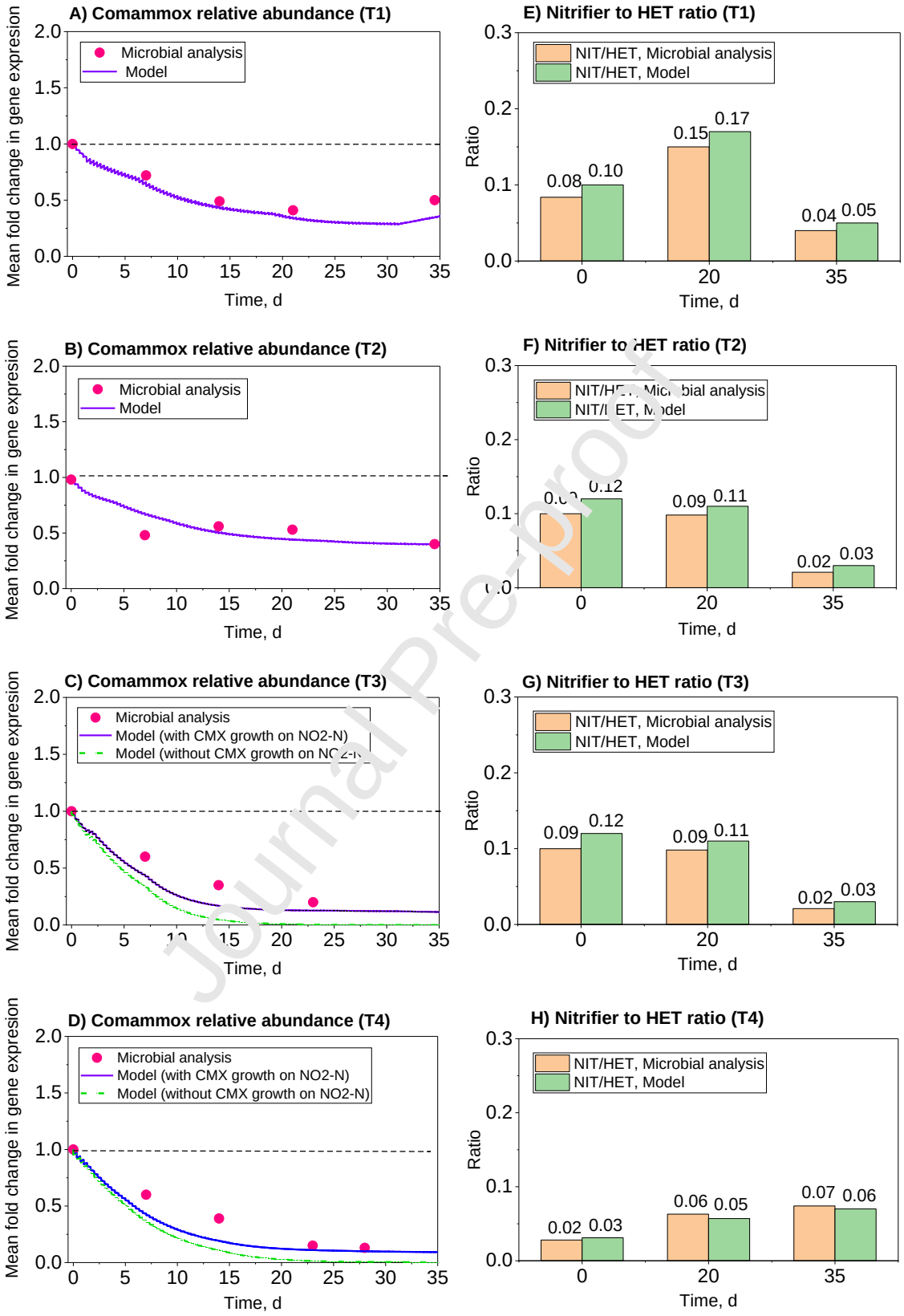

Figure 6. Observed vs. predicted relative adundances of comammox bacteria (A-D), and the ratio of nitrifiers to heterotrophs (E-H) for trials T1-T4

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 *Nitrospira*	NOB 2 *Nitrobacter*	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	0.50	-	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	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[e]		Literature
Y_H	gCOD/gCOD	-	-	-	-	0.6[d]	Literature
Correction factor, θ							
for μ	-	1.09[g]	1.11[g]	1.11[g]	1.09[g]	1.03[g]	Literature
for b	-	1.029[g]	1.029[g]	1.029[g]	1.029[g]	1.029[g]	Literature

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

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		0.8[illegible]	3.58	4.32	1.95

3.5. Contribution of accompanying processes in N conversion

Figure 7 shows the N conversion pathways in trial T2 with NH_4^+-N substrate. The relative contributions of some bacteria groups were significantly shifted during the trial. The canonical NOB1 and comammox bacteria continuously reduced their abundances - from 43 to 21% (NOB1) and from 5% to 1% (comammox). The contribution of NOB2 was negligible (<0.1%). The AOB increased their contribution from 38% at the beginning to 50% at the end of the trial. The contribution of denitrifying heterotrophs, mediating two steps of denitrification, increased from the initial 12.5% to 28% at the end of the trial.

Figure 8 shows the N conversion pathways in trial T3 with NO_2^-N substrate. The contribution of NOB1 was substantially decreased from 79% to 7%, while the NOB2 contribution had an increasing trend (4% to 89%) in the course of the trial. The comammox bacteria and denitrifying heterotrophs decreased their contributions, respectively, from 4% to <1% and from 16% to 3%.

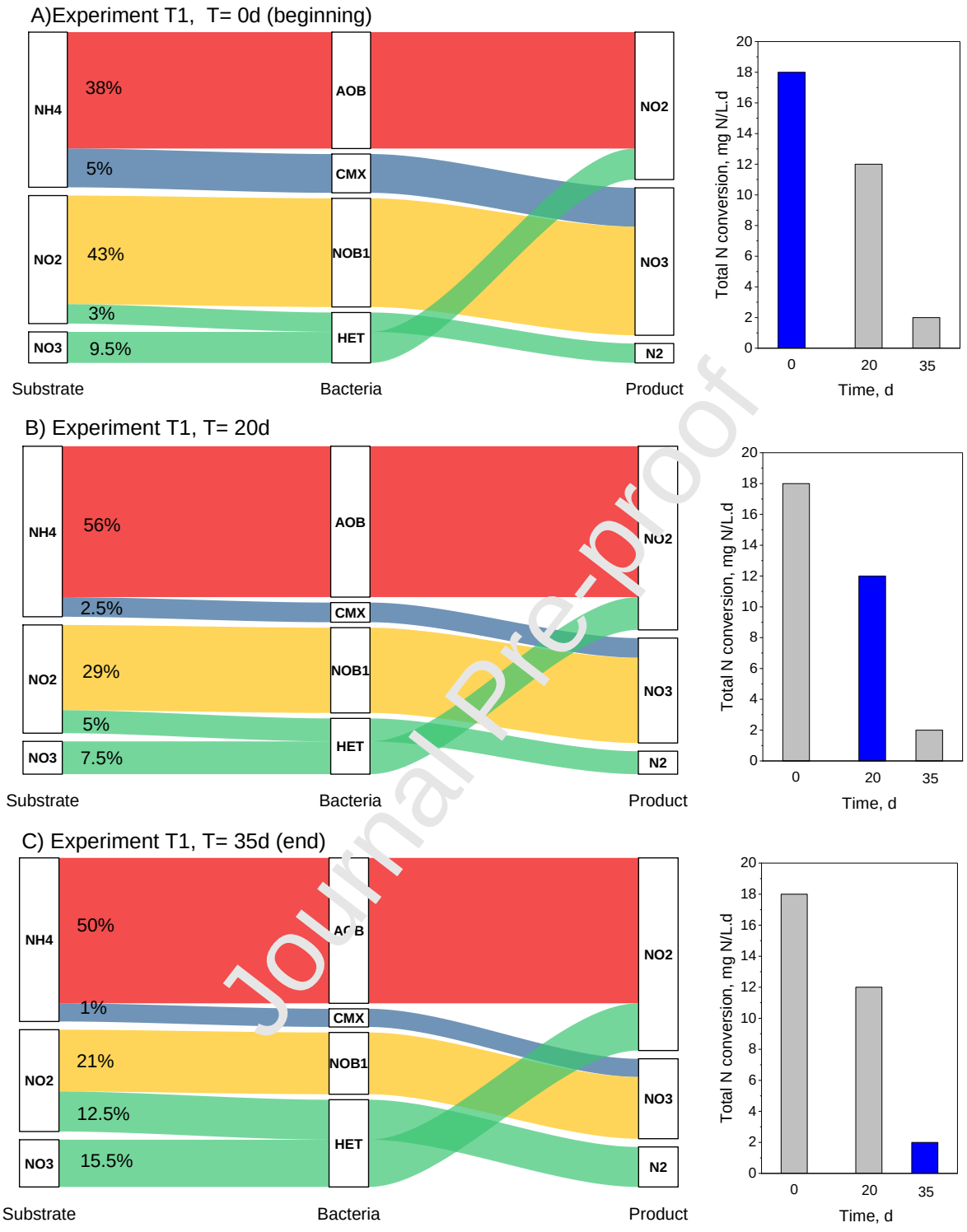

Figure 7. Sankey diagram showing the N conversion pathways in trial T1 (% represent the shares in the total conversion rate (mg N/L·d): A) 0 d, B) 20 d, C) 35 d

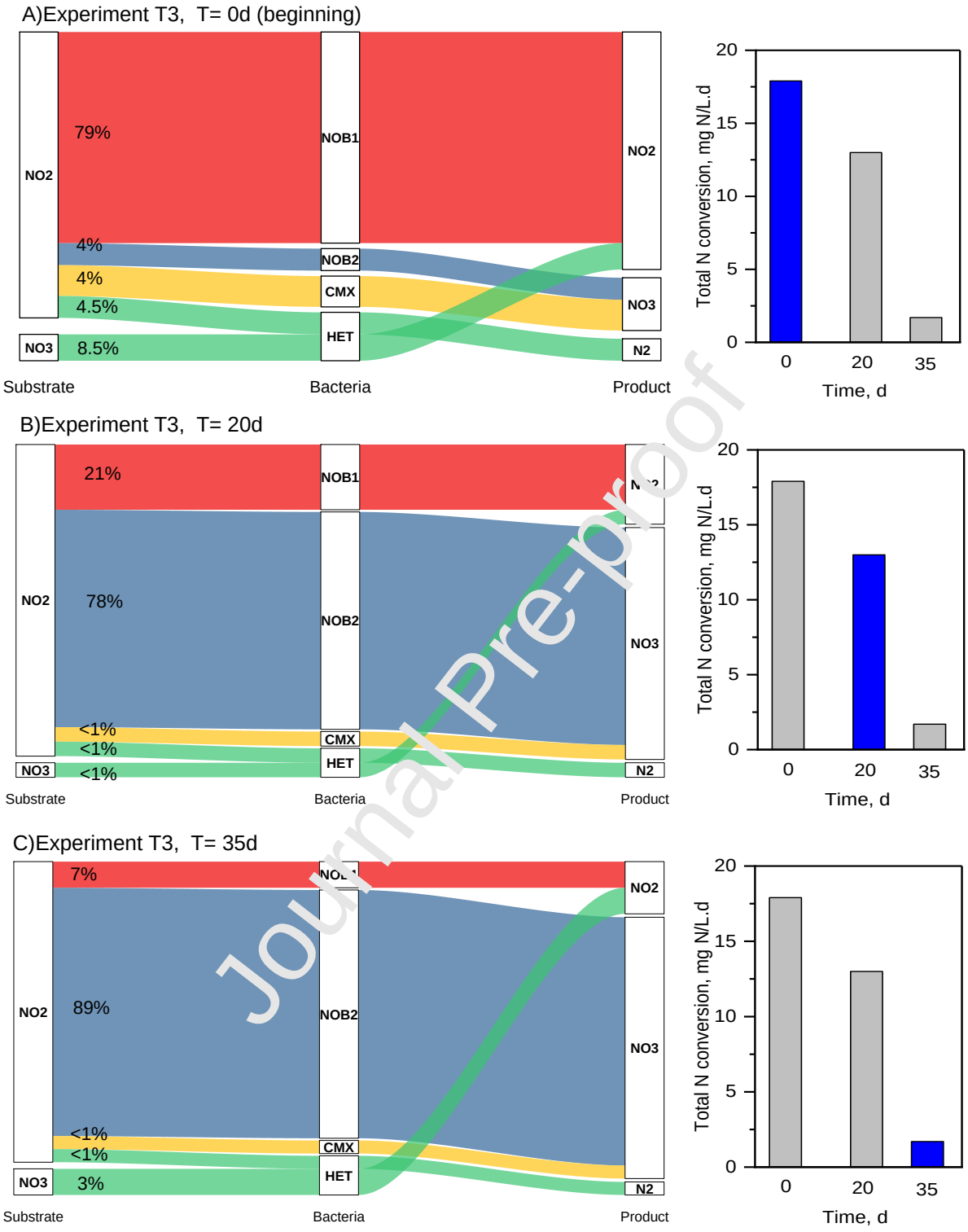

Figure 8. Conversion pathways of the N species for trial T3 (% represent the shares in the absolute total N conversions (mg N/L·d): A) 0 d, B) 20 d, C) 35 d

4. DISCUSSION

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

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

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

The DO concentration is another important operational factor that influences *Nitrospira* abundance in WWTPs (Ushiki et al., 2017; Chang et al., 2019). Park et al. (2017) attributed a strong enrichment of *Nitrospira* (at the expense of *Nitrobacter*) in a DO- and NO_2^--N -limited SBR to the lower affinity constants of *Nitrospira* for both substrates (see Table S4). (Liu and Wang, 2013) found that in addition to low DO concentrations (0.2-0.4 mg O_2/L), extending the SRT from 10 d to 40 d resulted in the dominance of *Nitrospira* over *Nitrobacter*.

Li et al., (2021) found that the SRT of 10 d and shorter aeration times (1.5 h) allowed to completely wash out NOB from the system. Gradually prolonged aeration times (3-3.5 h), applied subsequently, did not result in enriching NOB. Both *Nitrospira* and *Nitrobacter* abundances remained stable, but at a low level, which resulted in maintaining long-term NO_2^--N accumulation in a wide temperature range (18-29 C).

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

Ushiki et al. (2017) hypothesized that *Nitrospira* had other characteristics to compete with *Nitrobacter* and other NOB in nitrite-limited environments. *Nitrobacter* has a cytoplasmic nitrite oxidoreductase (nxrA) that is responsible for the reverse transport of NO_2^--N and NO_3^--N through the inner membrane. On the contrary, *Nitrospira* encodes for periplasmic nxrB, which catalyses nitratation. The periplasmic oxidation is advantageous since it generates a larger specific proton motive force and avoids the transmembrane exchange of NO_2^--N and NO_3^--N (Pester et al., 2014).

Table S4 shows a literature review of the kinetic and stoichiometric parameters for *Nitrospira* and *Nitrobacter*. The ranges of μ for r-strategist *Nitrobacter* and K-strategist *Nitrospira* are 0.31-1.28 d^{-1} and 0.15-0.93 d^{-1}, respectively. In the present study, the estimated μ for *Nitrobacter* (0.6 d^{-1}) was three times higher than *Nitrospira* (0.21 d^{-1}). In addition, the experimentally obtained Ko

of 0.13 mgO_2/L for NOB1 (*Nitrospira)* is slightly lower than the overall ranges in Table S4 ($K_{O,Nitrobacter}$= 0.17-4.32 mgO_2/L, $K_{O,Nitrospira}$= 0.33-1.35 mgO_2/L), while the optimized Ko of 0.45 mgO_2/L for NOB2 is in the lower range (0.45-1.35 mgO_2/L) reported by Yu et al. (2020). It should be noted, however, that in the literature (O'Shaughnessy, 2016), the K_O values for *Nitrospira* and other NOB genera were as low as 0.04 d^{-1}.

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

Comammox bacteria have received a lot of attention in the literature (e.g. Mehrani et al., 2020; Maddela et al. 2021; Xu et al., 2021; Chen et al., 2023), but the extent of their contribution to nitrogen conversions remains ambiguous and strongly depends on the biomass retention method. The reported abundances of comammox bacteria have ranged from 0.02–3% to 1.8–19.4% of the total bacterial community in activated sludge systems and biofilms systems, respectively (Xu et al., 2021).

In the present study (with activated sludge), the abundances of the total *Nitrospira* population in the inoculum samples remained below 1.2%, which is in accordance with the literature data. Thus, it is not surprising that the contributions of comammox to nitrogen conversions were relatively low (<5%) as shown in the Sankey graphs (Figure 7 and Figure 8).

The role of comammox may increase in the biofilm systems with long SRTs. Under such conditions, comammox bacteria have revealed several competitive advantages over coexisting canonical nitrifiers, including the capability of thriving at low DO levels and a high substrate affinity (Maddela et al., 2021).

Maddela et al. (2021) noted that comammox bacteria have several competitive advantages over coexisting canonical nitrifiers, including the capability of thriving at low DO levels and a high substrate affinity. The high NH_4^+-N affinity of pure cultured comammox bacteria (*N. inopinata*) is indicated by extremely low half-saturation constants, which are 4- to 2500-fold lower than the values reported for AOB. Comammox bacteria also show a lower NO_2^--N affinity than canonical NOB. Moreover, the abundances of comammox bacteria *Nitrospira inopinata (N. inopinata)* showed significant positive correlations with canonical NOB rather than AOB, which may suggest that comammox bacteria more actively mediate NO_2^--N oxidation rather than NH_4^+-N oxidation. The authors concluded that switches between the modes of NH_4^+-N and NO_2^--N oxidation in comammox bacteria remain unknown, and it is difficult to identify the environmental and operational factors determining the preferred nitrogen source.

The simulation results of the present study, strongly support the hypothesis that comammox bacteria can grow on NO_2^--N, even though NH_4^+-N appears to be a preferable substrate for them (Figure 6a-d). Following the discovery of comammox, it was thought that comammox-*Nitrospira*, do not possess this ability (Koch et al., 2019). Based on the findings of a previous investigation by Kits et al. (2017), this was explained by an extremely low NO_2^--N affinity. In comparison with canonical *Nitrospira*, *N. inopinata* (comammox bacteria) had a 50-fold lower NO_2^--N affinity (Kits et al., 2017). In a recent study, Shao and Wu (2021) found that the NO_2^--N affinities were variable among the comammox species. Under NH_4^+-N limiting conditions, some authors did not exclude the possibility of comammox bacteria using NO_2^--N as electron donors (Kits et al., 2017; Palomo et al., 2018; Roots et al., 2019).

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

4.3. Further developments of two-NOB models describing the competition with other NOB groups

The structure of the model developed in the present study may be used to describe competition of two groups of canonical NOB, i.e. K-strategists and r-strategists. The model coped well even when switching the NOB2 population from *Nitrobacter* to *Ca*. Nitrotoga in experiment T4 with NO_2^--N substrate at 12°C (Figure 5 and Figure 3d,h). In that case, the abundance of *Ca*. Nitrotoga increased from a negligible level to 1.22% at the end of the experiment (Figure S3d). This is in accordance with literature data (see below) implying a better adaptation of *Ca*. Nitrotoga to low temperatures in comparison with other canonical NOB.

In particular, the same model structure with NOB1 vs. NOB2 can also be used to describe a competition between *Nitrospira* and *Ca*. Nitrotoga. The latter genus has received growing attention as it has been detected as the main NOB, either alone or together with *Nitrospira*, in different full-scale WWTPs worldwide (Lücker et al., 2015; Saunders et al., 2016; Chen et al. 2020), and many pilot-scale systems (Figdore et al., 2018; Jiang et al., 2018; Persson et al., 2017; Wu et al., 2018; Zheng et al., 2019b). *Ca. Nitrotoga* can oxidize NO_2^--N at temperatures ranging from 4 to 28°C (Lantz et al., 2021), its optimum temperature (22-23°C) is lower compared to

other NOB genera (Zheng et al., 2020; Ishii et al., 2020). Nowka et al. (2015) observed that *Ca. Nitrotoga* outcompeted *Nitrospira* and *Nitrobacter* during a long-term cultivation at 5 and 10°C. Liu et al. (2021) calculated a very low temperature correction factor of 1.042 in the temperature range of 4-22 °C for NOB dominated by *Ca. Nitrotoga.* Speick et al. (2021) concluded that the adaptation of *Ca. Nitrotoga* to low temperatures may be beneficial for the recovery of nitratation during cold seasons.

With respect to the DO affinity, K_O for *Ca. Nitrotoga* was determined by Zheng et al. (2020) as 0.59 ± 0.11 mg O_2/L. This value is slightly higher than K_O for *Nitrobater,* and much higher than K_O for *Nitrospira* (see Table 3). Both *Nitrospira* and *Ca. Nitrotoga* resisted at intermittent aeration in a PN/A system (Gustavsson, 2020), whereas in another study, *Ca. Nitrotoga* was shown to have a lower DO affinity than *Nitrospira* (Zheng et al. 2020). Qian et al. (2021) found in a partial nitritation/anammox (PN/A) system that increasing the DO concentration from 0.4 to 1.8 mgO_2/L led to an increased abundance of *Ca. Nitrotoga* over *Nitrospira*. On the contrary, Jiang et al. (2018) found that a high DO of 2–2.5 mgO_2/L was sufficient for the washout of both *Nitrospira* and *Ca. Nitrotoga* in a PN/A system.

Higher substrate concentrations may be beneficial to *Nitrotoga* (Nowka et al. 2015). Kinnunen et al. (2017) discovered that at a NO_2^--N concentration of 1 mg N/L, *Ca. Nitrotoga* outcompeted *Nitrospira* in a biofilm community, but *Nitrospira* dominated at a tenfold lower substrate concentration. However, the reported K_{NO2} for *Ca. Nitrotoga* varied in a wide range of 0.345-1.68 mg N/L (Zheng et al., 2020; Kitzinger et al. 2018; Ishii et al. 2017; Wegen et al. 2019; Nowka et al. 2015). Ca. *Nitrotoga* was also shown to be much more resistant to free ammonia

(FA) and free nitrous acid (FNA) exposure than *Nitrobacter* and *Nitrospira* (Li et al. 2020, Zheng et al., 2021).

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

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

Based on the reported characteristics of the identified heterotrophs (section 3.1), there are three possible mechanisms of the surveillance and competitiveness of those bacteria in comparison with other heterotrophs under extremely short SRTs and inorganic feeding medium: (i) heterotrophic denitrification with soluble microbial products (SMP) and extracellular polymeric substances (EPS), generated by AOB and NOB (Nogueira et al. 2005; Sepehri and Sarrafzadeh 2019), (ii) heterotrophic nitrification (Chen et al. 2012), (iii) predation of heterotrophs on autotrophic bacteria (Dolinšek et al. 2013).

In particular, during the trials with NH_4-N as a sole N source, a wider range and more equally represented heterotrophic bacteria groups have been detected during the final stages. Similar to the present study, a noticeable occurrence of the *Acidovorax* and *Pseudomonas* representatives in the reactor fed with an inorganic medium was observed by Keluskar et al. (2013). The growth of those heterotrophs was supported by organic compounds, such as pyruvate, excreted by AOB (*Nitrosomonas*). An additional metabolic pathway enabling the survival of specific heterotrophs (*Hydrogenophaga*) in the systems operated under the limited carbon availability is

hydrogenotrophic denitrification with the utilization of hydrogen in the absence of organic compounds. Furthermore, representatives of *Rhodococcus* have been characterized as bacteria which are capable of performing simultaneous heterotrophic nitrification and aerobic denitrification (Chen et, al 2012).

In the present study, in the trials with NO_2-N as a sole N source, members of the *Comamonas* genera outcompeted other heterotrophs, and their relative abundances exceeded 50% in both trails (at 12 and 20°C) (Table S2). Representatives of the *Comamonas* are well known denitrifying heterotrophs, which are capable of utilizing a wide range of organic compounds, such as amino acids, carboxylic acids, steroids and aromatic compounds (Wu et al., 2018), thus due to wide metabolic properties were capable to develop substrate dependencies with *Nitrobacter* NOB.

Predation is another factor affecting the occurrence of specific heterotrophs and their interaction with the nitrifiers (Dolinšek et al. 2013). Daims et al. (2016) showed that abundances of *Nitrospira* were dramatically reduced due to the predation by *B. bacteriovorus*. In the present study, during the trials with NH_4-N, predator bacteria belonging to the *Bdellovibrio* genus were detected in the abundances > 5%. On the other hand, lower abundances of these bacteria were found during the trials with NO_2-N, when *Nitrobacter* was predominant. This finding suggests that predation may indeed reflect a selective pressure on specific NOB.

5. CONCLUSIONS

- A two-step nitrification model was extended with different NOB groups (competing r strategists vs. K strategists) to increase the prediction accuracy of modeling nitrifying systems under diverse NO_2^--N availabilities in the substrate.

- In addition to the standard prediction parameters (N species and biomass concentrations), microbiological indicators such as the relative abundance of comammox and the ratio of nitrifiers to heterotrophs, are useful target variables for calibration of mechanistic models as they revealed the dominant conversion pathways in the studied systems.
- A combination of microbiological analyses and modeling simulation results confirmed that comammox bacteria can grow on NO_2^--N as a sole N substrate.
- In contrast to canonical nitrifiers, comammox played a minor role in the study system. However, the impact of these bacteria needs to be further explored by modeling systems containing a higher abundance of *Nitrospira*.
- Even though comammox played a minor role in the studied system compared to the canonical nitrifiers, the impact of these bacteria should further be explored by modeling systems containing higher abundances of *Nitrospira*.

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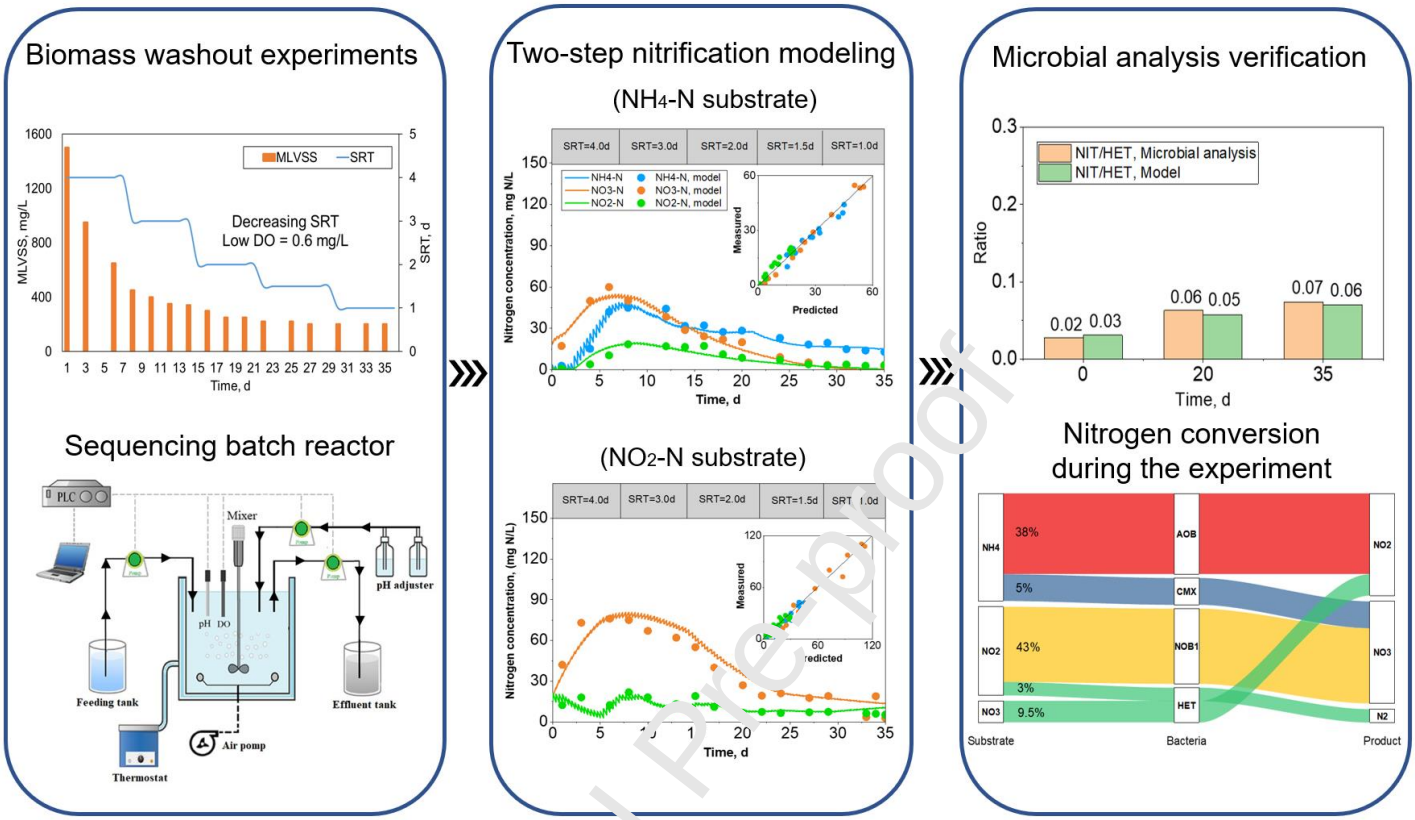
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical Abstract:



Highlights:

- A nitrification model was expanded with two groups of NOB and comammox bacteria
- Competition of r/k strategist and comammox NOB was assessed under decreasing SRTs
- The dominance of a specific NOB group depended on the availability of NO_2^--N
- Comammox bacteria were able to grow on both NH_4-N (preferably) and NO_2-N