



## Rearrangements of the nitrifiers population in an activated sludge system under decreasing solids retention times

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### ABSTRACT

Due to the key role of nitrite in novel nitrogen removal systems, nitrite oxidizing bacteria (NOB) have been receiving increasing attention. In this study, the coexistence and interactions of nitrifying bacteria were explored at decreasing solids retention times (SRTs). Four 5-week washout experiments were carried out in laboratory-scale ( $V = 10$  L) sequencing batch reactors (SBRs) with mixed liquor from two full-scale activated sludge systems (continuous flow vs SBR). During the experiments, the SRT was gradually reduced from the initial value of 4.0 d to approximately 1.0 d. The reactors were operated under limited dissolved oxygen conditions (set point of 0.6 mg O<sub>2</sub>/L) and two process temperatures: 12 °C (winter) and 20 °C (summer). At both temperatures, the progressive SRT reduction was inefficient for the out-selection of both canonical NOB and comammox *Nitrospira*. However, the dominant NOB switched from *Nitrospira* to *Ca. Nitrotoga*, whereas the dominant AOB was always *Nitrosomonas*. The results of this study are important for optimizing NOB suppression strategies in the novel N removal processes, which are based on nitrite accumulation.

### 1. Introduction

Conventional nitrification-denitrification processes have historically been the most commonly applied method of nitrogen (N) removal in municipal wastewater treatment plants (WWTPs). Due to the interest primarily focused on the N removal efficiency, both processes have been described as one-step conversions (Henze et al., 2000). This simplified approach is not sufficient anymore for the description of nitrification, with a shift in the interest on the development of sustainable, and energy-efficient processes, such as nitrite shunt (nitritation-denitrification) and deammonification (Gao and Xiang, 2021). Those processes require nitrite accumulation and thus the suppression of nitrite oxidizing bacteria (NOB) activity has been receiving increasing attention.

In practice, the dissolved oxygen (DO)-limited conditions coupled with short solids retention times (SRTs) have been recognized as one of the most common strategies for NOB suppression (Gao et al., 2014) and the effective NOB washout has been reported for the SRTs as low as 2.5–6.0 d (Regmi et al., 2014; Wu et al., 2016; Cao et al., 2017). On the other hand, high activities of NOB were observed in extensive washout

batch experiments carried out at the DO concentrations <0.7 mg O<sub>2</sub>/L (O'Shaughnessy, 2016) and a sequencing batch reactor (SBR) operated under intermittent aeration mode at the DO concentration of 0.2 mg O<sub>2</sub>/L (Roots et al., 2019).

Yu et al. (2020) provided a theoretical basis, based on the r/K selection theory, for the competition between ammonia oxidizing bacteria (AOB) and two NOB groups under different DO and SRT conditions. According to this theory, *Nitrospira* represents typical “K-strategist” NOB, while *Nitrobacter* is regarded as a member of r-strategist NOB, preferring high nitrite and DO concentrations (Cao et al., 2017). According to this theory, “K-strategist” NOB can survive under low DO concentrations along with ammonia oxidizing bacteria (AOB) (Yu et al., 2020). Moreover, under low substrate (nitrite) conditions, which are typically encountered in mainstream bioreactors of municipal WWTPs, the dominating NOB are members of the genus *Nitrospira* rather than *Nitrobacter* (Yao and Peng, 2017).

The increasing knowledge on two-step nitrification has resulted in some important revisions concerning the functional NOB. Firstly, *Nitrotoga* has been detected as another important bacteria, which can coexist in WWTPs together with *Nitrospira* under DO-limited conditions

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(Kitzinger et al., 2018). Secondly, the key role of *Nitrospira* in N removal systems may not only be attributed to its adaptation to DO-limited conditions, but also to its metabolic activities beyond nitrite oxidation (Koch et al., 2019). Most importantly, recently discovered *Nitrospira* can perform both nitrification steps in the process called complete ammonia oxidation (comammox) (Daims et al., 2016). Upon the discovery of comammox, high abundances of *Nitrospira* in full-scale WWTPs were initially attributed to that process (Wang et al., 2015; Daims et al., 2016). In contrast, the results of a few metagenomic studies (Gonzalez-Martinez et al., 2016; Annavajhala et al., 2018) indicated that comammox could play rather a minor role in N conversions in full-scale WWTPs. More recently, Roots et al. (2019) and Wang et al. (2020) found relatively high abundances of comammox *Nitrospira* and attributed the selective cultivation of those bacteria to the low DO concentrations and long SRT conditions. On the contrary, the detailed microbiological data are still unavailable for short SRT conditions.

In this study, the coexistence and interactions of comammox bacteria with canonical AOB and NOB were investigated in an SBR to verify a selective pressure of low-DO conditions and extremely short SRTs ( $\leq 1$  d) on nitrifying bacteria. The study aimed to test the hypothesis that under these unfavorable operational conditions, canonical NOB and comammox bacteria will efficiently be washed out from the reactor. With this approach, factors influencing the response of different nitrifier groups could be identified and very low growth rates of comammox bacteria could be verified. In the course of the experiments, complex monitoring of the microbial population structure and dynamics, including comammox *Nitrospira*, were analysed by implementing a combination of advanced microbiological tools. Those tools comprised 16 S rRNA high throughput sequencing and relative quantification of the comammox specified functional genes by the quantitative polymerase chain reaction (qPCR) technique. In order to obtain a broader perspective, the experiments were carried out (1) with inoculum biomass withdrawn from two different biological nutrient removal (BNR) activated sludge systems (low N-loaded continuous flow reactor vs. high N-loaded SBR) and in two operational periods (winter vs. summer conditions). The results of this study are important for optimizing NOB suppression strategies in the novel N removal processes, which are based on nitrite accumulation.

## 2. Material and methods

### 2.1. Origin of the inoculum biomass

Four long-term washout experiments were carried out with mixed liquor from Czajka (CZ) and Swarzewo (SW), two large WWTPs performing BNR in different process configurations and operational conditions. Both facilities meet the effluent standards of 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. Czajka WWTP in Warsaw (N: 52.3509, E: 20.960) is the largest facility in Poland with a hydraulic capacity of 435 000 m<sup>3</sup>/d and the pollutant load corresponding to approximately 2 million population equivalents (PE). The biological reactors run according to the alternating flow BioDeniPho (Veolia) system configuration.

The second plant, Swarzewo WWTP (N: 54.7687, E: 18.4063), is located in a touristic region on the Baltic Sea coast. The design hydraulic capacity of that plant is 18 100 m<sup>3</sup>/d, while the pollutant load corresponds to approximately 177 000 PE. In the summer months (June–September), the plant treats up to 15 000 m<sup>3</sup>/d of wastewater, while during the remaining period the flowrate drops to approximately 5000 m<sup>3</sup>/d. In addition to the domestic wastewater, the plant receives a substantial portion (~5%) of nitrogen-rich wastewater from the fish industry. The biological part consists of six parallel sequencing batch reactors (SBRs).

The mixed liquors were collected from the bioreactors either from an aerated compartment (Czajka) or during an aerated phase (Swarzewo). In both cases, mixed liquors samples were collected at approximately 2

m depth from the surface by a dedicated sampler. Approximately 20 L of the mixed liquors were collected in 25 L containers, thickened by sedimentation, tightly closed, and delivered to the laboratory. Until the beginning of the trials, the containers were stored in a refrigerator.

### 2.2. Experimental apparatus

The experiments were carried out in a fully automated Plexiglas SBR with the working volume of 10 L, connected with a feeding tank. The reactor was placed in a water coat, coupled with a thermostatic water bath, to keep the temperature set points close to the actual process temperatures, i.e. 12 °C and 20 °C, representing winter and summer conditions, respectively. The reactor was equipped with an automated aeration control system to maintain the selected DO set points.

### 2.3. Experimental procedures

The SBR was fed with ammonium-rich synthetic medium (Supplementary data Table S1) under specific operational conditions shown in Table 1. The DO concentration was set at  $0.6 \pm 0.1$  mg O<sub>2</sub>/L for all the washout experiments based on the highest nitrite (NO<sub>2</sub>-N) accumulation observed in preliminary batch tests with the mixed liquor from Swarzewo WWTP. At the beginning of each experiment, the SBRs were inoculated with fresh mixed liquor, collected from the full-scale bioreactors. Samples for the winter and summer series were withdrawn in January–February and July–August, respectively.

Each washout experiment lasted 36 days. At the beginning, the withdrawn mixed liquor was diluted in the reactor with tap water to adjust the initial mixed liquor suspended solids (MLSS) concentrations to approximately  $2000 \pm 200$  mg/L. The reactor was operated at the temperature set point of 12 °C (winter series) or 20 °C (summer series). During the washout experiments, the SRT was gradually reduced from the initial value of 4.0 d, through 3.0, 2.0, to 1.0 d. Only during the first (winter) series in Swarzewo WWTP, the ultimate SRT was 1.5 d. The volumetric nitrogen loading rates (NLRs) were kept at approximately  $0.02 \pm 0.01$  g N/(L·d) and  $0.05 \pm 0.01$  g N/(L·d), respectively, during the winter and summer series.

During each experiment, TN, NH<sub>4</sub>-N, NO<sub>2</sub>-N and NO<sub>3</sub>-N were monitored regularly (at least twice a week) at the beginning and end of the reaction phase. The first sample was withdrawn immediately after the feeding phase was completed, and then the samples of the mixed liquor (50 mL) were withdrawn from the reactor with the frequency of 60–90 min. The AOB activity was measured by the specific ammonium utilization rate (AUR). The NOB activity was measured by the specific rates of nitrate production (NPR) and nitrite utilization (NiUR). All the rates were calculated using the following formulas:

**Table 1**  
SBR Operational conditions during the washout experiments.

No.	WWTP	Temperature (°C)	DO, (mg O <sub>2</sub> /L)	SRT (initial → end), (d)	pH	Initial MLSS/MLVSS (mg/L)
1	Swarzewo	12	0.6 ± 0.1	4.1 → ≈ 1.5	7.5 ± 0.2	1890/1250
2	Czajka	12	0.6 ± 0.1	4.1 → ≈ 1.0	7.5 ± 0.2	1930/1350
3	Swarzewo	20	0.6 ± 0.1	4.1 → ≈ 1.0	7.5 ± 0.2	2150/1500
4	Czajka	20	0.6 ± 0.1	4.1 → ≈ 0.8	7.5 ± 0.2	2270/1570

$$AUR = \frac{\text{slope}(S_{\text{NH}_4^+-N, \Delta t})}{\Delta t \cdot X}, \text{ mg N/g VSS} \cdot \text{h} \quad (1)$$

$$NPR = -\frac{\text{slope}(S_{\text{NO}_3^- - N, \Delta t})}{\Delta t \cdot X}, \text{ mg N/g VSS} \cdot \text{h} \quad (2)$$

$$\text{NiUR} = -\frac{\text{slope}(S_{\text{NO}_2^- - N, \Delta t})}{\Delta t \cdot X}, \text{ mg N/g VSS} \cdot \text{h} \quad (3)$$

where:

$S_{\text{NH}_4^+-N}$

– concentration of ammonium nitrogen after time  $t_1$  or  $t_2$  (mg N/L),

$S_{\text{NO}_3^- - N}$

– concentration of nitrate nitrogen after time  $t_1$  or  $t_2$  (mg N/L),

$S_{\text{NO}_2^- - N}$

– concentration of nitrite nitrogen after time  $t_1$  or  $t_2$  (mg N/L),

$\Delta t$  – difference between the end and the initial time of the measurements (h),  $X$  – concentration of organic fraction of activated sludge (g VSS/L).

The nitrite accumulation ratio (NAR) was calculated based on the following equation:

$$NAR(\%) = \frac{(NO_2 - N)_{\text{eff}}}{(NO_2 - N)_{\text{eff}} + (NO_3 - N)_{\text{eff}}} \cdot 100 \quad (4)$$

where the  $(NO_2 - N)_{\text{eff}}$  and  $(NO_3 - N)_{\text{eff}}$  are the effluent nitrite and nitrate concentrations (mg N/L), respectively. For the uncertainty of the NAR, the 90% confidence ( $S_x$ ) was calculated to show the upper and lower error bands as follows:

$$S_x = \bar{X} \pm Z \frac{\sigma}{\sqrt{n}} \quad (5)$$

where the  $\bar{X}$  is a mean value,  $Z$  is a constant (= 1.645) for 90% confidence,  $\sigma$  is a standard deviation, and  $n$  is a population size.

## 2.4. Analytical methods

Before the analysis, samples of the mixed liquor were filtered under vacuum pressure through a 1.2  $\mu\text{m}$  pore size nitrocellulose filters MFV-3 (Millipore, USA). TN concentrations were determined using a TOC/TN analyzer (Shimadzu Corp., Japan). The analytical procedures, which were adopted by Dr. Lange and Shimadzu, followed the Standard Methods APHA (2002). The method detection limit is  $\leq 0.05$  mg TN/L. Concentrations of  $\text{NH}_4\text{-N}$  (detection limit 2.0–60 mg  $\text{NH}_4\text{-N/L}$ ),  $\text{NO}_3\text{-N}$  (detection limit 5.0–35 mg  $\text{NO}_3\text{-N/L}$ ), and  $\text{NO}_2\text{-N}$  (detection limit 2.0–90 mg  $\text{NO}_2\text{-N/L}$ ) were determined using cuvette tests in Xion 500 spectrophotometer (Hach Lange GmbH, Berlin, Germany). The MLSS and its volatile fraction (MLVSS) were determined by the gravimetric method, at detection limit  $\pm 0.001$  mg, following the Standard Methods APHA (2002).

## 2.5. Microbiological analyses

### 2.5.1. DNA extraction and high-throughput 16 S rDNA sequencing

Biomass samples for microbial analysis were collected from the studied SBRs three times: at the beginning (inoculum), in the middle phase, and at the end of the experiment. The samples from the initial

experimental stages were transferred to the 50 mL Falcon type tubes for sedimentation and thickening. Due to the low biomass concentration at the terminal stages, the samples were separated from the 5 L effluents by filtration through 0.22  $\mu\text{m}$  pore size filters. The DNA extraction from the biomass samples was performed with the FastDNA™ SPIN KIT (MP Biomedicals, USA) following the manufacturer's manual. Genomic DNA extracted from the duplicated samples was pooled together. The DNA acquired from purification was subsequently used for the Illumina Next Generation Sequencing protocol. High-throughput Illumina sequencing of the V3–V4 region of the 16 S rRNA gene protocol and following DNA sequencing data processing & analysis were conducted as described in our previous study (Al-Hazmi et al., 2021). The averaged sequencing depth was 50 000 reads per sample. The classification of the reads on each taxonomical level was carried out with the SILVA server ([www.arb-silva.de](http://www.arb-silva.de)) using the database release version 132 at the similarity level of 90% and operational taxonomic units (OTUs) clustering at 97%. Differences between the 16 S rRNA amplicon sequencing datasets were analysed using the Statistical Analysis of Metagenomic Profiles (STAMP v. 2.1.3) (Parks and Beiko, 2010) by performing the ANOVA test, followed by post-hoc Tukey-Kramer test at the 0.95 significance, and visualized as a Principal Component Analysis (PCA) plot. Sequencing data sets were uploaded to the MetaGenome Rapid Annotation Subsystems Technology (MG-RAST) (Meyer et al., 2008) to enable public access to the files under the accession numbers ranging from mgm4899014.3 to mgm4899025.3.

The biodiversity of the microbial communities was assessed with the Shannon (H) diversity index calculated by the following formula:

$$H = -\sum_{i=1}^R p_i \ln p_i \quad (6)$$

where,  $p_i$  is the ratio between the number of DNA sequences assigned to the particular  $i$ th genus to the total number of DNA sequences obtained from the sample.

An additional phylogenetic assignment of NOB was performed based on comparing the experimental DNA sequences of the 16S rRNA gene to the set of marker DNA sequences from the characterized *Nitrospira* and *Nitrotoga* species collected in the NCBI Gene Bank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The experimental and marker DNA sequences were initially aligned in the MEGA 6.06 program (Tamura et al., 2013) with ClustalW module and applied for building a phylogenetic tree using the neighbor joining method. To verify topology of the obtained dendrogram, a bootstrap for 500 repetitions was applied. The dendrogram was rooted with the sequence of *Nitrosomonas oligotropha* strain Nm45 (NR\_114770.1).

### 2.5.2. Comammox *Nitrospira* clade quantification by qPCR

In order to monitor the dynamics of the comammox bacteria population, qPCR with primers sets comaA-244 F & comaA-659 R and comaB-244 F & comaB-659 R was applied. The primer sets were used to detect *amoA* gene variants of comammox *Nitrospira* clades A and B in accordance with the protocol proposed by Pjevac et al. (2017). The real-time PCR reactions were performed on ABI 7500 real-time PCR thermocycler (Applied Biosystems) in MicroAmp™ Optical 96-well reaction plates (Applied Biosystems). Each sample was analysed in triplicate.

Due to unavailability of environmental matrix enriched with comammox *Nitrospira*, preparation of the standard curve for an absolute quantification of comammox bacteria was not possible. Instead of this, the relative quantification (RQ) method of Livak and Schmittgen (2001) was applied to analyse trends in the comammox occurrence during the experiments in relation to the inoculum samples. A fragment of the 16 S rRNA gene of the total bacterial population, amplified with primers 341 F & 515 R (Wang and Qian, 2009), was applied as a reference gene. The RQ was calculated as follows:

$$RQ = 2^{-\Delta\Delta Ct} \quad (7)$$

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

### 3. Results and discussion

#### 3.1. Long-term washout experiments in the SBR

##### 3.1.1. Nitrogen removal performance and process rates

The behavior of the inorganic N forms ( $\text{NH}_4\text{-N}$ ,  $\text{NO}_2\text{-N}$ ,  $\text{NO}_3\text{-N}$ ) during all the washout experiments is shown in Fig. 1, whereas the operational conditions, SRTs and biomass concentrations, can be found in the Supplementary data (Fig. S1). When decreasing the SRT from the initial 4 d to the ultimate values, the specific AURs at  $T = 20^\circ\text{C}$  increased consistently 3–4 times in both plants, reaching approximately  $10 \text{ mg N/g VSS} \cdot \text{h}$  at the end of the experiments. On the contrary, the specific AURs at  $T = 12^\circ\text{C}$  were stable around  $1 \text{ mg NH}_4\text{-N/g VSS} \cdot \text{h}$  (see: the Supplementary data).

Despite extremely low SRTs (0.8–1.5 d) and MLSS concentrations (250–400 g/L) at the end of the experiments, production of  $\text{NO}_3\text{-N}$  was not suppressed completely and the ultimate  $\text{NO}_3\text{-N}$  concentrations remained in the range 10–40 mg N/L. This indicates that the NOB and/or comammox bacteria were not washed out from the reactors.

Accumulation of  $\text{NO}_2\text{-N}$  began after 2–3 days of each experiment, and  $\text{NO}_2\text{-N}$  concentrations either persisted to the end (Swarzewo at  $12^\circ\text{C}$ , Czajka at  $20^\circ\text{C}$ ) or dropped nearly to zero at the final stage (after 30 days) (Swarzewo at  $20^\circ\text{C}$ , Czajka at  $12^\circ\text{C}$ ). The accumulation of  $\text{NO}_2\text{-N}$  hampered NOB suppression and the growth rates of NOB became higher in comparison with AOB. However, a distinct peak  $\text{NO}_2\text{-N}$  concentration (approximately 40 mg N/L) could only be distinguished in the case of Swarzewo at  $20^\circ\text{C}$ . Furthermore, in the summer series, the NiURs (Fig. S2 in the Supplementary data) and NARs (Fig. 1) were correlated and followed the trend of an inverted parabola.

#### 3.2. Phylogenetic composition and biodiversity of the general bacterial community during the washout experiments

Results and summary of the high-throughput DNA sequencing of the

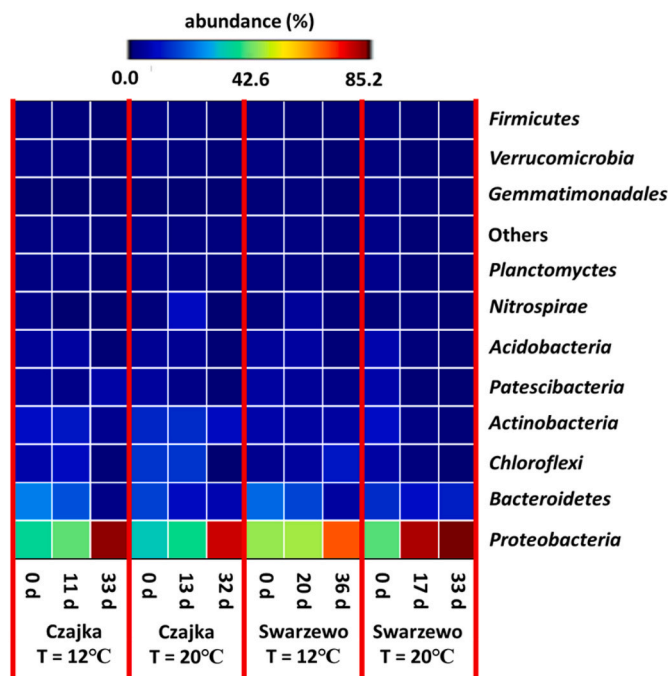


Fig. 2. Occurrence of the most abundant bacterial phyla (share in the total microbial community >1%) during the washout experiments with the biomass from both studied WWTPs.

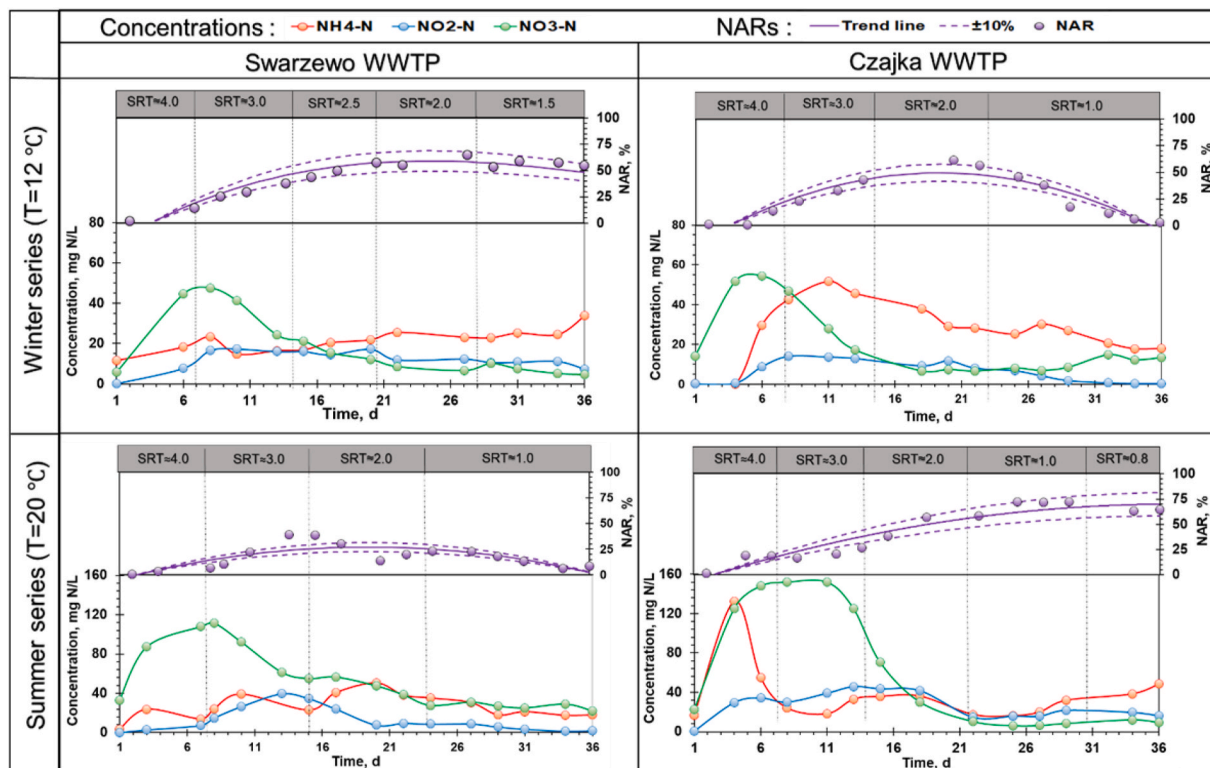


Fig. 1. Nitrite accumulation ratios (NAR) with a trend line including  $\pm 10\%$  error bands, and effluent nitrogen compounds during the long-term washout experiments.

16 S rRNA gene variants in the analysed samples of both studied WWTPs are shown in Fig. 2 and Table S2. The coverage of the datasets was verified with rarefaction curves obtained as data output from the SILVA server (Fig. S3 in the Supplementary data). The microbial community composition was typical for municipal plants (e.g., Yang et al., 2020). On average, representatives of the predominant *Proteobacteria* accounted for 35% and 44% of the microbial community from Czajka (CZ) and Swarzewo (SW) WWTPs, respectively. The main phyla (>8.0%) of the community comprised *Bacteroidetes*, *Chloroflexi*, and *Actinobacteria*. Less abundant (1–6%) phyla were affiliated to *Patescibacteria*, *Acidobacteria*, *Nitrospirae*, *Planctomycetes*, *Verrucomicrobia* and *Firmicutes*. Other phyla were represented by less than 1%.

In the course of the washout experiments, the microbial communities were subjected to the directed rearrangements at the genus level as well (Table S3 in the Supplementary data). Despite the application of synthetic wastewater dedicated to autotrophs (no organic carbon), a wide range of heterotrophic bacteria, reflecting the denitrifying capability, were abundant in the system under the lowest SRTs. A set of the predominant genera has changed along with the washout experiments and reflected similar patterns between the particular trials. In general, heterotrophs from genera *Dokdonella*, *Pelomonas*, *Saprosira*, and *Terrimonas* which prevailed in the initial biomass samples (shares  $\geq 1.6\%$ ), were subsequently outcompeted by the representatives of other genera, such as *Acidovorax*, *Brevundimonas*, *Comamonas*, *Flavobacterium* and *Pseudomonas*. This finding may be related to the growth of those heterotrophs on soluble microbial products (SMP) and extracellular polymeric substances (EPS) generated in the system (Sepehri and Sarrafzadeh, 2019).

The SRT of 2 d was an explicit boundary, which separated the microbial samples into two clusters (Fig. 3). Except for the summer series for Swarzewo WWTP, all the inoculum and intermediate samples (collected at the SRTs > 2 d) were grouped into the first cluster. Regardless of the biomass origin and process temperature, after decreasing the SRT below 2 d, the terminal samples reflected a relatively close genetic distance, which can be seen as another compacted cluster.

For the inoculum samples, the H index ranged from 4.6 to 5.1 (Table 2). Along with decreasing the SRT, a decreased biodiversity was observed. For the winter series, the values of H index were reduced by approximately 40% in the terminal stage of the experimental series. For the summer series, a similar decreasing trend of the biodiversity was observed. The decreasing biodiversity indicated the selection of specific bacterial groups with relatively high growth rates under the applied operational conditions.

### 3.3. Dynamics and phylogenetic composition of the nitrifying and comammox bacteria population

The nitrifier population structure was subjected to apparent changes under the specific operational conditions due to the aggressive SRT reduction (Fig. 4a).

In the inoculum samples, *Nitrosomonas* and *Nitrospira* have been identified as predominant AOB and NOB, respectively. For the SRTs > 2 d, both *Nitrosomonas* and *Nitrospira* tended to increase or stabilize their shares in the total microbial community, while the other NOB (*Ca. Nitrotoga*) balanced at the detection limit. After reducing the SRTs  $\leq 2.0$  d, the washout of AOB and *Nitrospira* was observed. For AOB, the washout intensity was related to the process temperature and the rate at 12 °C was higher in comparison with 20 °C. At the SRTs = 1.0 d, *Nitrospira* were nearly washed out from the system (abundance  $\leq 0.2\%$ ). In contrast, the decreasing SRTs were favorable for *Ca. Nitrotoga*, as its relative abundance increased to at least 2% under the extremely low SRTs. The population of the *Ca. Nitrotoga* was especially resistant to the washout at 20 °C at Swarzewo WWTP, when its relative abundance was approximately 19% in the total microbial population.

At the SRTs = 1.0 d, *Nitrospira* were nearly washed out from the system (abundance  $\leq 0.2\%$ ). In contrast, the decreasing SRTs were favorable for *Ca. Nitrotoga*, as its relative abundance increased to at least 2% under the extremely low SRTs. The population of the *Ca. Nitrotoga* was especially resistant to the washout at 20 °C at Swarzewo WWTP, when its relative abundance was approximately 19% in the total microbial population and led to the high NOB/AOB ratios (Fig. 4b).

The results of the relative quantification of comammox *Nitrospira* by qPCR are shown in Fig. 5. The *amoA* gene copies, specific for clade A and clade B comammox *Nitrospira*, were detected in all the analysed samples. However, due to relatively high values of the critical thresholds for the marker genes, the comammox shares were low in the bacterial consortia. For clade A, the critical thresholds remained at the detection limit from the beginning of the experiments. This proves the sensitivity of clade A to washout under the short SRTs, regardless of the actual process temperature.

Representatives of clade B were more resistant to washout compared to clade A, but their presence in the system was strongly related to the process temperature. At 12 °C, a continuous drop of clade B was observed along with the decreasing SRTs. At 20 °C, after the initial drop, further SRT reductions below 2.0 d did not result in the complete washout of clade B from the reactor. The share of its population tended to stabilize or even increase.

In both studied plants, the vast majority of 16 S rRNA gene DNA sequences specified for NOB reflected the highest similarity to representatives of the canonical *Nitrospira* from sublineage I (Fig. 6). At the

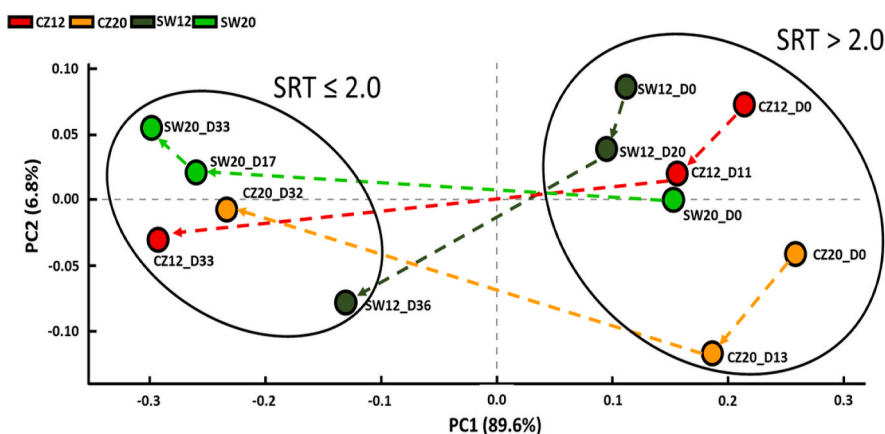


Fig. 3. PCA analysis of the genetic distance between the samples collected during the washout experiments performed at the phylum level, validated with the ANOVA test ( $P < 0.05$ ).

Table 2

H indices for the samples collected during particular experimental series with the biomass from Czajka and Swarzewo WWTPs.

WWTP	Czajka					
Temperature	12 °C			20 °C		
Sample name	CZ12_D0	CZ12_D11	CZ12_D33	CZ20_D0	CZ20_D13	CZ20_D32
Shannon's diversity (H)	4.7	4.8	2.6	4.9	4.6	3.3
WWTP	Swarzewo					
Temperature	12 °C			20 °C		
Sample name	SW12_D0	SW12_D20	SW12_D39	SW20_D0	SW20_D17	SW20_D33
Shannon's diversity (H)	4.6	4.7	3.6	5.1	3.5	3.2

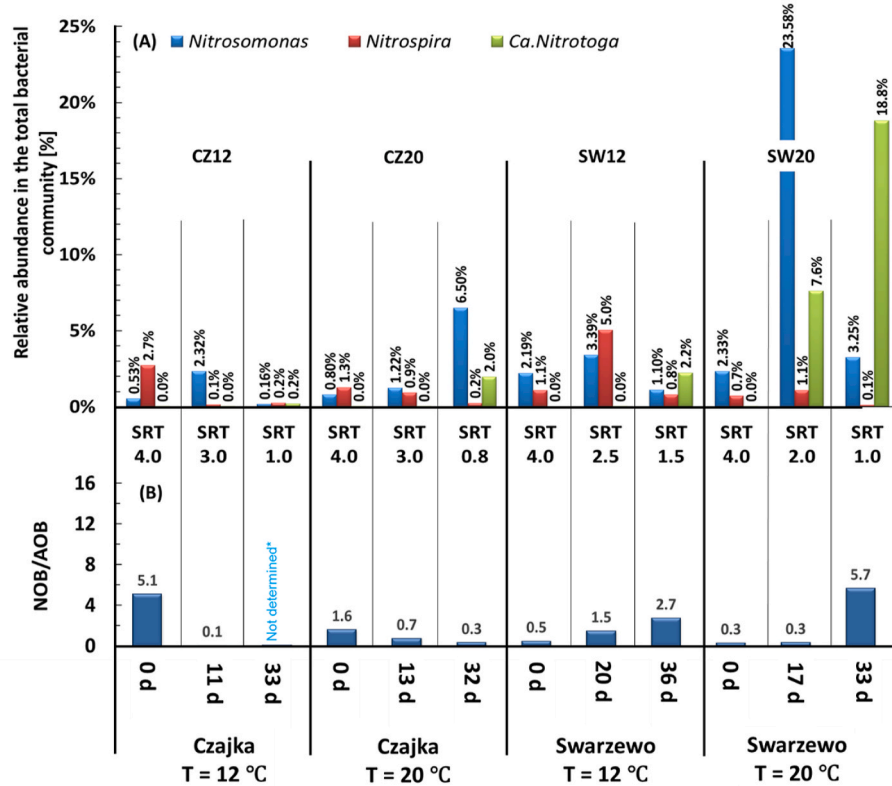


Fig. 4. Nitrifier population dynamics during the washout experiments in terms of the abundances of specific nitrifiers (*Nitrosomonas*, *Nitrospira*, *Ca. Nitrotoga*) (a), and NOB/AOB ratios (b) (\*Not determined due to the reads at the detection limits).

BLAST similarity level  $\geq 98\%$ , most of the reads were similar to the 16 S rRNA of *Nitrospira defluvii* (EU559167.1) and *Nitrospira moscoviensis* (AF155154.1).

DNA sequences of the 16 S rRNA gene, typical for comammox *Nitrospira*, were detected in considerable amounts (~100 reads per sample) in the samples from Swarzewo WWTP at the SRTs = 2.0–4.0 d. The DNA sequences showed  $>99\%$  similarity to the 16 S rRNA gene specific for *Ca. Nitrospira nitrosa*, which is regarded as a representative of comammox *Nitrospira* sublineage II clade B (Koch et al., 2019). The 16 S rRNA gene DNA sequences, initially assigned to *Nitrotoga* genus, have formed a diverse cluster along with the DNA sequences of *Ca. Nitrotoga* genus. The BLAST similarity level of the analysed DNA sequences ranged from 97.8% to 100% in relation to *Ca. Nitrotoga* AM1P (APO, 19547.1).

### 3.4. Coexistence of AOB and NOB in N removal systems

When comparing the nitrifying population of the inoculum samples for both WWTPs, significantly different abundances of NOB and AOB were detected. The predominance of NOB over AOB was observed in Czajka WWTP, where the NOB/AOB ratio ranged from 1.6 to 5.1. For Swarzewo WWTP, the average NOB/AOB ratio of 0.4 was more typical

for activated sludge.

In the course of the experiments, the NOB/AOB ratios (Fig. 4b) and NARs (Fig. 1) were used to monitor the nitrifier activities in terms of their population dynamics and metabolic activity, respectively. In the final stage of the experiments, the low NARs resulted from either high NOB/AOB ratio (5.7) (Swarzewo, 20 °C) or effective AOB washout (Czajka, 12 °C). At 20 °C, the NOB/AOB was low (0.3) at Czajka WWTP, and the NARs were high reflecting effective accumulation of  $\text{NO}_2\text{-N}$ . The dominant NOB was switching from *Nitrospira* to *Ca. Nitrotoga*. However, the growth of *Ca. Nitrotoga* may not exclusively be attributed to the nitrite metabolism, but also to the utilization of alternative electron donors which induce more energy efficient pathways (see: Section 3.5). This may also explain the notable NARs observed for Swarzewo WWTP at 12 °C, despite the elevated NOB/AOB ratios ( $>2.5$ ).

In theory, the ratio of NOB/AOB abundances in a system performing full nitrification could be calculated based on the respective yield coefficients of NOB ( $Y_{\text{NOB}}$ ) and AOB ( $Y_{\text{AOB}}$ ). However, values of those coefficients, reported in several handbooks and summarized by Makinia and Zaborowska (2020), have been highly variable, i.e.,  $Y_{\text{AOB}} = 0.03\text{--}0.15$  mg VSS/mg N,  $Y_{\text{NOB}} = 0.02\text{--}0.08$  mg VSS/mg N. In general,  $Y_{\text{AOB}}$  has been higher than  $Y_{\text{NOB}}$ , which implies that the ratio NOB/AOB

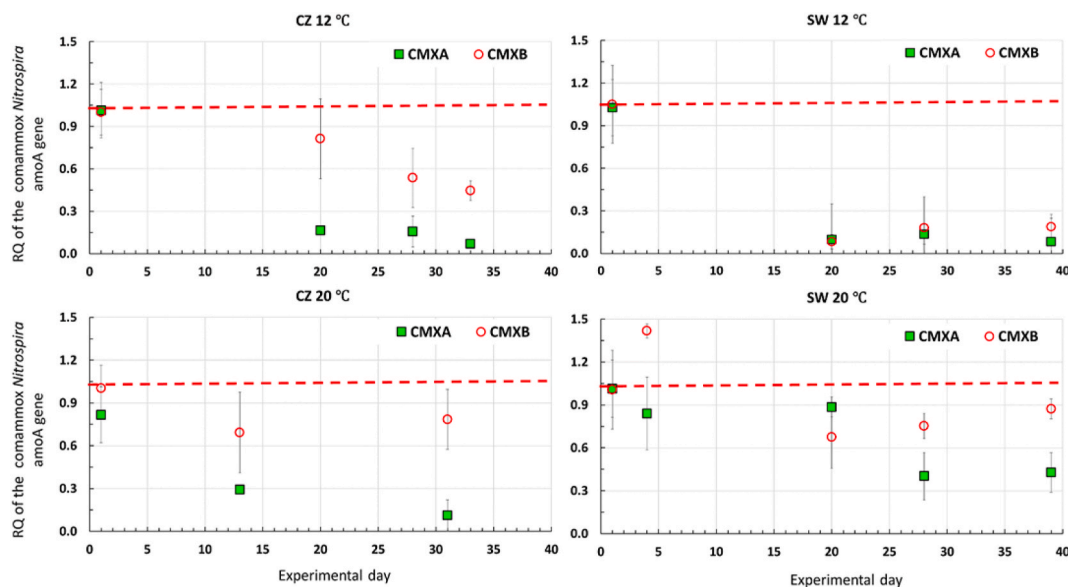


Fig. 5. Comammox *Nitrospira* clade A (CMXA) and clade B (CMXB) relative quantification (RQ) by qPCR.

should be  $< 1$  and AOB should be the most abundant in the microbial community in nitrifying systems. In practice, the abundances of AOB and NOB in WWTPs are highly dynamic even within one plant and depend on both process characteristics (e.g., influent wastewater composition, bioreactor configuration, effluent requirements) as well as local operational and environmental conditions. For example, Alejo et al. (2020) showed how the NOB/AOB ratio was changing in two deammonification moving bed biofilm reactors during a two-year operational period. The ratio varied in the range of 0.25–1.75 depending on three factors, i.e., the presence organic compounds ( $\text{COD} = 0$  or  $\text{COD} > 0$ ), process temperature (10–20 °C) and HRT (0.9–2.5 d).

In another study, Liu et al. (2019) showed that the DO concentration may not be the primary factor affecting the NOB/AOB ratios as those were found very similar at a low level (0.06–0.1) in two parallel laboratory scale SBRs. The reactors, termed  $R_H$  and  $R_L$ , were operated at 22 °C with the SRT = 10 d, but different DO levels, i.e., high DO  $> 2.0$  mg  $\text{O}_2/\text{L}$  ( $R_H$ ) vs. low DO = 0.3 mg  $\text{O}_2/\text{L}$  ( $R_L$ ). The AOB were the dominant nitrifiers in both reactors and the relative abundance of AOB was significantly higher in  $R_H$  (61.5%) compared to  $R_L$  (17.6%). Interestingly, different NOB species responded similarly to the DO changes, which resulted in the comparable NOB/AOB ratios.

Low NOB/AOB ratios have usually been achieved when controlling the NOB activity in nitrification systems. In general, the complete NOB elimination has rarely been achieved. Different operational strategies, including SRT reduction, aeration control, adding specific inhibitors or applying ultrasonic pre-treatment, resulted in either full or partial NOB suppression (i.e., NOB were present in the system, but inactive or less active than AOB).

Higher NOB/AOB ratios (0.8–1.5) have been reported for full-scale municipal WWTPs (Ramdhani et al., 2013) or even 3–4 for a laboratory aerobic granular reactor (Winkler et al., 2012). A similar range was reported in one study (Aqeel et al., 2019), in which the NOB/AOB ratios of 0.5 and 3.0, respectively, were found in conventional activated sludge flocs and granules (with *Nitrobacter* as the dominant NOB). Laurenzi et al. (2019) found different NOB/AOB ratios in the floc phase (0.3) and biofilm (2.0) in a MBBR performing deammonification. When the DO set point was decreased from 1.2 to 0.17 mg  $\text{O}_2/\text{L}$ , the NOB/AOB ratio remained unchanged in the biofilm, whereas it decreased to 0.04 in the floc phase.

The increasing NOB/AOB ratios have been observed in response to specific environmental conditions due to either inhibiting AOB (salinity) or favouring NOB (high influent nitrite concentrations). Fu et al. (2019)

found that with the increase in salinity from 0 to 4.5‰, the NOB/AOB ratio changed from 0.05 to 4. A lower increase (from 1 to 1.4) was caused by changing the influent nitrite concentration from 2.5 to 20 mg N/L and favouring the NOB activity (Liu et al., 2019).

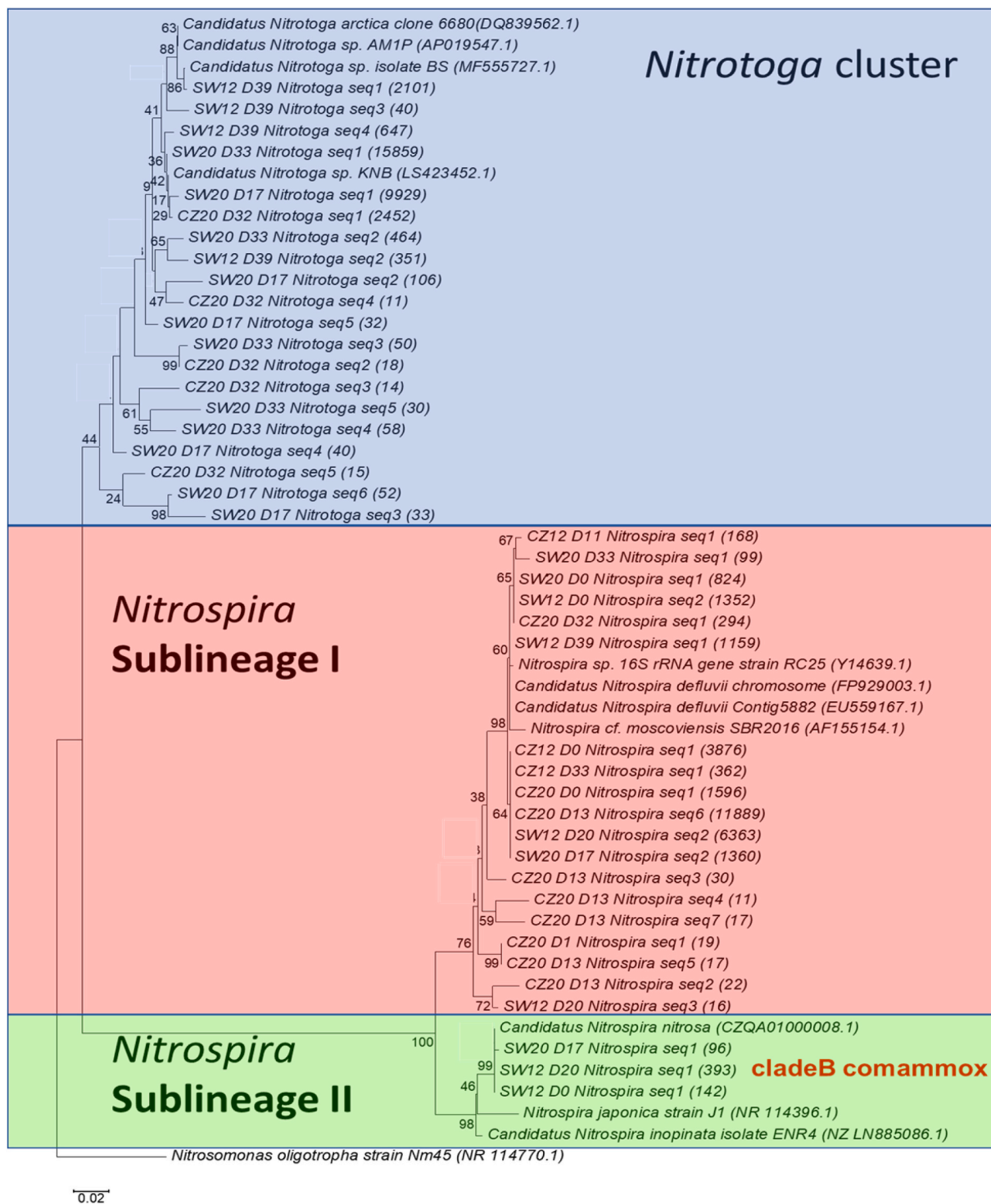
### 3.5. New insights into the NOB population in N removal systems

The results of the present study and literature data confirmed that the NOB population is more diverse than described by the traditional model of two-step nitrification. The characteristics of the dominant NOB representatives (e.g., adaptation under different operational/environmental conditions) and interactions between them and with AOB are the key aspects for understanding nitrogen removal pathways via nitrite.

*Nitrospira* is the most common representative of NOB. Its versatile metabolism may explain the competitive advantages under dynamic conditions in WWTPs. Beyond the nitrification process, members of the *Nitrospira* genus are capable of growing on formate and hydrogen under aerobic conditions, and reducing nitrate to nitrite under anoxic conditions (Koch et al., 2019). Most of *Nitrospira* representatives, which dominate in WWTPs, have belonged to lineages I or II (Yang et al., 2020). Due to the difficult cultivation of the pure or enriched cultures, a few studies have reported the *Nitrospira* activity and its kinetic parameters (Table 3). Lineage I *Nitrospira* have a higher DO affinity and a lower nitrite affinity compared to lineage II (Park et al., 2017). Based on the meta-analysis of literature data, Mehrani et al. (2020) identified the most favorable conditions (occurring simultaneously) for ensuring the highest *Nitrospira* abundances in N removal systems, including high influent  $\text{NH}_4\text{-N}$  ( $> 20$  mg N/L) and DO ( $> 3.0$  mg  $\text{O}_2/\text{L}$ ) vs. low pH ( $< 7$ ) and temperature ( $< 15$  °C).

Another important factor promoting NOB prevalence in the mainstream wastewater treatment is a long SRT ( $> 10$  d) (Cotto et al., 2020). The authors attributed this to slow growth rates of comammox bacteria compared to canonical AOB and NOB. The results of our studies are in line with this finding with respect to clade A comammox bacteria, which were systematically washed out from the system along with decreasing the SRTs. Representatives of clade B comammox *Nitrospira* reflected similar behavior under winter conditions. However, at 20 °C, these bacteria were present in the system under the extremely low SRT (0.8 d), which may suggest slight metabolic differences between clade A and clade B comammox *Nitrospira*.

High NOB/AOB ratios due to high abundances of *Nitrospira*-like NOB could be the evidence that some *Nitrospira* can perform comammox



**Fig. 6.** Phylogenetic analysis of the 16 S rRNA gene DNA sequences (at sequencing depth 50 000 reads per sample) obtained during the washout experiments (Clustering method-Neighbor joining at bootstrap 500). The data in the parentheses: accession numbers from the Gene Bank for the marker sequences, repetition number of the specified DNA sequence obtained from the analysed biomass. The names of the DNA sequences groups consist of: a) origin – Swarzewo (SW) or Czajka (CZ), b) temperature applied during experiment 12 vs 20 °C, c) day of the experiment.

(Wang and Li, 2015; Daims et al., 2016). That process has overthrown “a century-old dogma of nitrification research” (Koch et al., 2019). The comammox *Nitrospira* is a single microorganism that can perform the two nitrification steps, with a higher yield coefficient compared to the canonical NOB. The suitable conditions for the growth of these complete oxidizers generally comprise low DO conditions, low substrate diffusion gradients, and low mixing conditions in biofilms (Mehrani et al., 2020).

There is also a limited number of kinetic studies specifically focused on pure cultures of comammox *Nitrospira*, which reported a very low  $\mu_{max}$  of 0.15 d<sup>-1</sup> (Lawson and Lückner, 2018),  $K_{NH4}$  of 0.009–0.012 mg N/L, and high  $K_{NO2}$  of 5.21–6.29 mg N/L (Koch et al., 2019) (Table 3). In contrast, in a very recent study, Sakoula et al. (2021) found low values of both  $K_{NH4}$  (0.0006 mg N/L) and  $K_{NO2}$  (0.175 mg N/L) for an enriched culture of comammox bacteria.



**Table 3**  
Stoichiometric and kinetic parameters of Nitrospira and Ca. Nitrotoga reported in the literature.

Bacteria	$\mu_{max}$ (1/d)	Y (g VSS/g N)	$K_{NH4}$ (mg N/L)	$K_{NO2}$ (mg N/L)	$K_O$ (mg O <sub>2</sub> /L)	Remarks	Reference
Nitrospira	$0.69 \pm 0.10$	$0.14 \pm 0.02$		$0.52 \pm 0.14$	$0.33 \pm 0.04$	22 °C, Enriched culture, planktonic Nitrospira spp., lineage I	Park et al. (2017)
	$0.148$ $0.45-0.52$	$0.12 \pm 0.04$	$0.012$ $0.009$	$0.9-1.1$ $6.29$ $5.21$ $0.13-0.39$	$0.54$	15-30 °C, Enriched culture Pure culture, Comammox-Nitrospira, lineage II 37 °C, Pure culture, Comammox- Nitrospira, clade I, lineage II 28-37 °C, Pure culture, Nitrospira defluvii, Moscoviciensis, and Strain BS10, intrinsic kinetics 29 °C, pure culture, Nitrospira lineage I and II	Blackburne et al. (2007) Koch et al. (2019) Lawson and Lückner (2018) Nowka et al. (2015)
Ca. Nitrotoga				6 (Lineage I) 10 (Lineage II)			Ushiki et al. (2017)
				1.25		24- 28 °C, poor activity at <20 °C, optimum pH of 7.1-7.6, Ca. Nitrotoga fabula, (Kitzinger et al., 2018) (Ishii et al., 2017)	(Kitzinger et al., 2018) (Ishii et al., 2017)
				0.345		16 °C, Ca. Nitrotoga" sp., optimum pH of 8.3, Strain AMIP (enrichment culture)	Wegen et al. (2019)
				0.62 0.84 0.82		17 °C, Nitrotoga sp. HAM-1 22 °C, Nitrotoga sp. HW29 17 °C, enrichment culture, Nitrotoga arctica	Nowka et al. (2015)

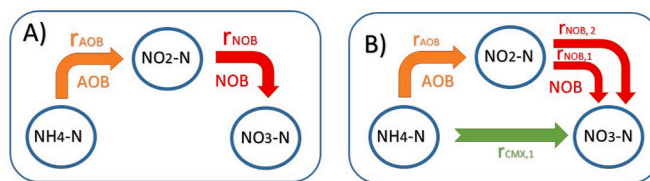
$\mu_{max}$ : Maximum growth rate, Y: Yield coefficient,  $K_O$ : DO affinity constant,  $K_{NH4}$ : Ammonia affinity constant,  $K_{NO2}$ : Nitrite affinity constant.

Apart from *Nitrospira*, *Ca. Nitrotoga* has recently been detected as another important NOB that together with *Nitrospira* or alone can dominate the NOB populations in WWTPs (Lückner et al., 2015; Kitzinger et al., 2018). The abundances of *Ca. Nitrotoga* was reported as 1.0–2.0% of the total microbial community at 20 different WWTPs (Lückner et al., 2015). Kitzinger et al. (2018) explained the coexistence of *Ca. Nitrotoga* and *Nitrospira* in low DO continuous flow bioreactors by different affinities for NO<sub>2</sub>-N (higher for *Nitrospira*) and DO (higher for *Nitrotoga*). However, the reported values of the affinity constants ( $K_O$  and  $K_{NO2}$ ) in other studies (Table 3) do not unambiguously confirm those specific metabolic properties of both bacteria. *Ca. Nitrotoga* was also found to be significantly more resistant to free ammonia (FA) exposure (FA at ~220 mg NH<sub>3</sub>-N/L) than the other NOB (*Nitrobacter* and *Nitrospira*) (Li et al., 2020). The experiments were carried out in a high DO (4.0 mg O<sub>2</sub>/L) SBR at 22 °C and pH of 7.2.

Furthermore, Kitzinger et al. (2018) isolated *Nitrotoga* from activated sludge and reported moderate optimum temperatures (24–28 °C), but the poor activity below 20 °C, and optimum pH of 7.1–7.6. For activated sludge systems, Liu et al. (2019) found that the *Ca. Nitrotoga* had the same optimum temperature range (pH not mentioned). A similar optimum temperature (23 °C), but higher optimum pH (8.3), was found by (Ishii et al., 2020). Despite the high sensitivity of *Ca. Nitrotoga* to the low temperatures, Liu et al. (2019) estimated a very low temperature correction factor ( $\theta = 1.042$  in the T range of 4–22 °C) for NOB predominated by both *Nitrospira* and *Ca. Nitrotoga*.

Lantz et al. (2021) provided further important insights into *Ca. Nitrotoga* ecophysiology. Those bacteria were capable of oxidizing nitrite at temperatures ranging from 4 to 28 °C. Moreover, the authors identified in the *Ca. Nitrotoga* strain CP45 genome proteins, potentially involved in counteracting the harmful effects of low temperatures, such as cold-shock proteins CspA. A wide metabolic potential of *Ca. Nitrotoga* may be a factor, which enables to outcompete *Nitrospira* and *Nitrobacter* under variable temperatures and short SRTs (similar to our study). Liu et al. (2021) reported similar findings for the long-term experiments in SBRs operated under long SRT (20 d) and variable temperatures in the range of 4–34 °C. Temperature was the key factor, which influenced niche occupation of *Nitrotoga*-related bacteria and favoured their predominance under low and moderate temperatures (4–22 °C).

In summary, the most common strategy for NOB suppression in practice, i.e., DO-limited conditions coupled with short SRTs (see the Introduction), may not always be successful. The main findings of the present study were (i) a high resistance of comammox bacteria for the washout, and (ii) the coexistence of *Nitrospira* and *Ca. Nitrotoga* under decreasing SRTs. These two aspects may be considered while expanding the traditional two-step nitrification models for simulation of the novel N removal processes. The expanded model, illustrated in Fig. 7, should incorporate two parallel pathways of nitrite oxidation to nitrate, catalyzed by two groups of NOB (e.g., *Nitrospira* and *Ca. Nitrotoga*), and a new pathway of the direct ammonia oxidation to nitrate by comammox *Nitrospira*. This concept can be used as a base for the development of a mathematical model with the available set of kinetic and stoichiometric parameters (Table 3). In addition, similar washout experiments should be planned with nitrite as a sole nitrogen source. Such experiments could provide an explanation for a preferable nitrogen source for



**Fig. 7.** The traditional two-step nitrification pathway (a), and the new conceptual model of the nitrification process (b) ( $r_{AOB}$  = *Nitrosomonas*,  $r_{NOB,1}$  = *Nitrospira*,  $r_{NOB,2}$  = *Nitrotoga*,  $r_{CMX}$  = comammox).

comammox bacteria and substrate dependences of the specific NOB groups, including the traditional r strategist NOB (*Nitrobacter*).

#### 4. Conclusions

The directed rearrangements of the general bacterial population, including nitrifiers, were observed in the studied SBR regardless of the examined temperature and origin of the inoculum. Both comammox bacteria and different canonical NOB (*Nitrospira* vs. *Ca. Nitrotoga*) were still present under extremely low SRTs, which suggests that the decreasing SRTs under DO-limited conditions may not be a versatile strategy for NOB suppression. In contrast to nitrite production mediated predominantly by *Nitrosomonas* AOB, the nitrate production pathways may be more diverse and include the metabolism of different canonical NOB (*Nitrospira* vs. *Ca. Nitrotoga*) and comammox bacteria. In particular, the short SRT was identified as an important selective factor that may favor the growth of *Ca. Nitrotoga* over *Nitrospira*. As a consequence of the new findings, a new conceptual model of two-step nitrification, including comammox and two nitrite oxidation pathways, should be considered for the description of the novel N removal processes.

E-supplementary data of this work can be found in online version of the paper.

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#### Declaration of competing interest

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.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2022.113753>.

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