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# Ecophysiology and dynamics of nitrogen removal bacteria in a sequencing batch reactor during wastewater treatment start-up

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## Abstract

Nitrogen removal communities performing wastewater treatment consist of ammonia oxidisers, nitrite oxidisers, denitrifiers, and anammox bacteria, and the proportion and activity of particular microbial groups depend not only on the physiochemical parameters of the bioreactor, but also on the composition of the inoculum. Nitrifiers and denitrifiers usually dominate in conventional wastewater treatment systems due to the fact that nitrification and denitrification are the most commonly used nitrogen removal processes. However, from the economical point of view in case of wastewater with high ammonia concentrations, anammox-based technologies are desirable for their treatment. The disadvantage of such systems is slow anammox bacteria growth, which extends an effective technological start-up. Thus, in this study, a fast start-up of the anammox process supported with an anammox-rich inoculum was performed in a sequencing batch reactor (SBR). Using anammox inoculation of SBR laboratory system, the start-up can be fastened to 85 days with 84.5% of nitrogen removal efficacy. The spatial distribution of nitrogen removal bacteria analysed with fluorescent in situ hybridisation revealed that anammox and nitrifiers are located side by side in the flocs and the relative number of ammonia and nitrite oxidisers decreased after 85 days of the experiment.

**Keywords** Anammox start-up · Nitrogen removal bacteria dynamics · Real Time PCR · FISH · TEM

## Introduction

Despite intensive research in the field of nitrogen removal using nitrification–denitrification from wastewater, there is still a need for the search of alternative, cheaper and more effective processes to remove this biogenic compound (Liu

et al. 2016). Since last century, it is known that not only nitrifiers and denitrifiers take part in the nitrogen cycle, both in nature and in engineered systems. There is a group of bacteria performing autotrophic ammonium removal to gaseous nitrogen, widespread in anaerobic environments. These anammox (anaerobic ammonium oxidation) bacteria and their efficient performance are the basis of many new technologies in wastewater treatment developed since 1990s (García-Ruiz et al. 2018). There is no need for aeration and an extra carbon source dosage in the anammox process; thus, anammox-based technologies are cheaper than the traditional nitrification–denitrification combination. Despite obvious advantages, there are several disadvantages of the anammox process: anammox bacteria are relatively sensitive to drastic changes of technological parameters, such as pH, temperature or free ammonia (Jin et al. 2012; Tomaszewski et al. 2017). Moreover, anammox bacteria grow slowly in comparison with other nitrogen removal bacteria [doubling time from 3.6 (Tsushima et al. 2007) to 18 days (Trigo et al. 2006)], which is the reason of the problems with a fast start-up of the process in a technological scale, both at the beginning of the performance and after the operational problems

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causing the process efficacy failure. Usually, it takes from 100 to 390 days to start-up of anammox reactor (Tomaszewski et al. 2017); thus, fastening the start-up process would be advisable especially from the economic reasons. In situation of inoculating systems with the anammox biomass, the fast start-up problem can be overcome, but still there is no sufficient knowledge about the ecological relationships existing in such a community among various nitrogen removal bacterial groups (Liu et al. 2012).

It is widely known that biochemical processes being a part of the nitrogen biogeochemical cycle do not exist in nature separately. Also in artificial environments, such as wastewater treatment bioreactors, there is a large number of these processes linked together and the bacteria responsible for these biochemical reactions coexist in the complex community. The performance of nitrifiers, denitrifiers and anammox bacteria depends on technological parameters settled for the bioreactor. Thus, the studies at such community dynamics and relationships are crucial from the perspective of the fast reactor start-up, especially in a situation where a new bioreactor is inoculated with a mixture of the regular activated sludge and the anammox biomass.

In case of nitrogen removal systems, a wide range of physiochemical parameters can influence the community structure and bacterial ecological relationships in activated sludge. Among these parameters, substrates and oxygen concentration, temperature or inoculating microorganisms can play an important role (Liu et al. 2012; Third et al. 2005; van de Vossenberg et al. 2008). Information gained from the research on the dynamics of the nitrogen removal bacterial community performed during the start-up of the anammox system can be of the utmost importance for future fast start-ups and for maintaining the effective bioreactor performance.

In this study the relationships among nitrifiers, denitrifiers and anammox bacteria were monitored for 85 days of the sequencing batch reactor (SBR) start-up. The reactor was inoculated with activated sludge with the dominance of anammox bacteria to accelerate the start-up process to check faster start-up possibility and to present the microbial community relationships in a newly created environment. In order to present an insight into the bacterial community dynamics and to describe how particular nitrogen removal bacteria behave in new environment, fluorescent *in situ* hybridisation (FISH) and Real Time PCR analysis were used. The presence of anammox bacteria, whose intracellular structure differs from the regular bacterial cell by possessing a membrane structure known as anammoxosome, was confirmed by the transmission electron microscopy (TEM).

## Materials and methods

### Experimental settings, physiochemical analysis and activated sludge sampling

A 20L sequencing batch reactor inoculated with activated sludge from a municipal wastewater treatment plant and anammox sludge (1:1) was operated at the temperature of  $32 \pm 3$  °C (mean  $\pm$  SD, standard deviation) with pH  $7.8 \pm 0.3$  (mean  $\pm$  SD). The dissolved oxygen (DO) was below  $0.1$  mg L<sup>-1</sup>, and volatile suspended solids (VSS) was  $1.6 \pm 0.4$  g L<sup>-1</sup> (mean  $\pm$  SD). The reactor was fed with a mineral medium adapted from van de Graaf et al. (1996). The total nitrogen loading rate (NLR) was regulated by the addition of NH<sub>4</sub>Cl and NaNO<sub>2</sub>, while other elements were dosed in constant concentrations:  $0.048$  g KHCO<sub>3</sub> L<sup>-1</sup>,  $0.041$  g KH<sub>2</sub>PO<sub>4</sub> L<sup>-1</sup>,  $0.228$  g MgSO<sub>4</sub>·7 H<sub>2</sub>O L<sup>-1</sup>,  $0.007$  g FeSO<sub>4</sub>·7 H<sub>2</sub>O L<sup>-1</sup> and  $0.004$  g EDTA L<sup>-1</sup>. pH was corrected using 10% HCl or 10% NaOH.

Regular measurements of ammonium, nitrite and nitrate nitrogen were conducted using fast photometric tests (MERCK Millipore) with a photometer (MERCK Spectroquant® NOVA60). Temperature and pH were monitored by JUMO tecLine HD—the pH combination electrode. The DO concentration was measured by ELMETRON Conductivity/Oxygen Meter CCO-505 with the ELMETRON COG-1 oxygen sensor. Concentrations of VSS were measured according to the standard method (APHA 2005).

Activated sludge samples were collected at 2-week intervals to 50mL sterile Falcon flasks and frozen at  $-20$  °C until further procedure.

### DNA isolation

The activated sludge samples were washed thrice with  $1 \times$  PBS to remove potential PCR inhibitors. Total bacterial DNA was isolated with a mechanical method. The samples (0.2 g) were suspended in 1 mL of the extraction buffer (100 mM Tris–HCl, 100 mM EDTA, 1.5 M NaCl, pH = 8.0), and then, a mixture of bead beating glass pearls, in sizes: 1.25–1.55 mm and 0.4–0.6 mm (Roth, Germany) was added. The mixture was shaken intensively with a vortex and mixed horizontally for 20 min at 1400 rpm using a thermomixer (Eppendorf). In the next step 200  $\mu$ L of 10% SDS (Sigma) was added to the samples, which were incubated with 1400 rpm mixing at 65 °C for 30 min. The samples were centrifuged twice (10 min, 13,000 rpm), and the 500  $\mu$ L of supernatant was placed at silica columns (A&A Biotechnology). The columns were washed twice with the A1 buffer (A&A Biotechnology), and DNA was eluted from the column with 30  $\mu$ L of MilliQ water. DNA concentration

was measured with a Qubit Fluorometer (Invitrogen) and kept frozen at  $-20\text{ }^{\circ}\text{C}$  for further analysis.

### Transmission electron microscopy

The activated sludge samples from inoculum were fixed with 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4) (5 day at  $4\text{ }^{\circ}\text{C}$ ) and postfixed with 1%  $\text{OsO}_4$  in a 0.1 M phosphate buffer (30 min at room temperature). After dehydration in increasing concentrations of ethanol (30, 50, 70, 90, 95, and 100%, each for 5 min), a mixture of 100% ethanol and acetone (1:1, 5 min), and acetone ( $2\times 5$  min), the material was embedded in the epoxy resin (Epoxy Embedding Medium Kit, Sigma). Ultrathin sections were cut on a Leica ultracut UCT25 ultramicrotome. Ultrathin sections (50 nm thick), mounted on the formvar-covered grids (50 mesh), were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined with a transmission electron microscope (Hitachi H500 at 75 kV).

### Fluorescent in situ hybridisation (FISH)

In order to estimate the amount and identify the active part of the bacterial community, fluorescent in situ hybridisation was used. The biomass samples were fixed by adding three volumes of the 4% (w/v) paraformaldehyde solution and incubated for 3 h in  $4\text{ }^{\circ}\text{C}$ . Then, the samples were washed thrice with phosphate-buffered saline ( $1\times \text{PBS}$ , pH = 7.5) and resuspended in a 1:1 mixture of phosphate-buffered

saline and absolute ethanol. Fixed samples were stored at  $-20\text{ }^{\circ}\text{C}$  for the further analysis. Selected probes which hybridise with the 16S rRNA have been used to collect data on the nitrogen removal bacteria community in samples collected from the bioreactor (Table 1). All oligonucleotide probes were obtained from Bionovo, Poland.

For fluorescent in situ hybridisation,  $10\text{ }\mu\text{L}$  of a fixed sample was applied on a well of glass slide (Thermo Scientific), dried and sequentially dehydrated in solutions of 50%, 80% and 97% ethanol (v/v) in PBS for 3 min each. To start hybridisation,  $10\text{ }\mu\text{L}$  of hybridisation buffer (containing the appropriate formamide concentration for each probe, Table 1) and  $1\text{ }\mu\text{L}$  of fluorescently labelled specific probes and  $1\text{ }\mu\text{L}$  of the EUB probe targeting most of bacteria (at a concentration of  $5\text{ pmol}/\mu\text{L}$  for Cy3 and  $8.3\text{ pmol}/\mu\text{L}$  for FLUOS) were added to the well. The hybridisation was conducted for 90 min at  $46\text{ }^{\circ}\text{C}$  in a humidified chamber. Following hybridisation, a stringent washing step was performed for 15 min at  $48\text{ }^{\circ}\text{C}$  in a buffer with the appropriate NaCl concentration (Table 1), 20 mM Tris-HCl, pH = 7.0, 5 mM EDTA and 0.01% SDS. FISH signal was evaluated after adding the mounting agent—Citifluor (Citifluor, London, UK). The signal obtained from specific probes for the nitrogen removal bacteria community was compared with the signal obtained from the EUBMIX probe targeting most of the bacteria. In order to estimate the number of individual nitrogen removal bacterial groups on the 3rd and 85th day of the experiment, the measurements were performed in at least 20 replicates for each group. Statistical analyses

**Table 1** Details of the fluorescent in situ hybridisation (FISH) probes' procedure used in the experiment

Aim	Probe	Sequence (5'–3')	Formamide concentration (%)	5 M NaCl concentration ( $\mu\text{L}$ )	References
All bacteria	EUB MIX (EUB 338 + EUB 338 II + EUB 338 III)	GCT GCC TCC CGT AGG AGT + GCA GCC ACC CGT AGG TGT + GCT GCC ACC CGT AGG TGT	0–50	100–9000	Amann et al. (1990)
All known <i>Planctomycetes</i>	Pla 46f	GAC TTG CAT GCC TAA TCC	30	1020	Neef et al. (1998)
Anammox bacteria	Amx 368	CCT TTC GGG CAT TGC GAA	15	3180	van der Star et al. (2008)
	Amx 820	AAA ACC CCT CTA CTT AGT GCC C	40	460	Schmid et al. (2000)
Betaproteobacterial ammonia-oxidising bacteria	Nso190	CGA TCC CCT GCT TTT CTC C	55	100	Mobarry et al. (1996)
	Nso1225	CGC CAT TGT ATT ACG TGT GA	35	700	
NOB (Phylum <i>Nitrospirae</i> )	Ntspa 712	CGC CTT CGC CAC CGG CCT TCC	50	180	Daims et al. (2001)
Potential denitrifiers	Pae 997	GCT GGC CTA GCC TTC	0	9000	Crocetti et al. (2000)
	PAR 651	ACC TCT CTC GAA CTC CAG	40	460	Neef et al. (1998)



of the obtained data were performed with Statistica 12.0 Software (StatSoft). Shapiro–Wilk test was used to test the data normality, and then, based on the statistic results, the Mann–Whitney  $U$  test was used to examine the significance of the differences between the results obtained from 3rd and 85th day of the experiment;  $p$  values  $< 0.05$  were considered statistically significant.

## Real Time PCR

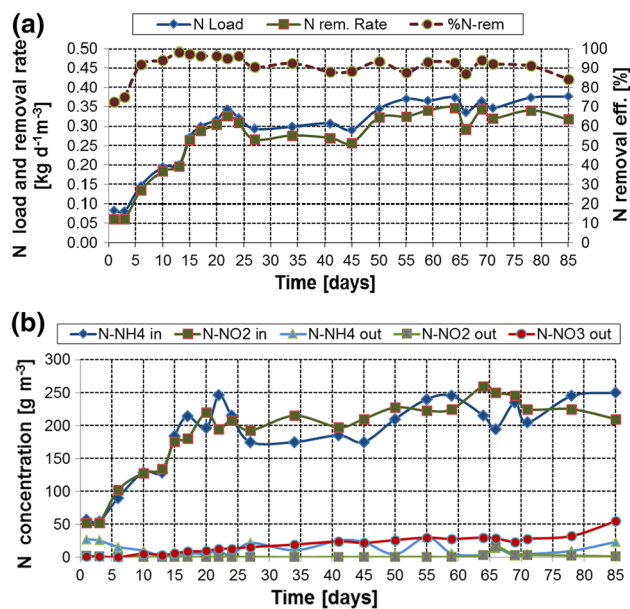
On the basis of total bacterial DNA isolated from the activated sludge samples, Real Time PCR for functional genes for AOB, NOB, anammox and denitrifiers was performed using Real Time PCR 2xRT PCR Mix SYBR A (A&A Biotechnology). Five ng of the DNA templates was used in the total reaction volume of 20  $\mu\text{L}$ . The reaction was lead in Stratagene Mx3005P Thermocycler (Agilent Technologies) in the SYBR Green system in triplicates. The Real Time PCR was performed using primers presented in Table 2 according to the procedure described previously for each primer set (Ferris et al. 1996; Li et al. 2012b; Gerbl et al. 2014; Kim et al. 2011). Relative quantification of functional genes was calculated in relation to the 16S rRNA reference gene representing the total bacterial community and presented as a mean value of triplicated data set. The values were calculated by the use of formula  $2^{\Delta C_t}$ , where  $\Delta C_t = C_{t_{\text{ref}}} - C_{t_{\text{anal}}}$ .  $C_{t_{\text{ref}}}$  is  $C_t$  of reference gene (bacterial 16S rRNA gene), while  $C_t$  is  $C_t$  value for analyzed gene.

## Results and discussion

To present an insight into the newly created community of the activated sludge of SBR reactor, two molecular methods: FISH and Real Time PCR were used. These analyses were supported with physiochemical measurements during

the total length of the experiment to present the effectiveness of the nitrogen removal. As it is presented in Fig. 1, ammonium, nitrite and nitrate concentrations were analysed.

The feeding strategy during the first 22 days was based on the increase in the NLR when the nitrogen removal efficiency was stable. NLR was gradually increased from 0.082 to  $0.343 \text{ kg N m}^{-3} \text{ day}^{-1}$ . At same time, the nitrogen removal rate (NRR) increased from 0.061 to  $0.326 \text{ kg N m}^{-3} \text{ day}^{-1}$  on day 22. After this period, the reactor was operated with the average NLR  $0.340 \pm 0.031 \text{ kg N m}^{-3} \text{ day}^{-1}$  (mean  $\pm$  SD) and NRR  $0.310 \pm 0.030 \text{ kg N m}^{-3} \text{ day}^{-1}$  (mean  $\pm$  SD). An almost complete nitrogen removal was demonstrated

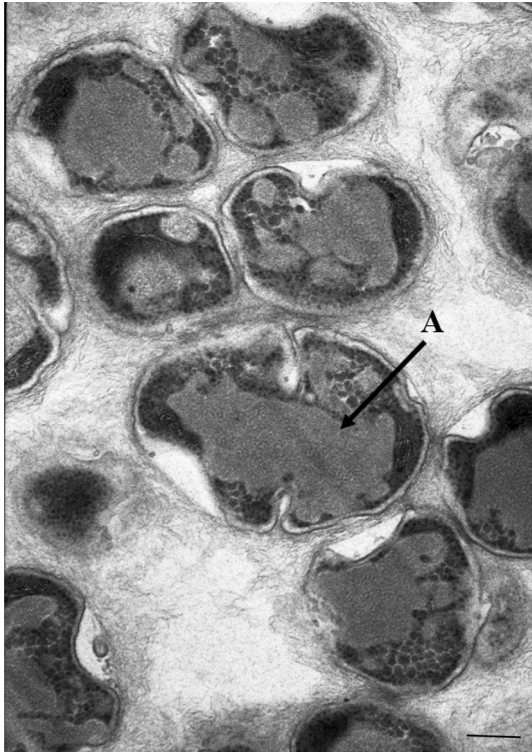


**Fig. 1** Physiochemical performance of SBR reactor during 85 days of the experiment; **a** total nitrogen loads and their removal rates, **b** ammonia, nitrite and nitrate concentrations

**Table 2** Primers used for Real Time PCR in this study

Aim	Target gene	Primers	Sequence 5'–3'	Bibliography
Bacteria	16S rRNA	1055F 1392R	ATGGCTGTCTCAGCT ACGGGCGGTGTGTAC	Ferris et al. (1996)
Ammonia oxidisers	<i>Amo</i>	amoA-1-F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAGCCTTCTTC	Li et al. (2012b)
Nitrite oxidisers	<i>Nxr</i>	nxrA-RT-F nxrA-RT-R	GTG GTC ATG CGC GTT GAG CA TCG GGA GCG CCA TCA TCC AT	Gerbl et al. (2014)
All known <i>Planctomycetes</i>	<i>Hzo</i>	hzoC11f1 hzoC11r2	TGYAAGACYTGCAAYTGG ACTCCAGATRTGCTGACC	Kim et al. (2011)
Denitrifiers	<i>NirS</i>	nirS 1f nirS 3r	TACCACCCSGARCCGCGCGT GCCGCCGTCRTGVAGGAA	
	<i>NirK</i>	nirK876 nirK1040	ATYGGCGVCAYGGCGA GCCTCGATCAGRTRTRTGTT	

between days 15 and 24 ( $96.7 \pm 1.1\%$ , mean  $\pm$  SD), but it was probably improved by the endogenous denitrification, which is typical during the anammox process start-up (Li et al. 2012a; Yu et al. 2013). However, after the 71 days of the experiment, the nitrate concentration in the effluent increased and the nitrogen removal efficiency dropped to 84.5% on day 85, which can be an indicator of the end of a successful anammox process start-up (Li et al. 2012a; Yu et al. 2013).

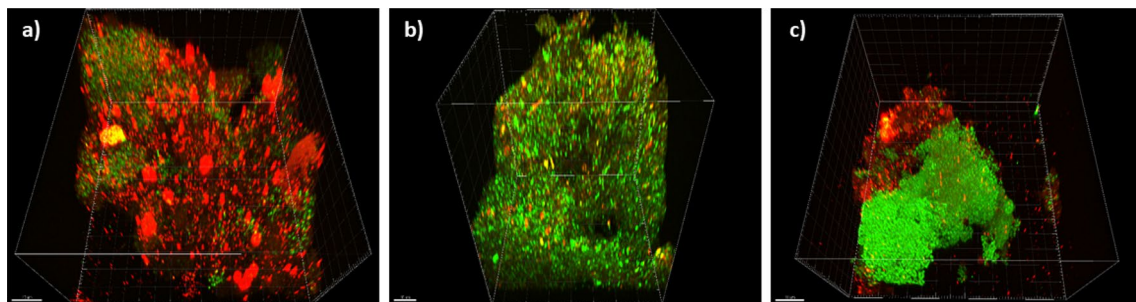


**Fig. 2** Anammox bacteria cells in activated sludge samples visualised in transmission electron microscopy (TEM); A—anammoxosome. Bar = 0.27  $\mu$ m

From the beginning of the experiment, anammox bacteria were present in the bioreactor and it was possible to prepare transmission electron microscopy pictures of their cells (Fig. 2). The cell structure of anammox bacteria differs from the commonly known bacterial ultrastructure. Their cell possesses three independent compartments, known as the paryphoplasm, riboplasm and anammoxosome. The last one is a structure in which energy conservation occurs (van Teeseling et al. 2013). In case of this experiment, the anammoxosome was visible in a cross section of the anammox bacteria cell (indicated as A, Fig. 2).

Fluorescent in situ hybridisation (FISH) was used for this study in order to measure the abundance of particular bacterial groups as a per cent of the total bacterial community during the experiment. This analysis presents information about the active part of the community as the oligonucleotide probes target rRNA molecules in activated sludge. FISH enables also to present the spatial distribution of the particular bacterial groups in activated sludge flocs, which helps to explain the mutual relationships among nitrogen removal bacteria (Fig. 3).

The seeding activated sludge consisted of both floccular and granular sludge with the dominance of anammox bacteria. As it was assumed the granules of anammox bacteria seem to dominate in the flocs (granules) with NOB, they are localised outside the flocs (Fig. 3a). AOB are located outside the NOB (Fig. 3b), but the observation of anammox and AOB together (Fig. 3c) leads to the statement that these two groups were side by side in the flocs (granules). These results are comparable with these obtained by Liu et al. (2012) working on the CANON reactors, and Pynaert et al.'s (2003) analysis of the spatial distribution of bacteria in a one-stage rotating biological contactor. Such a close location of AOB and anammox was explained in two ways: anammox bacteria are protected by AOB, which consume oxygen and produce nitrite, or AOB can perform some sort of anaerobic metabolism (Pynaert et al. 2003; Liu et al. 2012). We cannot also exclude the changes in the spatial



**Fig. 3** Fluorescent in situ hybridisation on confocal microscope performed to present the spatial distribution of the particular nitrogen removal bacteria in activated sludge flocs using probes: **a** AMX820 for anammox bacteria in Cy3 (red) and Ntspa712 for NOB in FLUOS

(green); **b** Ntspa712 for NOB in Cy3 (red) and Nso1225 for AOB in FLUOS (green); **c** MX820 for anammox bacteria in Cy3 (red) and Nso1225 for AOB in FLUOS (green)

distribution of particular nitrogen removal groups during fixation and samples preparation as the samples from the beginning of the experiment were partially granular, but the technological parameters settled in SBR lead to disintegration of the granules into floccular sludge.

By calculating the ratio of the signal for specific probes for AOB, NOB and potential denitrifiers to the signal obtained for most bacteria via the EUBMIX probe, a semi-quantitative analysis of nitrogen removal bacteria groups was conducted (Fig. 4). As it had been suspected, the relative number of AOB and NOB decreased after 85 days of the experiment from 18.4% to 15.5% and 21.02% to 14% of the total bacterial community, respectively. It is important to mention that activated sludge samples are highly heterogeneous. In case of this analysis, the heterogeneity of the samples was even higher due to the fact that the seeding sludge was derived from two different technological systems: a full-scale wastewater treatment plant (floccular sludge), and a nitrification–anammox laboratory-scale fixed bed reactor (granular sludge; partially disintegrated during the bioreactor operation). That is why the analysis using FISH was performed in at least twenty repetitions per analysis, but still the statistical analysis performed on these samples points at high diversity of the samples composition. The proportion of anammox bacteria as well as denitrifiers also slightly decreased, but these results can be explained by the change in the reactor volume and the adaptation phase of the community.

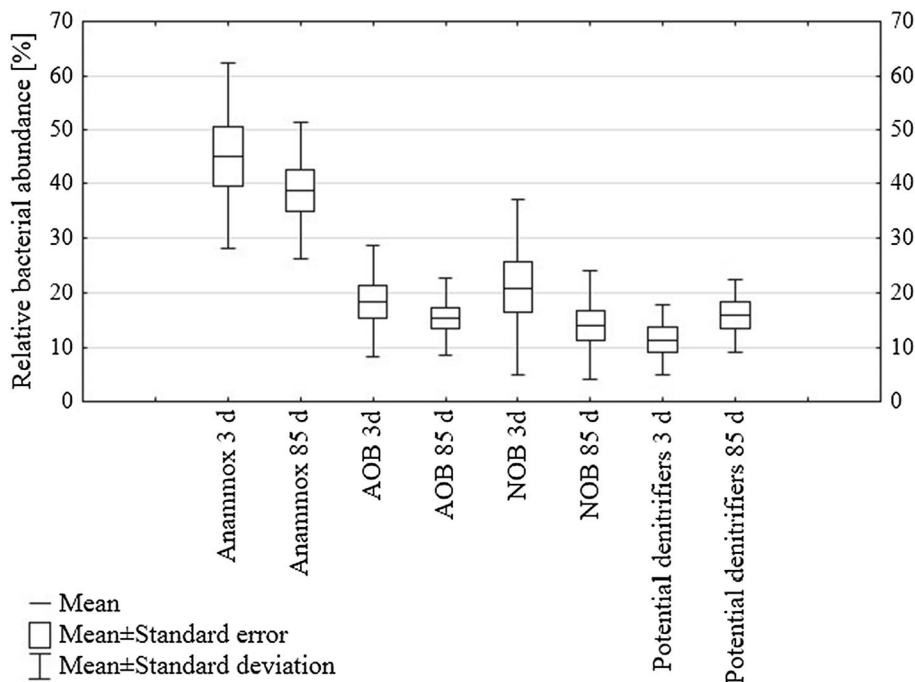
During the start-up in the SBR, microorganisms needed to adapt to a smaller volume of the reactor as well as to the newly created ecosystem. As it can be seen by comparing

these results to the physiochemical analysis, the decrease in the number of active bacteria did not influence negatively the reactor performance. This result stands along with the results obtained by Liu et al. (2012). In their work there was also no direct relationship between the performance of the reactor and the quantity of individual bacterial groups.

Interestingly, the number of bacterial functional genes analysed with the Real Time PCR decreased only in case of NOB; for AOB it was kept at a similar level (Table 3). Also the number of AOB detected with Real Time PCR in comparison with other nitrogen removal bacteria is the lowest. This analysis was performed at DNA level; thus, it is impossible to state that at least part of AOB community was active. An explanation for such a situation could be that SBR during the start-up of the anammox process was kept anoxic to create an anammox-friendly environment and the anoxic conditions caused that AOB remained inactive. Also a previous study (Hellinga et al. 1998; Liu et al. 2012) stated that the maximum specific growth of ammonia oxidisers is higher than that of nitrite oxidisers in temperatures over 25 °C, as well as in lower dissolved oxygen concentrations (De Clippeleir et al. 2011); thus, in this experiment, with temperature of  $32 \pm 3$  °C (mean  $\pm$  SD) and DO below  $0.1 \text{ mg L}^{-1}$  AOB and anammox could outcompete NOB. These results stay along with the results from FISH, and it is highly probable that the process settings caused partial elimination of NOB from the system.

The anammox bacteria number calculated as the *Hzo* gene activity per one copy of 16S rRNA gene increased during the experiment from  $3.07$  to  $3.64 \times 10^{-1}$ . These results are in line with the physiochemical data, according

**Fig. 4** Comparison of the relative abundance of individual nitrogen removal bacterial groups on the 3rd and 85th day of the experiment analysed using fluorescent in situ hybridisation (FISH). Abundance of the bacterial group was estimated as a comparison of the signal for specific probes for anammox bacteria, AOB, NOB and potential denitrifiers to the signal obtained for most bacteria via the EUBMIX probe





**Table 3** Relative quantification of functional genes for AOB, NOB, anammox and denitrifiers in the activated sludge samples obtained with Real Time PCR

Day of the experiment	Relative quantification of functional genes per one copy of 16S rRNA gene				
	<i>AmoA</i>	<i>NxrA</i>	<i>Hzo</i>	<i>nirS</i>	<i>nirK</i>
3rd	$6.14 \times 10^{-6}$	$7.63 \times 10^{-4}$	$3.07 \times 10^{-1}$	$4.74 \times 10^{-2}$	$4.82 \times 10^{-2}$
85th	$6.55 \times 10^{-6}$	$5.87 \times 10^{-4}$	$3.64 \times 10^{-1}$	$5.04 \times 10^{-2}$	$4.70 \times 10^{-2}$

The values present a mean value from a triplicate analysis and were calculated as copy number of the target genes per one copy 16S rRNA reference gene

to which the anammox process was introduced and worked effectively (Fig. 1). The relative abundance of the anammox bacteria presented as FISH results decreased from 45.14 to 38.68%. However, this difference in the anammox bacteria abundance is not statistically significant ( $p=0.35$ ), and it should be emphasised that the samples were highly heterogeneous, which hindered the analysis. As it could be suspected, the anammox bacteria group used as a seeding sludge was relatively abundant in the inoculum (Fig. 4) and the relative number of the *Hzo* gene is much higher from the beginning of the experiment than the functional genes of the other nitrogen removal bacteria (Table 3). The inoculum derived from both the communal wastewater treatment plant (WWTP) and the lab-scale anammox reactor, thus in these environments a large number of ecological niches were potentially available. When directed to a laboratory-scale bioreactor, although with optimal anammox parameters, the number of ecological niches decreased drastically and the number of the anammox bacteria dropped.

The results of FISH and Real Time PCR for denitrifiers seem to be convergent. Although the average relative abundance of this bacterial group increased from 11.41 to 16%, as in case of anammox bacteria, this difference is not statistically significant ( $p=0.19$ ). Both abundance of denitrifiers during the reactor start-up and the *NirS* and *NirK* gene number indicate that the activity of these bacteria remains relatively constant.

It could be suspected that as in case of the anammox bacteria, the new environment caused a sort of specialisation due to the lower niche number, and their number decreased slightly but the activity visible as physiochemical analysis was high. Interestingly, the number of denitrifiers remains at a similar level at the beginning and the end of the start-up of the process, which emphasises the possibility of the presence of endogenous denitrification, which was supported with physiochemical data (Fig. 1). These results support the thesis that nitrogen removal bacteria coexist in wastewater treatment systems and are linked with physiological and ecological relationships highly dependent on technological parameters which are responsible for their dominance. Studies on the dynamics of particular groups during technological processes could be helpful in optimising the process to reach high efficacy.

## Conclusion

The anammox-rich inoculum enables to shorten the start-up of the anammox process to 85 days, when bioreactor reached the level of 84.5% nitrogen removal efficacy.

According to the FISH results, it could be stated that particular nitrogen removal bacteria stay in a close relationship with each other and they cooperate in the community performing a particular nitrogen removal process which is the most suitable under the actual technological parameters. The obtained results support the thesis that AOB and anammox bacteria coexist in a close relationship in WWTPs system and AOB protective role is possible.

The number of anammox bacteria increased slightly during the experiment, but their performance was at a high level which let us to suspect that the bioreactor size caused microbial specialisation for lower ecological niche number. Nonetheless, the other nitrogen removal bacteria are still present in the system, and probably, a smaller bioreactor volume can still serve as a reservoir of ecological niches suitable for them.

The studies on the dynamics of particular groups during technological processes could be helpful in optimising the process to reach its high efficacy and a faster bioreactor start-up.

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