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DNA vs RNA based studies of nitrogen removal bacteria genes via qPCR

Anna Banach-Wiśniewska*, Filip Gamoń, Aleksandra Ziembińska-Buczyńska

Silesian University of Technology, Faculty of Power and Environmental Engineering, Environmental Biotechnology Department, Gliwice, Poland

*Corresponding author's e-mail: anna.banach-wisniewska@polsl.pl

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Abstract: Improvements in water quality requires the removal of nitrogen compounds from wastewater. The most promising and cost-effective methods for this purpose are biological ones based on activated sludge microorganisms such as nitrifiers, denitrifiers, and anammox bacteria. Due to the most of the nitrogen removal bacteria are uncultivable in a laboratory, the application of the molecular tools is required to investigate microorganisms involved in the nitrogen removal. In case of this study for the analysis of relative genes abundance of nitrogen removal bacteria, quantitative PCR (qPCR) based on bacterial DNA and qPCR preceded by reverse transcription (RT-qPCR) based on bacterial mRNA as a template, were used with specific bacterial functional genes (*amoA, nrxA, nirS, nirK, hzo*). Samples from four anammox sequencing batch reactors (SBRs) were analyzed, while the nitrogen removal process and bacteria growth were supported by biomass immobilization and nanoparticles addition. There were statistically significant differences between results obtained in the case of mRNA and DNA ($p<0.05$). Statistically significant positive correlations were found between results obtained with those two approaches. In case of mRNA analysis, positive results were obtained only for *hzo, amoA* and partly for *nirS* genes, despite additional purification and removal of inhibitors from samples prior to reaction.

Introduction

The protection of water resources is one of the main challenges of contemporary environmental management. The increase in the concentration of biogenic elements such as nitrogen and phosphorus in water reservoirs can cause eutrophication and changes in the diversity of aquatic organisms (Tekile et al. 2015, Dodds and Smith 2016). Thus, improvements in water quality require reductions of these compounds (Conley et al. 2009). The most common and promising methods for nitrogen removal are biological methods based on activated sludge. The activated sludge system is a widely applied microbial-based type of biological treatment of wastewater due to its relatively easy implementation and operation cost-effectiveness (Zhang et al. 2017). Nitrogen removal communities performing wastewater treatment consist of denitrifiers, ammonia oxidizers (AOB), nitrite oxidizers (NOB), and anammox bacteria. The abundance and activity of particular microbial groups may affect nitrogen removal processes efficiency and reactor performance. However, most of the nitrogen cycle microorganisms present in the technological systems are uncultivable in a laboratory. Therefore, to study them, it is necessary to use molecular biology tools (Stewart 2012). In the last decades, RNA and DNA based molecular biology tools have become the most relevant identification and detection methods in the investigation of complex microbial communities in wastewater treatment systems (Calli et al. 2006; Sharma et al. 2005; Abzazou et al. 2018, Zahedi et al. 2019; Ding et al. 2020; Wang et al. 2020). One of the techniques, that has become the most frequently used for the detection and quantification of specific bacterial genes in environmental samples is qPCR (quantitative Polymerase Chain Reaction). This method allows determining the number of copies of a particular DNA sequences using sequence-specific primers and also determining the number of copies of particular RNA sequences, when qPCR is preceded by reverse transcription (RT-qPCR). RT-qPCR is a powerful tool for analyzing gene expression. In the last decades, many PCR primers have been designed for nitrogen removal bacterial groups investigation (Gerbl et al. 2014; Jiang et al. 2020; Wang et al. 2020). For relative gene quantification, the expression of a target gene is measured in relation to one or multiple reference genes (Regier and Frey 2010). In our study 16S rRNA coding gene was chosen as a reference gene. In turn, functional genes, which encode enzymes involved in nitrogen conversion pathways and which are widely applied as genetic markers for nitrogen cycle bacteria studies, were selected as a target genes: *hzo* (gene encoding hydrazine oxidoreductase, specific

for anammox bacteria), *nirS* and *nirK* (nitrite reductase genes, specific for denitrifiers), *amoA* (gene encoding ammonia monooxygenase, used in AOB determination), *nxrA* (gene encoding nitrate oxidoreductase (III), specific for NOB) (Kim et al. 2011; Li et al. 2012; Smith et al. 2015; Wang et al. 2016; Zhang et al. 2019; Yang et al. 2018).

The experiment aimed to compare the results of qPCR on the DNA template and RT-qPCR on the mRNA template, isolated from environmental samples. Since it is known that DNA extracted from environmental sample represents all genes in a targeted biocenosis and RNA only the genes that are actively involved in nitrogen transformation, working with both matrices reveals two different pictures. Thus, our objective is to investigate these differences between the results of downstream analyses performed on both matrices extracted from activated sludge environmental samples. qPCR and RT-qPCR reactions were conducted for functional genes specific for anammox bacteria, denitrifiers, and nitrifiers. The results were presented as relative gene abundance expressed as the ratio of the cDNA copy number per one DNA copy of the 16S rDNA gene and mRNA (cDNA) copy number per copy of reference gene isolated from bacterial RNA. Samples were collected from the 4 anammox SBRs (sequencing batch reactors) operated at different temperatures: 23°C and 18°C. The experiment conditions such as SBR setup, immobilization and nanomaterial addition were selected in a way to support the anammox process carried out at temperature lower (23°C and 18°C) than temperature considered as optimal for anammox bacteria occurring in technological systems (30°C). Our previous studies revealed that immobilization in alginates and addition of reduced graphene oxide (RGO) may enhance the stability and effectiveness of the nitrogen removal processes in temperature close to the temperature of the main stream of wastewater (Tomaszewski et al. 2019; Banach-Wiśniewska et al. 2020a).

Materials and methods

Experiment settings and sampling procedure

Samples for molecular analyses were collected from 4 SBRs, the volume of 1 L, while the process was performed at 23°C and 18°C (samples 2–9). The SBRs were inoculated with equal amount of anammox biomass from the wastewater treatment plant in Germany (WWTP) (sample 1), the VSS (Volatile Suspended Solid) was 1.432 ± 0.634 g/L. In this study, three methods were used to support the biological nitrogen removal: biomass with reduced graphene oxide (RGO) addition (20 mg/gVSS), biomass immobilized in 4% sodium alginate (SA), biomass immobilized in SA with RGO addition. Immobilization procedure were described elsewhere (Banach et al. 2018). During the experiment, the reactors were operated at a pH level of 7.5 ± 0.4 and the pH was corrected using 10% NaOH and 10% HCl. The hydraulic retention time was equal 2 d and the dissolved oxygen (DO) concentration was below 0.2 mg/L. DO concentration was measured by ELMETRON Conductivity-Oxygen Meter CCO-505 with ELMETRON COG-1 oxygen sensor. The reactor was fed with a mineral medium containing: 0.048 g/L $KHCO_3$, 0.041 g/L KH_2PO_4, 0.228 g/L $MgSO_4$ $7H_2O$ and 0.007 g/L $FeSO_4$ $7H_2O$. The nitrogen concentration was regulated using NH_4Cl and $NaNO_2$. The SBRs operated with two cycles per day. Each cycle consisted of filling, reaction settling, drawing and idle phase. Samples for the molecular biology analysis were collected from 4 SBRs during the idle phase. The reactors scheme is presented in Figure 1. The physicochemical parameters of the reactors: ammonium, nitrite and nitrate nitrogen were monitored regularly during the experiment with fast photometric tests (MERCK Millipore, Germany).

Genetic material isolation

Genetic material (DNA and RNA) was isolated from the same samples previously protected by RNALater in a 1:10 ratio (Sigma) to avoid degradation of RNA. The DNA extraction was performed by the mechanical method and was described elsewhere (Ziembińska-Buczyńska et al. 2014).

The total bacterial RNA was extracted according to the manufacturer's instructions with the Total RNA Mini Plus Kit (A&A Biotechnology, Poland). Genetic material was treated by Antyinhibitor Kit (A&A Biotechnology, Poland) to remove potential inhibitors of the PCR reaction and purified with Clean up Kit (A&A Biotechnology, Poland). The concentration and the purity of extracted genetic material (both, DNA and RNA) were measured spectrophotometrically using Qubit Fluorimeter (Invitrogen). Isolated and purified total bacterial RNA was used for reverse transcription to synthesize the cDNA (TranScriba Kit (A&A Biotechnology, Poland)). Before the reverse transcription procedure, the deoxyribonucleases were inactivated with RQ1 RNase-Free DNase (Promega, Germany) according to the manufacturer's instructions. Extracted DNA and obtained cDNA were used for PCR amplification.

qPCR

qPCR analysis was based on the following genes: *hzo* (gene encoding hydrazine oxidoreductase, specific for anammox

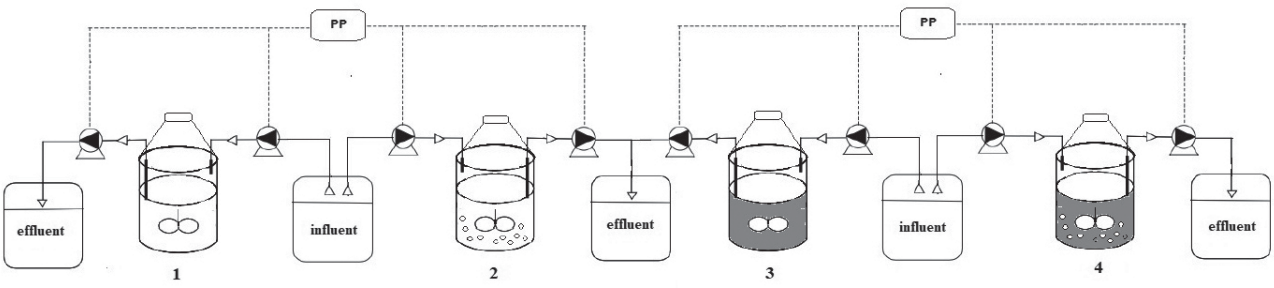

Fig. 1. Scheme of SBRs used in the experiment: 1 – control, 2 – biomass immobilized in SA, 3 – biomass with RGO addition, 4 – biomass immobilized in SA with RGO addition; PP – peristaltic pump

bacteria), *nirS* and *nirK* (nitrite reductase genes, specific for denitrifiers), *amoA* (gene encoding ammonia monooxygenase, used in AOB determination), *nxrA* (gene encoding nitrate oxidoreductase (III), specific for NOB). Sequences of the primers were presented in Table 1, while details for the genetic material isolation, qPCR procedure were described elsewhere (Ziembińska-Buczyńska, et al. 2019, Banach-Wiśniewska et al. 2020).

Relative genes abundancy (q) was calculated according to the following formula:

$$q = 2^{\Delta C_t} \quad (1)$$

where $\Delta C_t = C_{tref} - C_{tanal}$, C_{tref} is C_t of the reference gene (bacterial 16S rRNA gene), while C_t is C_{total} value (threshold cycle) for analyzed gene (Livak and Schmittgen 2001).

Statistical analysis

To test data normality and the equality of variances, Shapiro--Wilk and Leven's tests were conducted, respectively. Then, to investigated the significance of the differences between the DNA and mRNA based results, Student's t-test with Cochran--Cox adjustment or Mann Whitney U test were performed. In addition, the Spearman Rank Correlation coefficients were calculated between the obtained results.

Results and discussion

The relative abundance of genes responsible for the pathways of nitrogen transformation: *nxrA, amoA, nirK, nirS* and *hzo* genes were determined via qPCR using isolated bacterial DNA as a template and via RT-qPCR using synthesized cDNA from isolated bacterial mRNA as a template (Figure 2). All qPCR reactions were conducted at least in triplicates and efficiencies were closed to 1.0. The results are presented as the ratio of the cDNA copy number (and thus mRNA) of the gene tested per one DNA copy (and accordingly mRNA) of the 16S rDNA gene as a reference gene.

In case of DNA analyses, positive results were obtained for all investigated samples and genes. The highest relative gene abundance was calculated for anammox bacteria (*hzo* gene), AOB (*amoA* gene) and denitrifiers (*nirS* and *nirK* genes). As it has been reported previously these three group of bacteria was detected as coexisting microorganisms in anammox biomass (Ziembińska-Buczyńska et al. 2019).

For some of the investigated samples, the Ct values were similar in the case of qPCR an RT-qPCR (Table 2). In the case of *nirS, nirK* and *nxrA* genes the RT-qPCR reaction results were negative. Such results may indicate that the analyzed gene does not yield expression, the expression level is too low (and is not detectable: ND), or/and mRNA is unstable. For samples 4, 6 and 7 positive results were obtained in the case of *nirS* gene and mRNA template. However, these quantities were marked with a significant error, because in the samples tested contamination in the form of genomic DNA is a 10–50% matrix for amplification, thus we considered this results as a qualitative (Figure 2, Table 2: +).

There were statistically significant differences between results obtained in the case of reactions based on bacterial cDNA and DNA ($p<0.05$). However, there is worth to notice that results obtained for *hzo* and *amoA* gene with DNA template are correlated with results based on cDNA (synthesized from mRNA) template (Table 3).

The positive results and high relative *hzo* gene abundance for anammox bacteria was obtained for all of the investigated samples with both qPCR and RT-qPCR method (Figure 2). The biomass with RGO addition presented higher *hzo* abundance in comparison to the control reactors operated both at 23°C and 18°C. The Wang et al. (2013) pointed that graphene oxide addition may efficiently stimulate the increase of EPS (extrapolimeric substances), which may favor cell agglomeration and indirectly affect microbial activity and process efficiency. However, in case of our studies the difference in the *hzo* gene abundance was not statistically significant ($p=0.23$ for DNA and $p=0.12$ for mRNA results). In Tomaszewski et al. (2019) addition of RGO to the anammox biomass caused similar results of nitrogen removal at 13°C, as at 30°C (temperature optimal for anammox bacteria).

Table 1. Primers used for qPCR analysis

Specificity	Primers	Sequence 5'–3'	Target gene	Encoding enzyme
Bacteria	1055F	ATGGCTGTCGTCAGCT	16S rRNA	universal bacterial marker gene used as reference gene
	1392R	ACGGGCGGTGTGTAC		
Ammonia oxidizers	amoA-1-F	GGGGTTTCTACTGGTGGT	*amo*	ammonia monooxygenase
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC		
Nitrite oxidizers	nxrA-RT-F	GTG GTC ATG CGC GTT GAG CA	*nrx*	nitrite oxidoreductase
	nxrA-RT-R	TCG GGA GCG CCA TCA TCC AT		
All known *Planctomycetes*	hzoCl1f1	TGYAAGACYTGYCAYTGG	*hzo*	hydrazine oxidoreductase
	hzoCl1r2	ACTCCAGATRTGCTGACC		
Denitrifiers	nirS 1f	TACCACCCSGARCCGCGCGT	*nirS*	nitrite reductase
	nirS 3r	GCCGCCGTCRTGVAGGAA		
	nirK876	ATYGGCGGVCAYGGCGA	*nirK*	
	nirK1040	GCCTCGATCAGRTTRTGGTT		

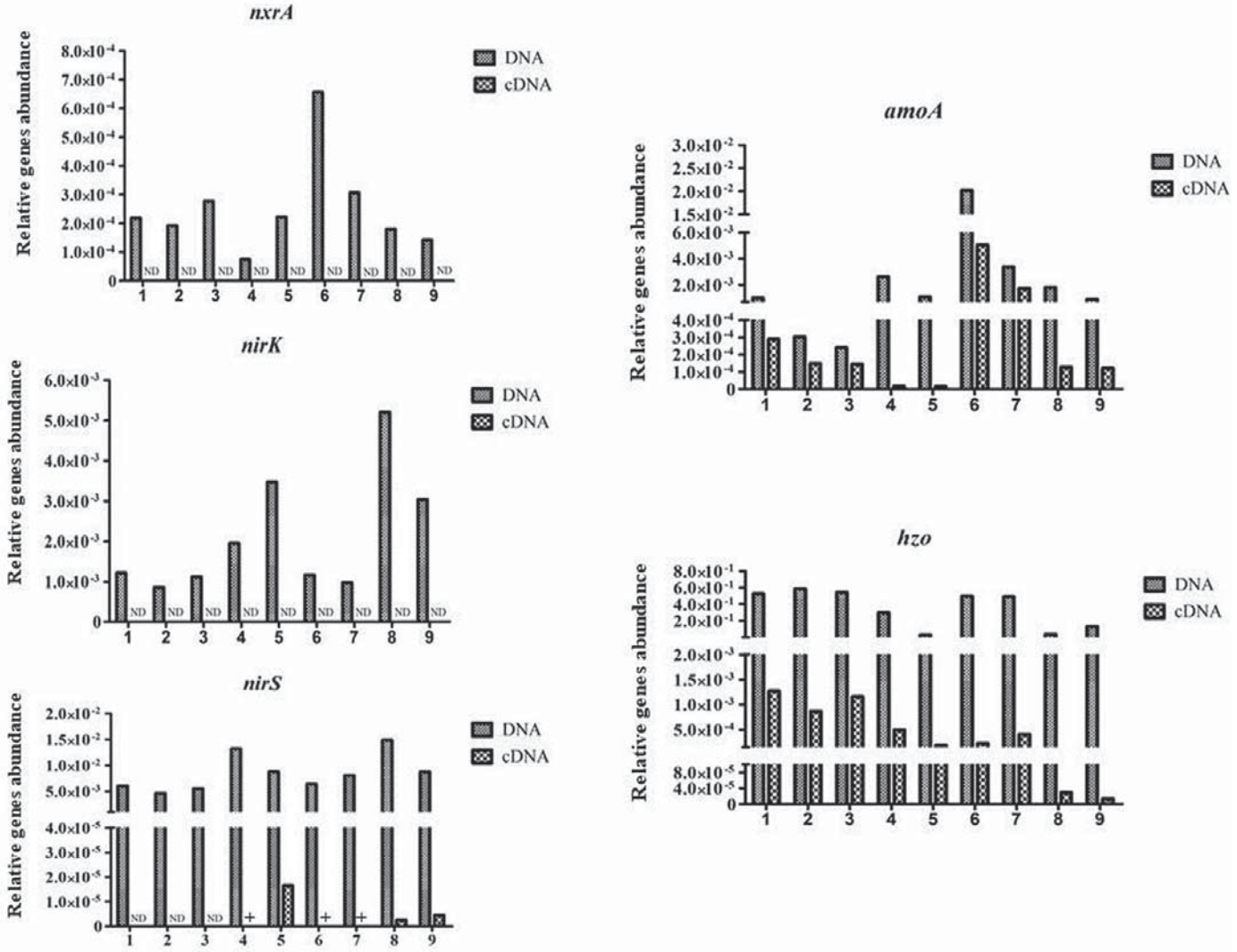

Fig. 2. Relative gene abundance calculations for *nxrA*, *amoA*, *nirK*, *nirS* and *hzo* genes based on cDNA and DNA templates. 1 – inoculation: activated sludge from WWTP, 2 – control, 23°C; 3 – biomass with RGO addition, 23°C; 4 – immobilized biomass, 23°C; 5 – immobilized biomass and RGO addition, 23°C; 6 – control, 18°C; 7 – biomass with RGO addition, 18°C; 8 – immobilized biomass, 18°C; 9 – immobilized biomass and RGO addition, 18°C; + qualitative results; ND – not detectable.

Table 2. C_t values obtained via qPCR based on DNA and cDNA for 16SrDNA, nirS, nirK, amoA, nxrA, hzo; + qualitative result; ND – not detectable

sample	DNA							cDNA					
	16SrDNA	nirS	nirK	amoA	nxrA	hzo		16SrDNA	nirS	nirK	amoA	nxrA	hzo
1	14.11	21.49	23.80	24.07	26.28	15.04		14.04	ND	ND	25.80	ND	23.66
2	14.33	22.09	24.53	26.03	26.68	15.11		15.02	ND	ND	27.75	ND	25.20
3	14.46	21.97	24.27	26.49	26.28	15.34		14.61	ND	ND	27.37	ND	24.36
4	15.52	21.76	24.52	24.11	29.24	17.26		14.45	+	ND	30.25	ND	25.42
5	13.10	19.94	21.27	22.97	25.24	18.27		13.11	28.99	ND	29.19	ND	25.51
6	14.61	21.90	24.37	20.24	25.18	15.62		11.47	+	ND	19.11	ND	23.59
7	14.95	21.91	24.96	23.17	26.63	15.98		11.60	+	ND	20.78	ND	22.86
8	14.36	20.43	21.94	23.48	26.81	19.10		8.70	27.25	ND	21.64	ND	23.76
9	15.05	21.89	23.41	25.17	27.84	17.97		10.36	28.12	ND	23.37	ND	26.50

Table 3. Spearman rank correlation coefficients; values considered as statistically significant were bolded and marked with*; α=0.05

		cDNA		*DNA*				
		hzo	***amoA***	***hzo***	***amoA***	***nxrA***	***nirS***	***nirK***
cDNA	***hzo***	1.000						
	amoA	-0.226	1.000					
DNA	***hzo***	**0.711***	0.308	1.000				
	amoA	-0.387	**0.968***	0.179	1.000			
	nxrA	-0.199	**0.946***	0.330	**0.897***	1.000		
	nirS	**-0.636***	-0.255	**-0.772***	-0.128	-0.430	1.000	
	nirK	**-0.642***	-0.318	**-0.935***	-0.197	-0.316	**0.784***	1.000

Due to the fact that investigated SBRs were inoculated with activated sludge conducting anammox process, where anammox bacteria were predominant, as it could be suspected, the relative abundance of the *hzo* gene is significantly higher than abundance calculated for others investigated functional genes. However, it should be underlined, that even when anammox process is dominant in the technological system, the nitrogen removal bacteria coexist with each other, and with anammox bacteria presence often other nitrogen cycle bacteria are a part of the activated sludge community.

Despite additional pre-reaction treatment and purification, positive results in mRNA-based analyses were obtained only for the *hzo*, *amoA* and partly for *nirS* genes. Besides anammox bacteria, AOB was probably the most active fraction in the nitrogen removal community. The possible activity of AOB bacteria in anammox biomass and the presence of the nitrification was confirmed in our previous studies (Ziembińska-Buczyńska et al. 2019; Banach-Wiśniewska et al. 2020).

In contrast to the results for AOB, the signal for NOB via RT-qPCR was below detection threshold. The low expression level of the *nrxA* gene in combination with low abundance of *nxrA* gene calculated on the basis of DNA may indicate that NOB were presented in investigated reactors, but were less abundant and/or less active part of the microbial community. It is worth to notice that in WWTP systems for nitrogen removal the theoretical ratio of AOB to NOB should be 2:1, according to thermodynamics and electron transfer calculations. Thus, AOB should be the dominant bacterial group over NOB in a microbial community where nitrification is present (Li et al. 2007, Winkler et al. 2012, Yao and Peng 2017). Gilbert et al. (2015) pointed that AOB outcompeting NOB leads to partial nitrification. Results of qPCR on the basis on DNA matrix obtained for *amoA* and *nxrA* genes revealed that both at 23°C and 18°C, higher relative genes abundancy was obtained for *amoA* gene in all samples. This situation might be caused by process conditions. AOB convert ammonium into nitrite in aerobic condition, however, the presence of AOB bacteria in anaerobic anammox reactors was previously shown in some studies. Meanwhile, NOB bacteria were less abundant (Schmid et al. 2000, Ziembińska-Buczńska et al. 2019). The values calculated for AOB and NOB were correlated to each other (Table 3), what may indicate that except the dominance of AOB over NOB, there is a relationship between the abundance of those two bacterial groups. The calculated correlation coefficients confirming the interaction and ecological association between the nitrogen transformation genes. It is also worth to mention, that the higher relative genes abundance for both, *amoA* and *nxrA* results based on DNA was obtained for control probes at 18°C (2.02×10^{-2} and 6.56×10^{-4}, respectively) than at 23°C (3.02×10^{-4} and 1.92×10^{-4}, respectively). It follows that nitrifying bacteria prefer temperatures similar to prevailing in the main sewage treatment plant (Whang 2019).

There are four microbial enzymes that mediate denitrification: nitrate reductase (*nar*), nitric oxide reductase (*nor*), nitrous oxide reductase (*nos*), and nitrite reductase (*Nir*). Because, not all denitrifiers contain full combination of genes involve in complete denitrification, and nitrite reductase genes (*nir*) are commonly used to indicate the presence of denitrifying bacteria (Lindeman et al. 2016; Wallenstein et al. 2006). In case of our study two functionally homologous *nir* genes: *nirS* and *nirK* were chosen for denitrifiers investigation. Our qPCR results obtained for these genes were correlated to each other (Table 3). However, the differences between gene abundance were significant and qPCR and RT- qPCR results were not convergent (Figure 2). This might suggest that denitrifiers have been presented in activated sludge, but the same as NOB, they were less active groups due to the process conditions: reactors were fed with medium free of organic compounds required for denitrifiers development. However, an increase in relative gene abundance in the samples taken from the reactors supported with immobilization may suggest that denitrifiers may start to use inactive part of biomass or immobilization reagents as an organic carbon source (i.e. sodium alginate).

The discrepancies between *nirK* and *nirS* results based on both, DNA and RNA may also indicate that denitrifiers in the investigated biomass used the *nirS*, not the *nirK* pathway for nitrate reduction. A similar conclusion was presented by Yoshida et al. (2012), wherein the activity of denitrifiers in rice paddy soil by DNA- and RNA-based was analyzed.

Differences between *nirS* and *nirK* abundance based on RNA might also result from the degradation time of mRNA. Härtig et al. (1999) reported the half-life of *nirS* transcripts to be 12.6 min, indicating that mRNA may be degraded during extraction (Yoshida et al. 2012). A similar problem could be the reason for the lack of results of the *nxrA* gene abundancy, however, no information was found about the half-life of *nxrA* gen.

Problems with the amplification of genetic material based on RNA from environmental samples was previously presented

by several authors (Härtig et al. 1999, Yoshida et al. 2012, Barnes et al. 2016). Low stability of mRNA and its degradation during isolation procedure are given as the main reasons for lack of positive results.

Conclusions

In the past decades, qPCR has been considered as a widely used method in the nitrogen removal bacteria abundance and activity studies. Depending on the template used in the qPCR (bacterial DNA or cDNA synthesized via reverse transcription form bacterial mRNA), the obtained results may provide different scientific information. In this study, despite calculated correlations, the differences between the obtained results of qPCR and RT-qPCR were significant. Two probable reasons for divergent molecular biology results may be suggested:

i) even if the genetic material of nitrogen removal bacteria groups was present in investigated samples, the investigated microorganisms were not active and the gene expression was low.
ii) In case of environmental samples, such as activated sludge, RNA analyses pose several difficulties in material isolation as well as obtaining positive reaction results.

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DNA i RNA-zależna analiza genów bakterii przemian azotowych metodą qPCR

Streszczenie: Z uwagi na to, że większości bakterii przemian związków azotowych nie można wyizolować w postaci czystych kultur, do ich zbadania konieczne jest zastosowanie metod biologii molekularnej. Jedną z najczęściej stosowanych w tym celu jest ilościowa reakcja łańcuchowa polimerazy (ang. Quantitative Polimerase Chain Reaction, qPCR). Celem eksperymentu było porównanie wyników analizy wybranych genów funkcyjnych bakterii przemian związków azotowych przy pomocy metody qPCR wykonanej na matrycy DNA i RNA (po odwrotnej transkrypcji). Względna liczebności genów funkcyjnych analizowana była z zastosowaniem metody qPCR (na matrycy DNA) oraz RT-qPCR (ang. Reverse Transcription-qPCR) (na matrycy RNA). Analizę przeprowadzono w oparciu o geny: amoA, nrxA, nirS, nirK i hzo. Próbki osadu czynnego pobrano z czterech sekwencyjnych reaktorów porcjowych, w których proces usuwania azotu i wzrostu bakterii wspomagano za pomocą immobilizacji biomasy i dodatkiem nanocząstek. Wykazano statystycznie istotne różnice między wynikami uzyskanymi w przypadku badań mRNA i badań opartych na DNA ($p<0,05$). Wyniki uzyskane za pomocą zastosowanych narzędzi biologii molekularnej (qPCR, RT-qPCR) były skorelowane pozytywnie. W przypadku analizy opartej na mRNA pozytywne wyniki uzyskano tylko dla genów hzo, amoA i częściowo dla genów nirS, pomimo dodatkowego oczyszczania i usuwania inhibitorów z próbek przed reakcją. Należy podkreślić, że w zależności od matrycy zastosowanej w qPCR (bakteryjne DNA lub cDNA zsyntetyzowane z bakteryjnego mRNA w procesie odwrotnej transkrypcji) uzyskane wyniki mogą wskazywać na różne informacje naukowe. Pomimo znaczących różnic pomiędzy wynikami otrzymanymi za pomocą dwóch metod, obliczone współczynniki korelacji Spearmana wskazują na wzajemne powiązanie pomiędzy otrzymanymi wynikami oraz powiązania ekologiczne pomiędzy bakteryjnymi genami przemian związków azotowych.