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Short Communication

Changes in Microbial Communities of Nitrifying Immobilized Biomass: the Role of Operational Conditions

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Abstract

Our research determined the impact of operational conditions on the quantity of total and ammonia-oxidizing bacteria (AOB) in immobilized biomass. The experiment was conducted for two kinds of wastewater differing in organic carbon concentration at HRTs of 1.5 h, 1.0 h, and 0.5 h. The evaluation of bacteria number was accomplished by using the real-time PCR targeting ammonia monooxygenase (*amoA*) and 16S rDNA genes. In the absence of organic carbon in the influent, the shortening of HRT from 1.5 h to 0.5 h positively affected the AOB number in immobilized biomass, which reached even 30%. But their activity was limited. In the presence of organic carbon in the influent, the shortening of HRT below 1.5 h resulted in a decline of the abundance of ammonia-oxidizing bacteria and nitrification efficiency.

Keywords: AOB population modeling, immobilized biomass, nitrification, real-time PCR

Introduction

Ammonia oxidation is the limiting step in nitrogen removal from municipal wastewater. Maintaining slow-growing nitrifiers in the activated sludge chamber of wastewater treatment plant requires a circulation of biomass, long hydraulic retention time (HRT), and sludge age. Using reactors with immobilized biomass is one of the methods that enable successful culturing of nitrifiers. High biomass concentration in such systems creates the possibility of operation at a high volumetric reaction rate and, in consequence, at short HRT. Physical retention of biomass and a small amount of bacterial suspension in the effluent entailed long sludge age [1]. As a result of complete biomass retention, technological systems with immobilized biomass have a lower food:microorganisms ratio (F:M) than conventional activated sludge [2]. Immobilization of biomass prevents nitrifiers from being washed out from the system, and secures complete ammonia oxidation at short HRT [3]. In reactors with immobilized bacterial communities, part of the biomass is attached to the support, the other part (e.g. < 9±11% in a fluidized bed) is suspended [4]. According to Kariminiaae-Hamedaani et al. [5], immobilized systems can cope with high flow rates. Intensive wastewater flow due to increased internal circulation capacity in reactors favors mainly the growth of bacterial species with greater attachment affinity, whereas the percentage of free biomass is minor. For this reason, immobilization is a valuable method of retaining massive populations of biomass inside bioreactors.

The efficiency of pollutant removal in biological systems depends on the microorganisms involved in the process. Understanding microbial activity and growth rates provides crucial information that can improve the system of wastewater treatment [6]. Ammonia oxidation is preserved in wastewater treatment plants mainly by autotrophic

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ammonia-oxidizing bacteria (AOB). Studies on their physiological activity and abundance are essential for design and operation of efficient nitrogen removal [2, 7]. Besides, determination of the correlation between operational parameters of the wastewater treatment process and changes in the population of AOB allows the wastewater process control in order to keep this critical group of microorganisms in the reactor and encourage their high abundance and species differentiation. Monitoring changes in the nitrifier community in relation to plant operational conditions can assist plant operators in preventing nitrification failure and the washing out of these essential bacteria from the system [8]. Operational parameters such as COD/N, solids retention time, organic loading, or reactor type influence microbial consortia in wastewater treatment facilities, changing species diversity and the abundance of AOB [9-11]. Therefore, it may be possible to affect the microbial communities involved in wastewater treatment by manipulating the operational parameters, thus creating environmental conditions favouring the intensive growth of particular microbial groups [12, 13].

There are numerous reports on the impact of operational parameters on the AOB community, but there is still a lack of data concerning these communities in immobilized biomass. In the present study we focused on determining the effect of HRT and wastewater composition on the number of total bacteria and ammonia-oxidizing bacteria (AOB) in biomass immobilized in a porous ceramic support. The evaluation of their abundance was accomplished by using the real-time PCR technique. Since classic microbiological techniques are unable to fully investigate the species richness of environmental samples, in this research molecular techniques were used to gain insight into changes in bacterial communities in the immobilized biomass in relation to applied operational parameters.

Experimental Procedures

Activated sludge was immobilized in a ceramic support of cylinder shape (TAMI Industries, Germany). The support (external diameter 10 mm, height 1178 mm) had three holes through the whole length and a number of macropores inside the ceramics. Hydraulic diameter of the support, as the ratio of 4-fold cross-sectional area and wetted perimeter of the cross-section equalled 3.6 mm. Pore diameters ranged from 4 μm to 6 μm and the material porosity was 35-40%. The support comprised the stationary filling of the reactor with internal circulation. The internal surface of the support was 0.04 m^2 and the total volume – 0.1 l. The total volume of the reactor was 0.7 l so the free space outside the support equaled 0.6 l. The bioreactor was continuously aerated and the dissolved oxygen concentration in the effluent was at the level of 2 mg O_2/l. The general reactor scheme is shown by Zielińska and Wojnowska-Baryła [14]. The capacity of an internal circulation was 60 l/h. Activated sludge collected from a municipal wastewater treatment plant with simultaneous nitrification and denitrification was immobilized by circulating for 24 h through a ceramic bed.

The experiment was conducted in two modes differing in wastewater composition. In mode A, carbonates and hydrocarbonates were the carbon sources, whereas urea and ammonium chloride were the sources of ammonia. The average pollutant concentrations were: 53.3±19.3 g COD/m^3, 60.4±5.0 g TKN/m^3, 23.7±1.8 g N-NH_4/m^3. In mode B, sodium acetate was introduced to the above-mentioned wastewater that was then characterized by 411.5±35.9 g COD/m^3. The TKN and N-NH_4 concentrations were the same as in mode A. Wastewater was prepared based on Coelho's et al. formula [15]. The experiment was carried out for three HRTs: 1.5 h, 1 h, and 0.5 h.

The experiment was carried out consecutively in the same reactor. Before the start, inoculum was immobilized in the support and used throughout the whole experiment. The adaptation period for each HRT and each mode was considered complete when the range of changes of particular parameters of the effluent (COD, TKN, N-NH_4) within 7 days did not exceed 5-10%. At each hydraulic retention time, after c.a. 40 days of biomass adaptation for the experimental conditions, the experiment was carried out for about 3 weeks. Wastewater samples were collected twice daily. The data presented in figures are the arithmetic means of the experimental results presented with standard deviations. The discussed correlations were expressed by Pearson's correlation coefficient. Biomass samples for molecular analyses were collected in two replicates at the end of each set of operational conditions tested.

The wastewater was assayed for the concentration of organic compounds (expressed as COD), total Kjeldahl nitrogen, ammonium nitrogen, nitrites and nitrates, according to APHA [16]. The efficiency of nitrification was calculated on the basis of ammonia consumption, including ammonia uptake for biomass synthesis. The oxidation rate of ammonium was estimated as the load of ammonium oxidized per volume of the support.

PCR reactions were carried on in a 7500 Real Time PCR System using MicroAmp optical tubes and caps (Applied Biosystems, Foster City, CA 94404, USA). Before the reactions, primer concentrations were optimized in accordance with the Applied Biosystems guidelines. Quantification of *amoA* gene was performed in a reaction mixture that contained 4 ng of template DNA (concentration 1 ng/μl), 12.5 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems), 50 nM of each primer (Table 1), 25 nM of KCl, and water to a final volume of 25 μl. The protocol for *amoA* quantification was as follows: 2 min in 50ºC, 10 min at 95ºC and 40 cycles consisting of 30 s at 95ºC, and 1 min at 60ºC. The reaction mixture for 16S rDNA amplification was as for *amoA* gene analysis, but the primer concentration was 100 nM and no KCl was added. The protocol for bacterial 16S rDNA quantification was as follows: 2 min at 50ºC, 10 min at 95ºC, and 40 cycles consisting of 15 s at 95ºC, 1 min at 50ºC, and 1 min at 60ºC. Each sample was amplified in triplicate in the presence of negative and positive controls. The fluorescence signal was normalized by dividing the SYBR emission by the passive reference dye (ROX). The threshold was defined as 10

Table 1. Applied primers and efficiency of real-time PCR reactions.

Sequence	Primers	Source	Efficiency of real-time PCR		
			Determination coefficient	Slope of curves for C_T vs log No. of gene copies	Range of linearity
16S rDNA	519f/907r	[17]	0.985	-3.59	$1.5 \cdot 10^5$-$6 \cdot 10^7$
amoA	amoA1F/amoA2R	[18]	0.986	-3.66	$3 \cdot 10^2$-$3 \cdot 10^7$

Table 2. Operational data.

HRT (h)	1.5		1.0		0.5	
Mode	A	B	A	B	A	B
VLR_N (kg TKN/(m^3·d))	1.0		1.4		2.9	
$N\text{-}NH_{4eff}$ (g/m^3)	5.2	3.2	1.0	3.1	26.8	28.7
$N\text{-}NO_{2eff}$ (g/m^3)	25.2	24.5	0.5	10.8	22.4	2.7
$N\text{-}NO_{3eff}$ (g/m^3)	19.9	1.7	47.2	27.9	4.5	0.3

times the standard deviation around the average intensity of background fluorescence from non-template controls. Data were analyzed with Sequence Detection Software, version 1.3 (Applied Biosystems). To ensure specificity of the reactions after the real-time amplification a denaturation step was introduced to confirm the melting temperature of the PCR product and products were electrophoresed in the presence of a molecular marker. For calculations of bacteria number it was assumed that the average number of 16S rDNA gene copies in bacterial genome is 7, and that the average number of *amoA* gene copies in AOB genome is 2.5 [19]. Results were expressed as the number of bacteria per reaction tube [20].

For more reliable results a standard curve for *amoA* quantification was generated with serial dilutions of a linearized plasmid with inserted *amoA* gene [20]. The standard curve for bacterial 16S rDNA was prepared with genomic DNA extracted from *Escherichia coli* JM109 (Promega) using Genomic Mini kit (A&A Biotechnology). The 16S rDNA copy number was calculated on the basis of genome size (4.64 Mbp) and seven copies of the 16S rDNA per *E. coli* genome. The characteristics of the obtained standard curves are given in Table 1.

Results and Discussion

In this research, we determined the dependence between number of total and ammonia-oxidizing bacteria in immobilized biomass and operational conditions: hydraulic retention time and type of substrate supplied to the reactor. During the experiment, the hydraulic retention time (HRT) was changed from 1.5 h, 1.0 h, to 0.5 h, which resulted in volumetric loading rate (VLR_N) 1.0, 1.4, and 2.9 kg TKN/(m^3·d). In mode A, the shortening of HRT from 1.5 h to 1 h induced a growth of ammonia oxidation rate from 0.8 kg $N\text{-}NH_4$/(m^3·d) to 1.4 kg $N\text{-}NH_4$/(m^3·d). Further HRT shortening to 0.5 h did not affect the ammonia oxidation rate. In mode B, the highest ammonia oxidation rate (1.0 kg $N\text{-}NH_4$/(m^3·d)) was obtained at HRT of 1 h. At the remaining HRTs, ammonia oxidation rate was 0.5-0.8 kg $N\text{-}NH_4$/(m^3·d). Comparing the effect of feeding conditions on oxidation rates, at the HRT of 1.5 h the supply of organic carbon to the reactor did not cause a decline in ammonia oxidation rates, as occurred at HRTs of 1 h and 0.5 h. Summarized operational data are given in Table 2. It was observed that at the shortest HRT of 0.5 h in both modes the concentrations of ammonium nitrogen in the effluent were significantly higher than in the other experimental conditions. This indicated that at VLR_N of 2.9 kg TKN/(m^3·d), independently of acetate presence in the influent, the limitation of nitrification occurred. Moreover, at discussed HRT in mode A, nitrites built up that were not noted in mode B. It can be concluded that the presence of acetate in mode B promoted nitrite reduction in denitrification. In general, as can be seen from the mass balance, the sum of nitrogen species in the influent was lower in mode B than in mode A, confirming the thesis about the positive effect of volatile fatty acids in the influent on the nitrogen removal in bioreactors.

In order to carry out the detailed analysis of the performance of the reactor with immobilized biomass, the number of bacteria was determined. It was observed that in mode A the number of total bacteria in immobilized biomass decreased with shortening of HRT (Fig. 1a) and changed from $3.18 \cdot 10^5$ cells/100 ng DNA at HRT 1.5 h to $1.4 \cdot 10^5$ cells/100 ng DNA at HRT 0.5 h. The supply of organic carbon (acetate) resulted in a rise of total bacteria quantity that is obvious under such feeding conditions. In mode B, a shortening of HRT from 1.5 h to 0.5 h induced

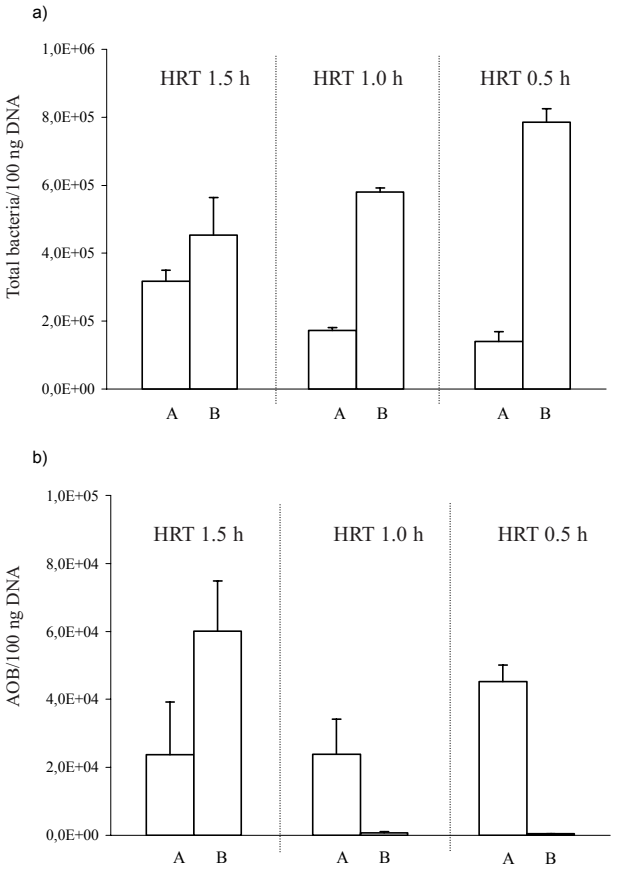

Fig. 1. The number of total bacteria (a) and AOB (b) at three different HRTs in modes A and B.

the increase in the number of total bacteria to $7.85 \cdot 10^5$ cells/100 ng DNA, which was caused by the increase in organic loading from 6.6 to 19.8 kg COD/(m^3·d).

Regarding the quantity of AOB versus operational conditions, the tendency depicted conversely. In mode A (inorganic carbon in the influent), the number of AOB at HRT of 1.5 h and 1 h was similar and reached $2.3 \cdot 10^4$ cells/100 ng DNA (Fig. 1b). Almost double the number of AOB was observed at HRT of 0.5 h. At the same loading of nitrogen but in the presence of organic carbon in the influent (mode B), a high number of AOB was noticed only at the longest HRT. An increase in the organic loading to 9.9 and 19.8 kg COD/(m^3·d) resulted in a significant decline of AOB quantity to the averaged value of $5 \cdot 10^2$ cells per reaction tube. Pholchan et al. [21] proved that changes in the operational conditions had a bigger effect on the AOB communities than on the total bacterial communities. Generally, the higher biodiversity, the better treatment performance. However, Pholchan et al. [21] stated that operating the reactors at high VLR enhanced diversity but not biodegradation performance.

In the absence of organic carbon in the influent, the shortening of HRT from 1.5 h to 0.5 h generated a decrease in the number of total bacteria and, simultaneously, an increase in the percentage of AOB in biomass. At the shortest applied HRT, the percentage of AOB in immobilized biomass reached over 30%. We explain it by the rising nitrogen loading from 1.0 to 2.9 kg TKN/(m^3·d) and lack of organics, potentially limiting the activity of slow growing ammonia oxidizers. Besides, according to Morgenroth et al. [22], it can be assumed that such changes in the proportion of total bacteria and ammonia-oxidizing bacteria were entailed by more rapid lysis of the heterotrophic bacteria cells, compared with autotrophic microorganisms, under the conditions of low organic carbon in the influent.

Zhang et al. [23] and Zhang et al. [2] suggested a relationship between the AOB population size and the quality of effluent, and a link between AOB presence and ammonia content in a membrane bioreactor treating municipal wastewater. In our research, at HRT of 1.5 h and VLR_N of 1.0 kg TKN/(m^3·d), the availability of organic carbon in the influent entailed the increase of the fraction of AOB (FN)

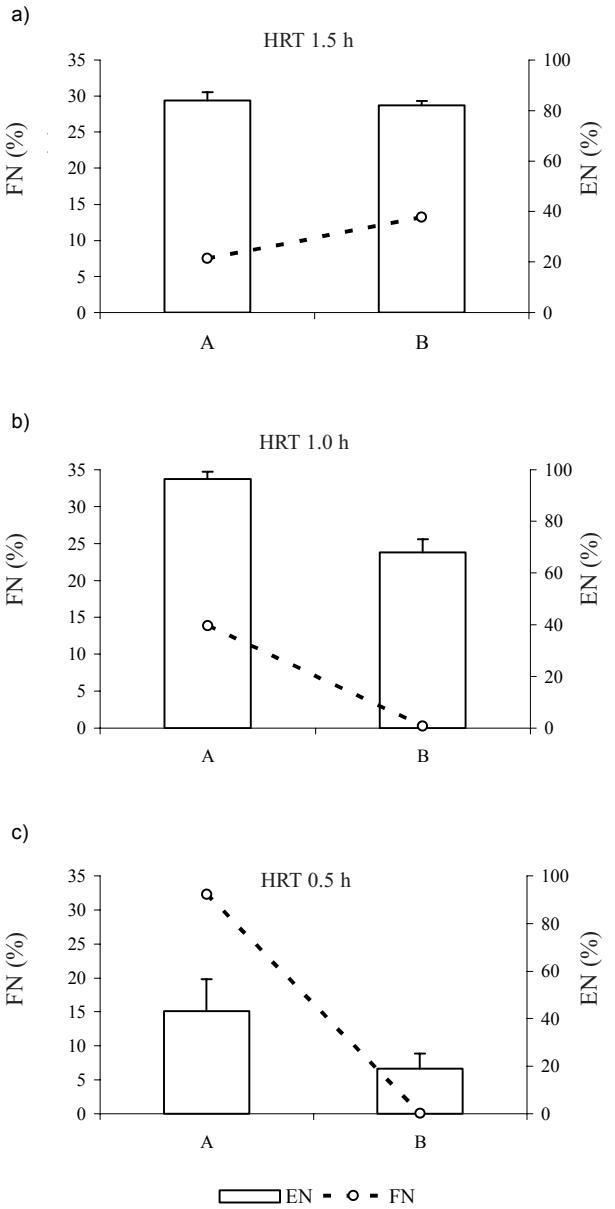

Fig. 2. Nitrification efficiency (EN) and percentage of AOB in activated sludge (FN) in modes A and B; a) HRT 1.5 h, b) HRT 1 h, c) HRT 0.5 h; nitrification efficiency (EN): the ratio of total Kjeldahl nitrogen removed minus nitrogen used for biomass synthesis to total Kjeldahl nitrogen in the influent.

in biomass from 7.5% to 13.2%, while the nitrification efficiency (EN) did not alter and remained at a level of about 83% (Fig. 2a). This can be explained by the fact that there was not any bacterial competition at the organic loading of 6.6 kg COD/(m^3·d) that could diminish ammonia oxidation. Nogueira et al. [9] claimed that a long HRT results in a decline in competition for oxygen between heterotrophic and autotrophic microorganisms, because heterotrophs grow mainly in suspension, whereas nitrifiers exist in biofilm and are retained in the bioreactor. Similarly, Pholchan et al. [21] stated that at the low organic loading, nitrification proceeded better and the diversity of AOB community is higher. Our research indicated that at the properly applied HRT, effective nitrification and maintaining a high abundance of AOB in biomass are possible in spite of the presence of organic carbon in the influent.

At HRTs of 1 h and 0.5 h, the supply of organic carbon into the influent inducing the growth of loading to 9.9 and 19.8 kg COD/(m^3·d) provoked the decrease in nitrification effectiveness; simultaneously the AOB participation in immobilized biomass declined. At HRT of 1 h the nitrification efficiency changed from 96.5% in mode A to 68% in mode B, while the AOB fraction changed from 13.8% to 0.3% (Fig. 2b). It can be explained by the fact that high organic loading induced the shift of the composition of immobilized biomass and heterotrophic nitrification could have been responsible for ammonia oxidation. Xia et al. [7] applied a compact suspended carrier biofilm reactor and revealed that wastewater composition, i.e. the ratio of organics to nitrogen, slightly alter the proportion between nitrifiers and denitrifiers in a biofilm. They proved that the relatively low concentration of organic carbon was beneficial to nitrifiers, whereas excessive organic carbon would inhibit their growth.

At the shortest HRT, with inorganic carbon in the influent (mode A), the abundant and active AOB population, accounting for 32%, was present. This indicates that the use of immobilized biomass with a proper choice of operational parameters would allow a high number of AOB to be maintained in the reactor. The supply of acetate induced the decrease of nitrification efficiency from 43.2% to 19%, which was accompanied by a drop in AOB participation to 0.06% (Fig. 2c). This indicated the strong competition between autotrophic and heterotrophic bacteria under the conditions of organic loading of 19.8 kg COD/(m^3·d). Introduction of organic compounds leads to the activation of heterotrophic bacteria that limit ammonia oxidation in favor of the oxidation of organic matter as stated by Geets et al. [24]. Authors noticed that the inhibition or elimination of nitrifiers as a result of the competition often leads to a decline of process capacity or even complete failure. Pholchan et al. [21] claimed that the high organic loading induced more diverse total bacterial community. For this reason the decrease in AOB diversity in the reactors operated at the high loading could be related to oxygen being present at limiting concentrations at the centre of the microbial aggregates (immobilized biomass), inducing competition for this resource, where the heterotrophs win over the AOB. Zhu and Chen [25] observed that the majority of ammonia-oxidizing bacteria form dense consortia with oxygen deficient interiors – this makes them prone for oxygen competition. This competition is mainly influenced by wastewater composition. Apart from the fact that at high organic loading there is higher oxygen consumption, there is also higher assimilation of cells [26], and in consequence higher uptake of ammonia for assimilation that decreases the amount of ammonia for oxidizers.

The research proved that the composition of microbial communities in immobilized biomass depends on HRT and the influent characteristics, especially the availability of organic carbon. Besides, this study revealed that altering these operational parameters makes it possible to shape the key microbial communities and influence the effectiveness of the treatment of wastewater in reactors with immobilized biomass.

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