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Nitrogen removal from wastewater and bacterial diversity in activated sludge at different COD/N ratios and dissolved oxygen concentrations

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Abstract

The impact of the organic carbon to nitrogen ratio (chemical oxygen demand (COD)/N) in wastewater and dissolved oxygen (DO) concentration on carbon and nitrogen removal efficiency, and total bacteria and ammonia-oxidizing bacteria (AOB) communities in activated sludge in constantly aerated sequencing batch reactors (SBRs) was determined. At DO of 0.5 and 1.5 mg O_2/L during the aeration phase, the efficiency of ammonia oxidation exceeded 90%, with nitrates as the main product. Nitrification and denitrification achieved under the same operating conditions suggested the simultaneous course of these processes. The most effective nitrogen elimination (above 50%) was obtained at the COD/N ratio of 6.8 and DO of 0.5 mg O_2/L. Total bacterial diversity was similar in all experimental series, however, for both COD/N ratios of 6.8 and 0.7, higher values were observed at DO of 0.5 mg O_2/L. The diversity and abundance of AOB were higher in the reactors with the COD/N ratio of 0.7 in comparison with the reactors with the COD/N of 6.8. For both COD/N ratios applied, the AOB population was not affected by oxygen concentration. Amplicons with sequences indicating membership of the genus *Nitrosospira* were the determinants of variable technological conditions.

Key words: nitrogen removal; activated sludge; COD/N ratio; oxygen concentration; ammonia-oxidizing bacteria; microbial diversity
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Introduction

Conventional nitrogen removal from wastewater by activated sludge is most often achieved by sequential aerobic and anoxic processes. The main factors affecting these processes include the ratio of carbon to nitrogen (chemical oxygen demand (COD)/N) in the influent and the concentration of dissolved oxygen (DO). The COD/N ratio influences the population of microorganisms since an increase in this parameter results in a decrease in nitrification efficiency and an improvement of denitrification efficiency (Gieseke et al., 2001). The control of DO concentration is also crucial as it determines the dominant processes in the reactor. A high DO inhibits denitrification, whereas a low DO causes a limitation of ammonia oxidation (Garrido et al., 1997). DO is easily controlled by manipulating the air supply and it strongly influences the cost of wastewater treatment resulting from aeration. A low COD/N ratio and a low DO promote partial nitrification, and this diminishes the oxygen demand by 25% and the COD requirement for denitrification by 40% (Ganigué et al., 2007; Pollice et al., 2002). According to Ma et al. (2009), a DO concentration of 0.4–0.7 mg/L favors nitrite accumulation in continuous-flow systems treating domestic wastewater.

Despite the fact that nitrification and denitrification require different conditions when it comes to DO and COD/N ratio, these reactions can occur concurrently in one reactor under the same operating conditions, for example in different zones of a single reactor (Guo et al., 2005) and they are called simultaneous nitrification and denitrification (SND). The course of these two processes in one environment is advantageous since a decrease of alkalinity caused by nitrifiers is partially neutralized by an increase in alkalinity due to denitrification, and there is no risk of nitrification failure for this reason (Pochana and Keller, 1999). The most important factors affecting SND are organic carbon, DO concentration and floc size (Third et al., 2005). Simultaneous growth of nitrifiers (autotrophs) and heterotrophs in a single reactor with a high COD/N ratio causes low nitrification efficiency due to competition between these two bacterial groups (Campos et al., 2007). According to Münch et al. (1996), the optimal DO concentration for effective nitrogen removal via SND equals 0.5 mg O_2/L.

For many years it has been known that the diversity of specific bacterial groups in activated sludge influences the functioning of the reactor and is crucial in maintaining the stability of the wastewater treatment system (Daims et al., 2001). Since ammonia oxidation to nitrites is the limiting step in nitrogen removal from wastewater, ammonia-oxidizing bacteria (AOB) are of universal

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importance in activated sludge technologies. The growth rate of AOB is slow and the activities of their population are strongly affected by operational conditions. Park and Noguera (2004) showed that at a low DO (0.12–0.24 mg/L) the AOB community in activated sludge was dominated by members of the *Nitrosomonas europaea* lineage, whereas at high DO (up to 8.5 mg/L) members of the *Nitrosomonas oligotropha* lineage were prevalent. However, with the passage of time the AOB community in the high-DO reactor shifted from the *Nitrosomonas oligotropha* lineage to the *Nitrosomonas europaea* lineage without loss of nitrification efficiency. These considerations suggest that AOB lineages include species showing different affinities for oxygen. According to Wittebolle et al. (2009), not only the presence of certain specific species but also community biodiversity and its dynamic are important indicators of good microbial functionality.

In conventional wastewater treatment, to remove organic and nitrogen compounds, alternating anoxic-aerobic conditions are commonly used, e.g. in flow reactors, with above 2 mg O_2/L in the aerobic tank. Nitrogen removal (nitrification, denitrification and biomass synthesis) in a single reactor under the same operating conditions allows for savings in aeration cost for nitrification and limits organic requirements for denitrification. In our research, the sequencing batch reactors (SBRs) were constantly aerated and their DO concentration was diminished. We investigated the impact of the COD/N ratio and DO concentration on the efficiency of carbon and nitrogen removal from wastewater. Using fluorescence *in situ* hybridization (FISH), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing we determined the diversity and abundance of ammonia-oxidizing communities in activated sludge to correlate them with nitrification efficiency. Such dependence can be helpful in the choice of operational conditions that favor maintaining nitrifiers in the reactors and, simultaneously, allow for denitrification.

1 Experimental

1.1 Process configuration

The experiment was carried out in two parallel SBRs (SBR A and SBR B), with a working volume of 5 L each. Activated sludge originated from a municipal wastewater treatment plant with simultaneous nitrification and denitrification. The SBRs were directly seeded without any enrichment processes of the sludge. The reactors were equipped with stirrers (rotation speed 50 r/min) and controlled air supply system. Air was supplied by porous diffusers placed at the bottom of the reactors. Gas flow rate was controlled by a thermal mass flow controller. The amount of air entering the sequencing batch reactors was automatically adjusted to a stable set-point.

Both SBRs were operated in a 24-hr cycle mode. Each cycle consisted of 15 min of feeding, 23 hr of aeration, followed by 30 min of settling and 15 min of decanting. The volumetric exchange rate (n) in both reactors equalled 0.5 day^{-1}. The pH in the system was maintained at a level between 6.5 and 7.5. The reactors were operated at a temperature of 20°C that was maintained by a water jacket.

Four experimental series were performed, differing in dissolved DO in the reactor and COD/N ratio in the wastewater. The COD/N represents the ratio of the concentration of organic compounds, expressed as COD, to the concentration of total Kjeldahl nitrogen (TKN). In SBR A, the COD/N ratio equalled 0.7 and DO was 0.5 mg O_2/L (series 1) and 1.5 mg O_2/L (series 2). In SBR B, the COD/N ratio equalled 6.8 and DO was 0.5 mg O_2/L (series 3) and 1.5 mg O_2/L (series 4).

The sludge retention time (SRT) was maintained at about 30 days by controlling the amount of excess sludge. The activated sludge concentration was 2.3 g total suspended solid (TSS)/L in SBR A and 2.9 g TSS/L in SBR B. The sludge stabilization ratio (volatile suspended solids (VSS)/TSS) in SBR A and SBR B was 0.53 and 0.59, respectively.

1.2 Wastewater feed

Synthetic wastewater used in this study was prepared according to Coelho et al. (2000), with some modifications. Wastewater contained ammonium chloride and urea as the nitrogen sources. Regarding carbon compounds, in series 1 and 2 only inorganic compounds (carbonates and bicarbonates) were introduced, while in series 3 and 4 both inorganic (carbonates and bicarbonates) and organic compounds (acetate) were present in the wastewater. In every series the average concentrations of TKN and ammonia nitrogen in the influent were 63.1 ± 4.6 mg/L and 26.6 ± 5.6 mg/L, respectively. The COD in the influent averaged 45.9 ± 12.8 mg/L (series 1 and 2) and 434.8 ± 63.4 mg/L (series 3 and 4).

1.3 Analytical methods

In every series the adaptation period lasted about 30 days and was considered complete when the range of changes of particular parameters in the effluent (COD, TKN, NH_4^+-N, NO_2^--N, NO_3^--N) within 7 days did not exceed 5%–10%. For established effluent parameters, the research was carried out to determine the COD and ammonia removal rates. In the working cycle of the reactor, sampling and measurements of COD and nitrogen compounds were carried out and biomass samples for molecular analyses were collected.

Daily measurements of the effluent of the reactors included: COD, TKN, NH_4^+-N, NO_2^--N, NO_3^--N. The activated sludge was analyzed for TSS and VSS. The analyses were performed according to APHA et al. (1992).

The method of FISH, as detailed by Amann et al. (1990b), was used to estimate the abundance of AOB in the activated sludge. FISH probes applied included EUB338 (Amann et al., 1990a; Chae et al., 2008) specific for all bacteria, and Nso190 (Mobarry et al., 1996), specific for the majority of AOB. The samples were visualized and images collected using a Nikon Eclipse epifluorescence microscope. Bacteria quantification was performed according to Crocetti et al. (2002). For each image collected,

the abundance of the ammonia-oxidizing bacteria was determined as the ratio between the area bound to the specific probe and that bound to the EUB338 probe (Rittmann et al., 1999). The areas were calculated using ImageJ software (National Institutes of Health, USA). To obtain reproducible and statistically correct results, 30 images were analyzed for each quantification (Hall et al., 2003) and the average percentage value was used as the abundance.

Genomic DNA was isolated in duplicate from 400 mg of centrifuged sludge sample using FastDNA® SPIN® Kit (Q-BIOgene, USA), mixed and frozen at –20°C. The quality and quantity of isolated DNA was measured spectrophotometrically using a BioPhotometer (6131, Eppendorf, Germany).

Primer sequences and annealing temperatures for each primer set are shown in Table 1. PCR reactions were performed in an Eppendorf® Mastercycler Gradient (5332, Eppendorf, Germany). The PCR mixture contained 1.7 ng/μL of extracted DNA, 0.5 μmol/L of each primer, 100 μmol/L of deoxynucleoside triphosphate mixture (Promega, USA), 1.5 U of GoTaq® DNA Polymerase (Promega, USA), 6 μL of 10× reaction buffer supplied with polymerase, 1.5 mmol/L $MgCl_2$ and sterile water to a final volume of 30 μL. The PCR amplifications were carried out in duplicate using the following program: 95°C for 5 min, 35 cycles consisting of denaturation at 94°C for 30 sec, annealing for 45 sec, extension at 72°C for 1 min, and a final elongation at 72°C for 5 min. The size of PCR products was estimated using 0.8% agarose gel in the presence of 100 bp O'GeneRuler ladder (Fermentas, Canada).

DGGE electrophoresis was performed and the gels were processed as described by Cydzik-Kwiatkowska et al. (2011). Representatives of *amoA* gene amplicons that were clear and had a high intensity were excised from the DGGE gel, reamplified using specific primers, however, with no GC clamp, and sequenced (www.oligo.ibb.waw.pl). The nucleotide sequences were compared with sequences in the GenBank using the BLASTn program (Altschul et al., 1997) and deposited in the GenBank under accession no. JN712256-JN712263. The sequences determined in this study were aligned and genetic relationships were determined (the Maximum Likelihood method) using MEGA5 software (Tamura et al., 2011).

1.4 Calculation methods

COD removal rate was described by pseudo first-order kinetics and defined by Eq. (1):

$$r = \frac{dC}{dt} = k \times (C_0 - C_e) \quad (1)$$

The solution for this could be fitted to the experimental data according to Eq. (2):

$$C_t = (C_0 - C_e) \times \exp(-k \times t) + C_e \quad (2)$$

Ammonia removal rate was described by zero-order kinetics and defined by Eq. (3):

$$r = -\frac{dC}{dt} = -k \quad (3)$$

the solution for this could be fitted to the experimental data according to Eq. (4):

$$C = C_0 - k \times t \quad (4)$$

where, r (mg/(L·hr)) is the rate of COD or ammonium removal, k (mg/(L·hr)) or (hr^{-1}) is the reaction rate constant, C_0 (mg/L), C_t (mg/L), C_e (mg/L) are the COD or ammonium concentrations at the beginning, after time t (hr), and at the end of the aeration phase, respectively.

Reaction rate constants were determined on the basis of the experimental data by non-linear regression with the use of Statistica 7 (StatSoft, USA).

The structural diversity of the microbial community was examined by the Shannon-Wiener index of general diversity - H' (Wiener, 1948; Shannon and Weaver, 1949). For each sample, H' was calculated as an average of the two measurements of the band intensities on the gel tracks. The intensity of the bands was reflected as peak heights in the densitometric curve. The Shannon-Wiener index (H') was calculated according to Eq. (5):

$$H' = -\sum (n_i/N)\ln(n_i/N) \quad (5)$$

where, n_i is the height of the peak, and N is the sum of all peak heights of the densitometric curve.

2 Results and discussion

2.1 Efficiency of COD removal

Changes in the COD concentration in the SBR cycle, at the volumetric exchange rate (n) of 0.5 day^{-1}, were described by first-order kinetics (Fig. 1). Independently of the feeding regimes (COD/N ratio) and DO concentration in activated sludge, after 3–4 hr of aeration the COD concentration reached the level that maintained constant to the end of the SBR cycle. Generally, at the COD/N ratio of 0.7 (series 1 and 2), the specific COD removal rates (r_{COD}) were more than 5-fold lower than at the COD/N of 6.8 (series 3 and 4) (Table 2). The COD removal efficiency (E_{COD}) did not exceed 50% in series 1 and 2, whereas in

Table 1 PCR primers used in the study

Primer	Sequence (5′–3′)	Annealing temperature (°C)	Target sequence	Reference
amoA-2R	ccc ctc tgc aaa gcc ttc ttc	60	*amoA*	Rotthauwe et al., 1997; Nicolaisen and Ramsing, 2002
amoA-1F	[a]ttt cta ctg gtg gt			
357F	[b]cct acg gga ggc agc ag	63	V3 16S rDNA	Muyzer et al., 1993
517R	[a]tt acc gcg gct gct gg			

[a] - cgc cgc gcg gcg ggc ggg gcg ggg gcg ggg; [b] - cgc ccg ccg cgc gcg gcg ggc ggg gcg ggg gca cgg ggg g.

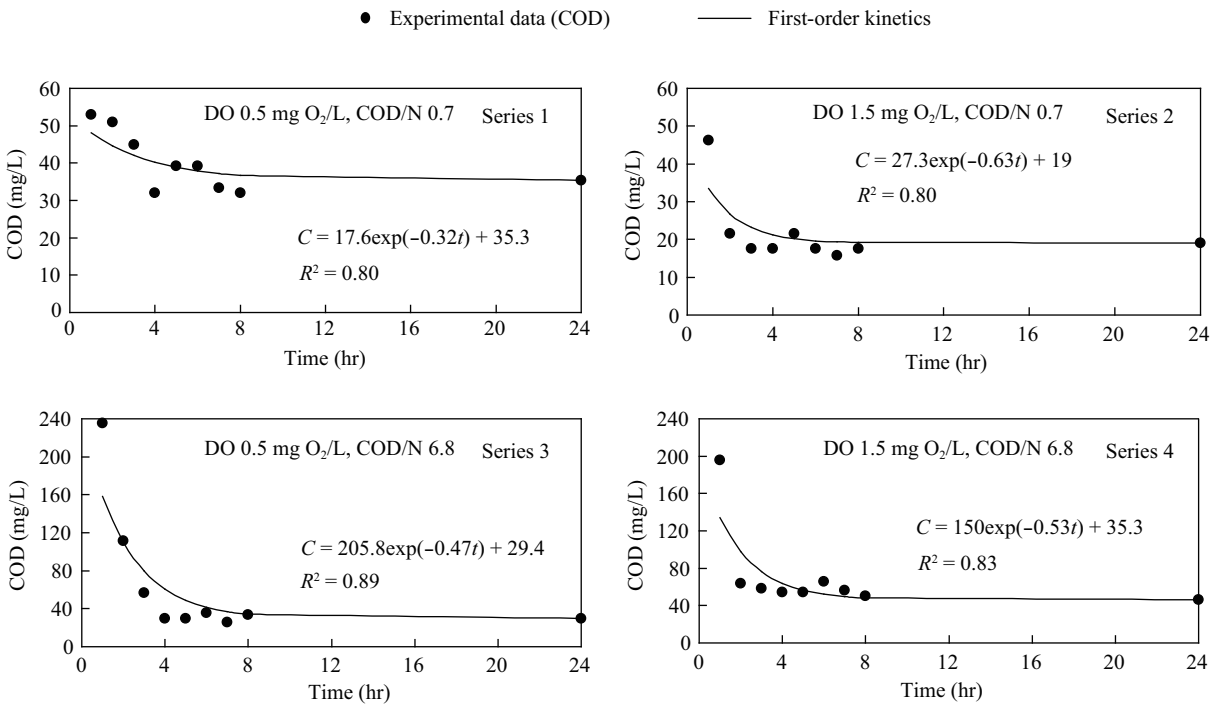

Fig. 1 Time profiles of COD concentration in SBR cycle of series 1–4.

series 3 and 4, E_{COD} was c.a. 93%. At the COD/N ratio of 0.7, despite lower organic carbon loading in the influent, the COD concentration in the effluent was higher by about 27%–55% than at the COD/N ratio of 6.8. An increase of the COD concentration in the effluent under conditions of very low COD/N ratio could have been the result of cell lysis (Texier and Gomez, 2004).

2.2 Efficiency of nitrogen removal

In each series, the ammonia concentration in the effluent did not exceed 0.5 mg/L (Fig. 2) and nitrates were the main oxidized nitrogen form. The nitrate concentrations were higher in series 1 and 2 (56.2 and 66.1 mg NO_3^--N/L, respectively), compared to series 3 and 4 (33.2 and 40.0 mg NO_3^--N/L, respectively). During the first few hours of aeration, nitrite accumulation to 8.1 mg NO_2-N/L at the COD/N ratio 0.7 and to 3.6 mg NO_2^--N/L at the COD/N ratio 6.8 was observed. However, the effluent concentration did not exceed 0.03 mg NO_2^--N/L. TKN and ammonia removal from wastewater in the SBR cycle followed zero-order kinetics. In our research, independent of the COD/N ratio in the wastewater, at a DO concentration of 0.5 mg O_2/L (series 1 and 3) the activated sludge needed 8 hours to achieve complete ammonia oxidation. At DO concentration of 1.5 mg O_2/L ammonia was fully removed from wastewater after 5–6 hr. In this study, the increase in the COD/N ratio from 0.7 to 6.8 influenced neither the rate of ammonia oxidation ($r_{NH_4^+\text{-}N}$) (Table 2) nor the efficiency of ammonia oxidation, whereas Komorowska-Kaufman et al. (2006) proved that the nitrification process in activated sludge was stable with efficiencies above 95% for COD/N ratios lower than 4. In our study, both at the COD/N ratio of 0.7 and 6.8, increasing the DO concentration from 0.5 to 1.5 mg O_2/L caused a growth of the specific $r_{NH_4^+\text{-}N}$ by 80% and 34%, respectively. Similarly, Orhon and Artan (1994) proved that the activity of nitrifying bacteria substantially decreases at lower DO levels. Campos et al. (2007) claimed that in a nitrifying activated sludge, ammonia was completely oxidised to nitrates at DO levels higher than 1 mg O_2/L, whereas at DO concentrations of 0.4 and 0.6 mg O_2/L ammonia and nitrite accumulation was observed. In our experiment, independent of operating conditions, the ammonia oxidation efficiency exceeded 90% with nitrates as the main product. We conclude therefore that the oxygen concentration of 0.5 mg O_2/L did not inhibit the efficient ammonia oxidation. The oxidation of ammonia may be inhibited by a high organic compound or ammonia concentrations in wastewater at both low COD/N ratio and DO. Under these conditions, nitrite accumulation can be observed (van Dongen et al., 2001). The studies revealed that partial nitrification is advantageous for nitrogen removal, however the final product, i.e. nitrites, is toxic to the environment and denitrification of nitrites may result in the production of greenhouse gases. In our study, degradation of organic compounds was finished after 4 hr of the reaction time. Simultaneously, ammonia oxidation occurred. At diminished COD concentration, ammonia oxidation was not limited by organics. Moreover, a long SBR working cycle and a relatively low ammonia concentration, typical for municipal wastewater, favored ammonia oxidation, even at the DO of 0.5 mg O_2/L.

Table 2 Organic compound removal (r_{COD}) and ammonia removal ($r_{NH_4^+\text{-}N}$) rates in series 1–4

	Series 1	Series 2	Series 3	Series 4
DO (mg O_2/L)	0.5	1.5	0.5	1.5
COD/N	0.7	0.7	6.8	6.8
r_{COD} (mg/(g VSS·hr))	5.2	13.0	63.0	41.5
$r_{NH_4^+\text{-}N}$ (mg/(g VSS·hr))	1.6	2.8	1.5	1.6
$r_{NH_4^+\text{-}N}$ (mg/(g AOB·hr))	15.4	27.8	17.4	23.4

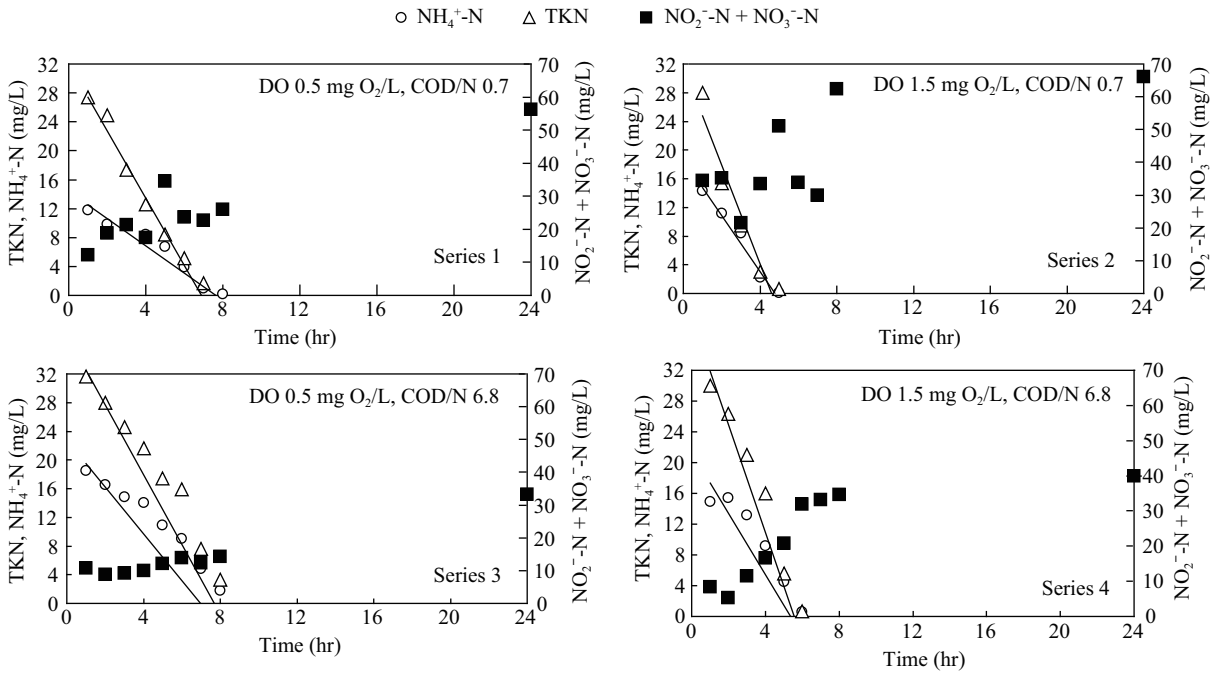

Fig. 2 Time profiles of total Kjeldahl nitrogen (TKN), ammonia, nitrite and nitrate concentrations in SBR cycle for series 1–4.

However, for both COD/N ratios applied in the experiment, an increase of DO concentration from 0.5 to 1.5 mg O_2/L resulted in an increase in the ammonia oxidation rate.

In the present experiment, during the SBR operation, the reaction was carried out under constant aeration, however, the nitrogen balance indicated that the denitrification process was noted in parallel with ammonia oxidation. Concurrent aerobic ammonium oxidation and anaerobic denitrification under identical operating conditions is termed SND and proceeds at low DO concentrations (Pochana and Keller, 1999). In our study, at the COD/N ratio of 0.7, denitrification was the only process responsible for nitrogen removal. The activated sludge production was almost zero, so the amount of nitrogen used for biomass synthesis could be taken as negligible. DO did not affect nitrogen removal efficiency, which averaged 17.5%. Chiu et al. (2007) demonstrated that under carbon deficit conditions, the nitrification and denitrification rates are not in equilibrium, resulting in a low SND effectiveness.

At the COD/N ratio of 6.8, DO concentration influenced nitrogen removal efficiency, which was 50.3% and 31.1% at 0.5 and 1.5 mg O_2/L, respectively. Under these conditions, nitrogen depletion resulted also from biomass synthesis that accounted for 40% and 54% of the total amount of nitrogen eliminated. Higher organic carbon concentration in the discussed series accelerated the activity of heterotrophic bacteria that utilize oxygen for COD oxidation. For this reason, high initial COD concentration may result in oxygen depletion zones in activated sludge that favor nitrogen removal. Zhu et al. (2007) also observed a linear relationship between SND and DO concentration, but SND was obtained even under high DO concentration (to 4.5 mg/L). The authors claimed that SND is favored by anoxic conditions that might be present in inner parts of biofilms or activated sludge flocs. Ma et al. (2009), in a continuous process for domestic wastewater treatment at DO in the aerobic zone of 0.4–0.7 mg O_2/L, obtained a contribution of SND to nitrogen removal of about 5%–8%, independently of the nitrate or nitrite pathway. Meng et al. (2008) applied an airlift internal circulation membrane bioreactor with synthetic wastewater of a composition similar to real domestic wastewater. The authors obtained complete SND and optimal removal of nitrogen and organic carbon at a COD/N ratio of 10.04, whereas a DO concentration of 0.75–1.0 mg O_2/L was the optimum range for this process. Our research showed that at the COD/N ratio of 6.8, and DO of 0.5 mg O_2/L, the efficiency of denitrification was > 2-fold higher than at DO of 1.5 mg O_2/L and that nitrification was not inhibited even at DO concentration as low as 0.5 mg O_2/L, which made SND possible.

2.3 AOB population in activated sludge

Analyses of microbial populations in activated sludge were performed using PCR-DGGE, FISH and sequencing. The PCR-DGGE pattern obtained for biomass samples collected during the experiment showed that the composition of the AOB communities in activated sludge depended on the COD/N ratio in wastewater and was not influenced by the different oxygen concentrations (Fig. 3a). Amplicons D, F, and G were common in activated sludge samples from all experimental series. Amplicon A occurred only in DGGE patterns characterizing the reactors working at the COD/N ratio of 6.8, while amplicons B, C, E, H and I were present only in activated sludge samples from the reactors operated at the COD/N ratio of 0.7. All the mentioned bands, except from band E, were successfully sequenced. On the basis of the sequences the phylogenetic tree was constructed (Fig. 4). Most amplicons which were common to all samples formed a distinct branch with other uncultured AOB. The amplicons characteristic for given substrate conditions, mainly bands C, H, I for the

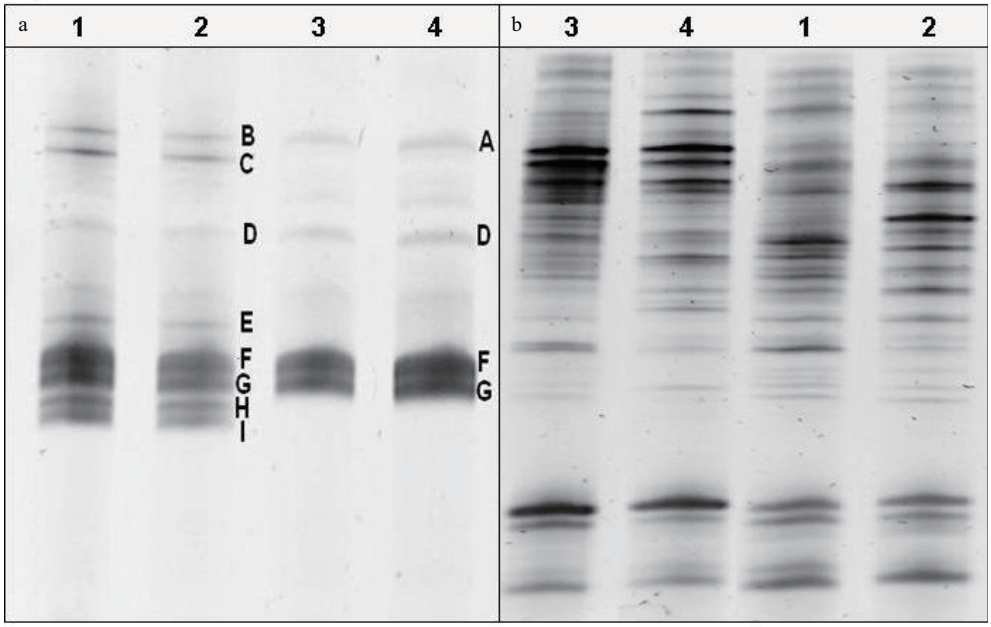

Fig. 3 PCR-DGGE analysis of *amoA* gene (a), and 16S rDNA (b) in activated sludge samples. Lane labels along the top represent experimental series. Sequenced bands are named with capital letters.

COD/N ratio of 0.7, and band A for the COD/N ratio of 6.8 showed an affinity with *Nitrosospira* sp. NpAV. This appears to indicate that bacteria from the *Nitrosospira* sp. NpAV lineage are sensitive to operational conditions and differentiate the AOB populations, thus providing a marker for the presence of organic carbon.

Values of Shannon-Wiener index calculated on the basis of 16S rDNA patterns (Fig. 3b) varied in the range from 2.90 ± 0.20 to 3.20 ± 0.10 (Fig. 5). For both COD/N ratios applied, the total bacterial diversity was slightly higher at

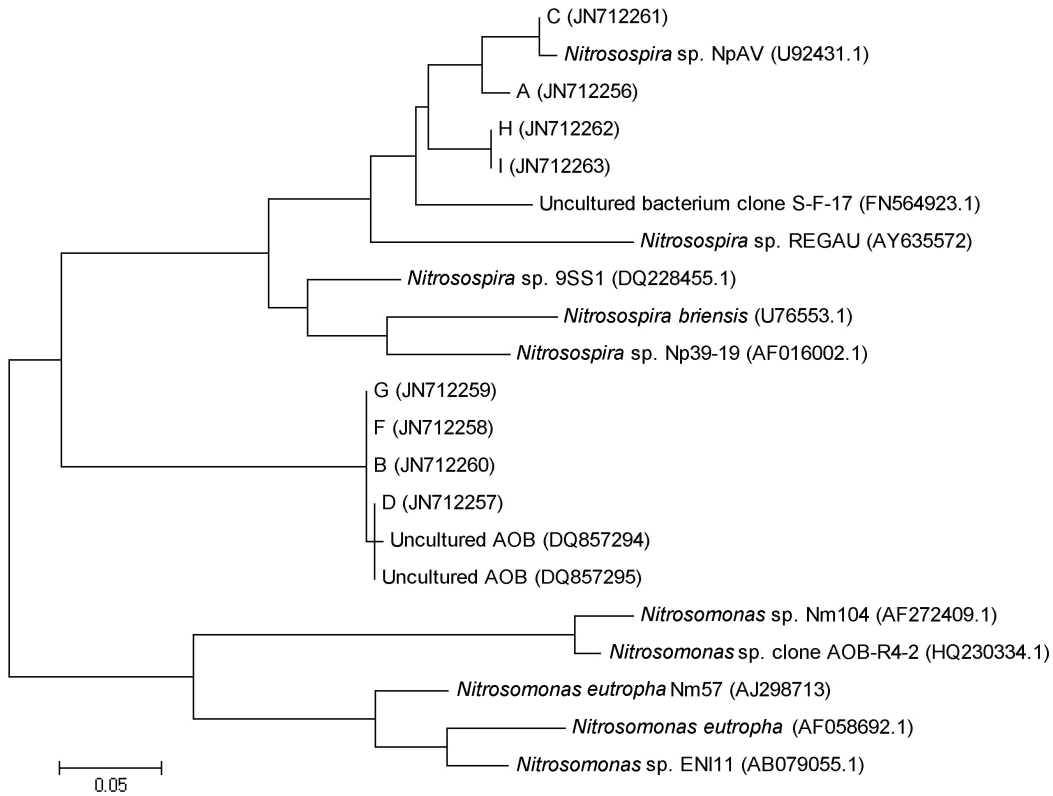

Fig. 4 Phylogenetic tree showing the relationships of partial *amoA* gene sequences to reference sequences from GeneBank database (accession numbers given in parentheses). The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically as follows: when the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise the BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

DO of 0.5 mg O_2/L. At lower oxygen concentrations its diffusion into the floc is limited and anoxic and anaerobic zones appear (Pochana et al., 1999). These zones behave as microenvironments that can be inhabited by microorganisms adapted to limited oxygen conditions. In general, higher bacterial diversity was observed at the COD/N ratio of 0.7. This dependence was less obvious in the total bacterial diversity estimations, and clearly visible in the AOB analysis. The values of H' for the AOB community in series 1 and 2 were similar 2.03 ± 0.12 and 2.07 ± 0.11 at oxygen concentrations in the reactors of 0.5 and 1.5 mg O_2/L, respectively. At the COD/N ratio of 6.8, H' was 1.25 ± 0.14 at DO of 0.5 mg O_2/L, and 1.36 ± 0.06 at the DO of 1.5 mg O_2/L. The H' values obtained for AOB showed that changing oxygen concentrations from 0.5 to 1.5 mg/L did not select for the different species composition in the reactors, however, increasing the COD/N ratio from 0.7 to 6.8 decreased AOB diversity probably because some species lost in the competition for oxygen and space with heterotrophs (Zhu and Chen, 2001).

The highest abundance of AOB was observed at the COD/N ratio of 0.7, independently of the DO concentration (Fig. 5). The AOB percentage equalled 10.3% and 10.2% in series 1 and 2, respectively. It has been shown that AOB can survive under conditions of low DO since they have a higher affinity towards oxygen than nitrite-oxidizing bacteria (Ma et al., 2009). Similar values of AOB proportions were obtained by You et al. (2003), treating wastewater of similar characteristics as in our work. They observed 10.3% of AOB in biofilm of a combined activated sludge, rotating biological contactor (RBC) process, 13.7% in activated sludge of a combined activated sludge, RBC process, and 5.2% in an anaerobic-anoxic-oxic (A2O) system. In our study, after increasing the COD/N ratio to 6.8, the AOB percentage in activated sludge remained relatively high, independently of oxygen concentration, and averaged 7.8% for series 3 and 4. Different results were obtained by Ballinger et al. (2002)

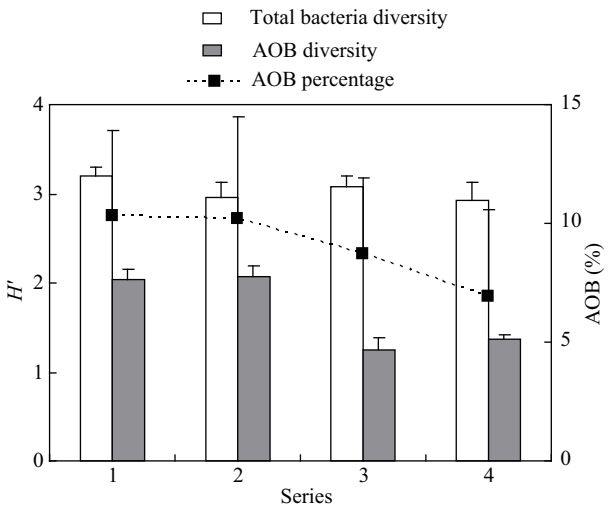

Fig. 5 Values of Shannon-Wiener index (H') calculated on the basis of DGGE patterns for *amoA* gene and 16S rDNA, and ammonia-oxidizing bacteria (AOB) percentage in activated sludge in series 1 (DO 0.5 mg O_2/L, COD/N 0.7), 2 (DO 1.5 mg O_2/L, COD/N 0.7), 3 (DO 0.5 mg O_2/L, COD/N 6.8), and 4 (DO 1.5 mg O_2/L, COD/N 6.8).

who performed a single sludge reactor fed with synthetic wastewater with C/N ratios of 2 and 5. At the C/N ratio of 2, ß-proteobacterial AOB were present in activated sludge, whereas they were not detected both at the C/N ratio of 5 and after the returning the C/N ratio to 2.

Park and Noguera (2004) demonstrated that a direct correlation between AOB phylogeny and DO concentration cannot be established at the lineage level. It is rather likely that different AOB lineages include species that are well adapted to low DO. Gieseke et al. (2001) reported that members of *N. europaea* and *N. oligotropha* lineages dominated the outer layers of a biofilm where the DO concentration was high, while members of the *N. oligotropha* lineage were found exclusively in deeper layers of the biofilm where DO was lower. On the other hand, Prinčič et al. (1998) showed that oxygen saturation levels of 1%, 7%, and 21% did not influence the AOB community in the reactors. Our research also showed that oxygen concentrations in the reactor at the level of 0.5 and 1.5 mg O_2/L did not significantly influence either the species composition or the number of AOB in activated sludge. It can be concluded that even at the lower oxygen concentration, the high diversity and participation of ammonia-oxidizing bacteria in activated sludge can be preserved. Since aerobic conditions in wastewater treatment facilities are mainly designed to ensure nitrification, a lower oxygen concentration can be maintained in the reactor without worsening the nitrogen removal efficiency and, consequently, result in savings in operational costs.

In our research, the abundance of ammonia-oxidizing bacteria demonstrated no relationship with nitrification performance. This can be explained by changes of the occurrence and activity of AOB in activated sludge, and, simultaneously, their high diversity. In investigations by Ebie et al. (2002), the ratio of AOB to *Bacteria* increased as nitrification progressed due to an excessive proliferation of nitrifying bacteria. The decrease in AOB abundance, despite good nitrification efficiency, revealed that the structure of the AOB community was complicated and changed. Even if the number of AOB was constant, the ratios of AOB were changed through temperature and load fluctuation. The authors concluded that, if the AOB number decreased and the mixed liquor suspended solids (MLSS) of activated sludge did not change, the decline of nitrification ability could not result from a decrease of AOB abundance but was caused instead by a drop in their activity or change of dominant species.

3 Conclusions

The research proved that at reduced DO concentration (0.5 and 1.5 mg O_2/L) in the aeration phase, total oxidation of ammonia nitrogen can be achieved with nitrates as the main products of nitrification. At the low DO applied, simultaneous nitrification and denitrification took place in the aeration phase, with the efficiency of nitrogen removal over 50% (COD/N ratio of 6.8 and DO of 0.5 mg O_2/L).

This study revealed that DO concentration of 0.5 mg O_2/L improved total bacterial diversity, while the

oxygen concentration did not influence the AOB community. A significant drop in AOB abundance was, however, observed at the higher COD/N ratio. The technological and molecular results point out that the application of DO at the level of 0.5 mg/L enables high nitrogen removal efficiency, retaining high AOB number and diversity in biomass and significant reduction of the costs of aeration at the same time.

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