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ORIGINAL PAPER

The Impact of Organic Carbon and Ammonia Load in Wastewater on Ammonia-oxidizing Bacteria Community in Activated Sludge

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Abstract

The experiment was carried out in two SBR reactors differing in ammonia load (reactor R1 – ammonia load of 130 mg $N\text{-}NH_4 \times d^{-1}$, reactor R2 – ammonia load of 250 mg $N\text{-}NH_4 \times d^{-1}$). Feeding conditions in the reactors were switched from favoring autotrophic nitrification through favoring heterotrophic processes and back to autotrophic conditions. Observations of ammonia-oxidizing bacteria (AOB) community changes were based on PCR-RFLP analysis of amplified *amoA* gene fragments and AOB genetic diversity was evaluated on the base of the number of different *amoA* gene forms. When only carbonates were introduced with wastewater restriction patterns established about day 23 and 28 at ammonia load of 250 and 130 mg $N\text{-}NH_4 \times d^{-1}$, respectively. In both reactors statistically higher number of different *amoA* gene forms was observed when only carbonates were present in wastewater in comparison to conditions in which sodium acetate was introduced to the reactors. The AOB participation in activated sludge was higher at ammonia load of 250 mg $N\text{-}NH_4 \times d^{-1}$ but their genetic diversity was lower in comparison with this observed at ammonia load of 130 mg $N\text{-}NH_4 \times d^{-1}$.

Key words: ammonia-oxidizing bacteria, microbial diversity, PCR-RFLP, SBR

Introduction

Stable and efficient ammonia oxidation is still one of the most challenging tasks in modern wastewater treatment technology. In wastewater treatment plants ammonia oxidation often fails because of high sensitivity of nitrifying bacteria to environmental conditions. It was suggested that to improve process stability and efficiency, a high diversity of AOB is necessary (Daims *et al.*, 2001). According to Briones and Raskin (2003) ecosystem stability is the outcome of functional redundancy, which is ensured by the presence of many species able to perform the same ecological function. Presence of many microorganisms which are able to conduct a specific process increases probability that a sudden change of environmental conditions does not worsen the effectiveness of wastewater treatment, because one of the species will manage to adapt and ensure maintenance of the specific metabolic pathway (LaPara *et al.*, 2002).

Research proved that in case of AOB, factor that influences microbial diversity is reactor design. Rowan *et al.* (2003) studied the diversity of AOB in two full-scale bioreactors: biological aerated filter (BAF) and a trickling filter, receiving the same wastewater. The research proved higher diversity of AOB in the trickling filter than in the BAF and that the performance of the trickling filter was better than the BAF. Widely used single sludge wastewater treatment designs (*e.g.* SBR) accomplish nitrification concurrent with removal of chemical oxygen demand (COD) in one reactor; activated sludge in these reactors contains both heterotrophs and nitrifiers. Organic carbon compounds in wastewater stimulate heterotrophic bacteria that compete for oxygen with nitrifiers and may produce intermediary metabolic byproducts, which inhibit AOB (Gieseke *et al.*, 2001).

The use of molecular techniques allows gaining an insight into changes in bacterial communities in activated sludge in relation to environmental conditions. In presented work, PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) technique was employed in order to monitor changes in AOB population. PCR-RFLP technique is relatively simple and can be commonly used in wastewater treatment plants. A functional gene-based PCR

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assay was used targeting a 675-bp stretch of the *amoA* gene which encodes the active-site polypeptide of the ammonia monooxygenase enzyme (McTavish *et al.*, 1993). In presented research AOB genetic diversity was evaluated on the base of the number of different *amoA* gene forms in activated sludge.

The objective of the study was to determine the impact of organic carbon and ammonia load in wastewater on AOB community formation and diversity in activated sludge during adaptation period and after stabilisation to different substrate conditions.

Experimental

Materials and Methods

SBR operation. In the experiment two 3.0 l sequencing batch reactors operating simultaneously were employed. Seed sludge was collected from a conventional municipal wastewater treatment plant in Olsztynek (Poland). Each SBR operated in 24-hour cycle, with the following operating strategy: filling (0.25 h), aeration (23 h), settling (0.50 h) and decantation (0.25 h). The mean solids retention time (sludge age) was about 20 days and the total suspended solids (TSS) averaged 2500 mg TSS$\times$l^{-1}. Reactors were operated at 2 mg$\times$l^{-1} of dissolved oxygen, the temperature was maintained at 20°C and pH was kept between 7 and 8. During the filling period, 1.0 l of synthetic wastewater was added to the reactor to make the final working volume of 2.5 l. The artificial wastewater composition was as follows: $Na_2HPO_4\times12H_2O$ (46.0 mg$\times$l^{-1}), NaCl (10.0 mg$\times$l^{-1}), KCl (4.7 mg$\times$l^{-1}), $CaCl_2\times2H_2O$ (4.7 mg$\times$l^{-1}), $MgSO_4\times7H_2O$ (16.7 mg $\times$l^{-1}), $FeCl_3\times6H_2O$, $MnSO_4\times H_2O$, $ZnSO_4$, $CuSO_4$ ($<$0.2 mg$\times$l^{-1}), (Coehlo *et al.*, 2000, modified). There was a difference in ammonia load between the reactors: 130 and 250 mg N-$NH_4\times$d^{-1} in R1 and R2, respectively. In order to investigate the effect of organic carbon on AOB diversity in each reactor, the experiment was carried on in three phases. In phases I and III (40 and 38 days, respectively) only inorganic carbon was added to the reactors while in phase II (160 days) both inorganic and organic carbon was supplied with wastewater. A given phase lasted until restriction pattern and effluent parameters did not stabilize in particular substrate conditions. In phase II, the organic carbon load was 660 mg COD$\times$d^{-1} that resulted in COD concentration at the beginning of aeration phase of about 260 mg COD$\times$l^{-1}. Carbonates in amount of 7.15 mg of alkalinity per 1 mg of N-NH_4 oxidized were introduced with wastewater (Villaverde *et al.*, 1997)

Analytical measurements. Every day sampling was made at the influent and effluent of one cycle. All samples were filtered using a 0.2 mm micro-pore filter before being assayed. The following parameters were determined in wastewater: total suspended solids by drying at 103–105°C, organic compounds expressed as total and dissolved COD (chemical oxygen demand), ammonia by Nesslerization method or distillation, nitrite and nitrate by colorimetric methods (APHA 1992). Nitrification efficiency was measured as the amount of ammonia oxidized in SBR cycle.

Total DNA extraction. Activated sludge samples were collected in 2–4-day intervals and kept in freezer in –20°C before analysis. Genomic DNA was isolated from 400 mg of centrifuged sludge sample using FastDNA® SPIN®Kit (Q-BIOgene, USA) according to manufacturer protocol. Quality and quantity of isolated DNA was measured spectrophotometrically using Biotech Photometer (WPA, UK). The extracted DNA was suspended in 80 µl of distilled water and frozen in –20°C for further analyses.

PCR-RFLP. PCR was carried out with oligonucleotide primers 301F (5'-GACTGGGACTTCTGGCTG GACTGGAA-3') and 302R (5'-TTTGATCCCCTCT GGAAAGCCTTCTTC-3') (Norton *et al.*, 2002). PCR was performed in Eppendorf® Mastercycler Gradient (Eppendorf, Germany). Each 30 µl of PCR amplification mixture contained 0.2 µg of extracted total DNA, 1 µl of each primer (20 pmol$\times$µl^{-1}), 1 µl of dNTPs mixture (10 mM of each dNTP) (Promega, Winsconsin, USA), 1 U of Taq DNA polymerase (Invitrogen, Life Technologies), 3 ml of reaction buffer supplied with the DNA polymerase, 1 µl of $MgCl_2$ (50 mM) and sterile water to a final volume of 30 ml. The standard PCR amplification was carried out using the following program: 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s and a single final elongation at 72°C for 5 min. The presence of PCR products was confirmed by analyzing 5 ml of the product on a 0.8 % agarose gel stained with ethidium bromide. Size and intensity of PCR products were estimated by comparison with molecular weight marker øX174 DNA/HinfI (Promega, USA) according to KODAK 1D Image Analysis Software instruction. The gel was viewed with an ultraviolet transilluminator and recorded with CCD camera (Eastman Kodak Company, USA). Bands were detected automatically from digital images of the gel using KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company, USA). Obtained PCR products were subsequently digested using CfoI, HaeIII, HinfI, RsaI and SinI restriction enzymes. Enzymatic digestion was carried out in 0.2 ml Eppendorf tubes. To each tube 8 µl of PCR product, 1 µl of digestion buffer, restriction enzyme proportionally to PCR product amount and sterile water to a final volume of 10 µl were added. The digestion was carried out at 37°C for 3 h. Digested PCR

fragments were electrophoresed for 1 h in 2.5 % high resolution agarose gel (PRONA Micropor Delta) stained with ethidium bromide. The gel was documented and digitalized as described above.

Analysis of RFLP patterns. The structural diversity of microbial communities was assessed on the basis of the bands on the gel tracks. For a given enzyme the sum of all band masses in one lane was divided by 675 (length of *amoA* amplicon). The result was rounded up to the nearest whole number. Computations were performed for the five restriction enzymes and the highest value was chosen as the minimal number of different *amoA* gene forms in a given day.

Statistical analyses. In order to find statistically significant differences between the number of different *amoA* gene forms in different experimental phases and series, t-tests and Kruskal-Wallis test were applied at the probability level of 95%. Statistical computations were performed using the program STATISTICA 6.0 (StatSoft, USA).

Results

Nitrification efficiency depended on the phase of the experiment and ammonia load (Fig. 1.). In phase I in both reactors, a gradual increase in nitrification efficiency was observed and from 25 day ammonia oxidation stabilized at the level of 95% and 70% in reactors R1 and R2, respectively. At the beginning of phase II, organic carbon introduction with wastewater did not noticeably decrease the nitrification efficiency in reactor R1, however, from day 65 of the experiment, the efficiency of ammonia oxidation begun to drop to about 25%. Organic carbon introduction to reactor R2 caused deterioration of ammonia oxidation to about 20%, and then nitrification efficiency gradually increased. At the end of phase II in both reactors ammonia was oxidized in above 90% and this nitrification efficiency maintained until the end of the phase III.

Restriction patterns obtained by enzymatic digestion of amplified *amoA* gene fragments together with the number of different *amoA* gene forms in successive days of the experiment are presented in Fig. 2 and 3. In phases when only inorganic carbon was introduced with wastewater, restriction patterns reflecting AOB species composition established about day 23 at ammonia load of 250 mg $N\text{-}NH_4 \times d^{-1}$ (reactor R2, Fig. 3), and about day 28 at ammonia load of 130 mg $N\text{-}NH_4 \times d^{-1}$ (reactor R1, Fig. 2). In these phases in both reactors at least 3 different forms of *amoA* gene were present in biomass. RFLP patterns obtained in phase II showed that in both reactors AOB community changed greatly in time. After organic carbon introduction the restriction patterns and the effluent parameters in both reactors changed in time and did not stabilize until about day 130 of phase II. In both reactors the lowest nitrification efficiency was observed in periods when only 2 different forms of *amoA* gene were present in activated sludge (total amount of AOB in activated sludge, estimated by densitometry scanning of PCR products, did not change noticeably in this time, data not shown).

On the base of molecular and chemical data statistical analyses were carried out. The result of the test

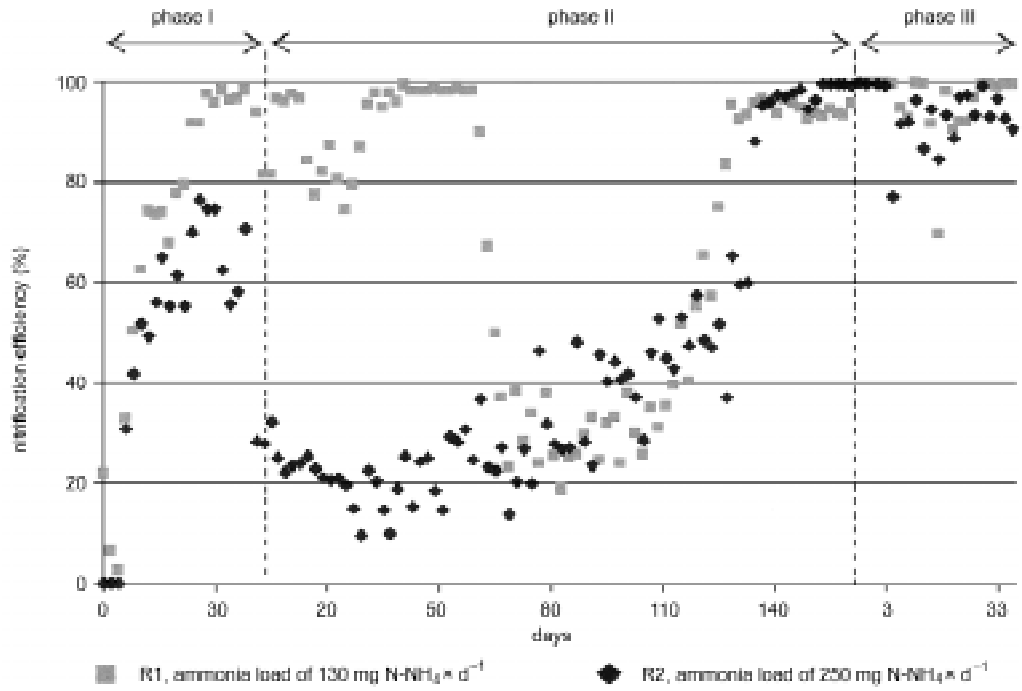

Fig. 1. Nitrification efficiency during experimental phases; R1, R2 – reactors 1 and 2, respectively; phase I and phase III – only inorganic carbon introduced with wastewater, phase II – inorganic and organic carbon introduced with wastewater.

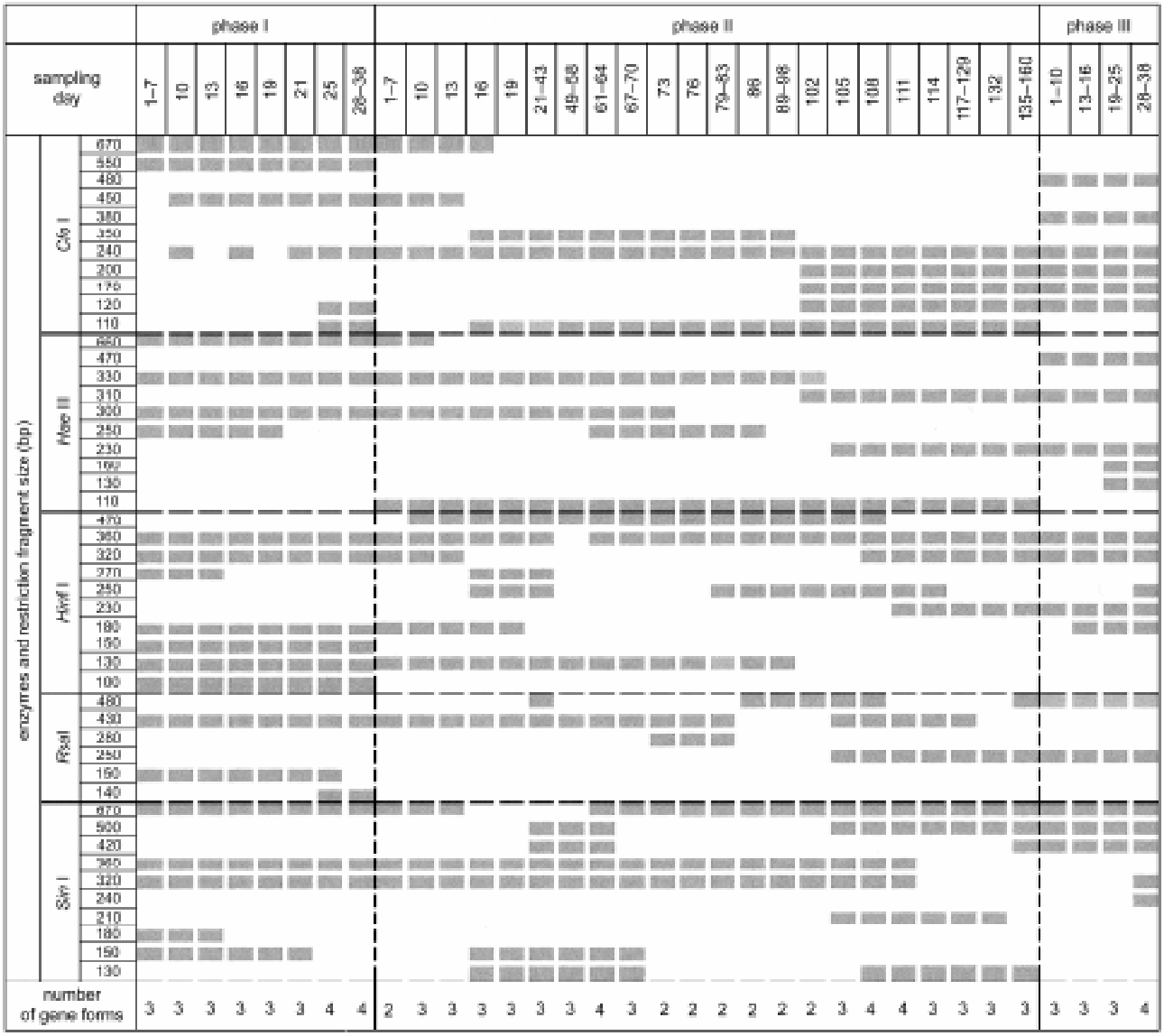

Fig. 2. RFLP patterns of AOB community from the reactor operating at ammonia load of 130 mg $N\text{-}NH_4 \times d^{-1}$ obtained from the digestion of amplified *amoA* gene fragments by five restriction enzymes. Periods of time between days in experimental phases in which restriction pattern remained unchanged are spaced with dashes.

($t_{162,\,81} = -3.15$; $p<0.002$) demonstrated that there was a statistically significant higher participation of AOB in activated sludge at ammonia load of 250 mg $N\text{-}NH_4 \times d^{-1}$ in comparison with ammonia load of 130 mg $N\text{-}NH_4 \times d^{-1}$ (Fig. 4). This analysis was confirmed by respirometric data (results not shown). The diversity of AOB was, however, higher in the reactor with lower ammonia load ($t_{2,\,81}=3.97$; $p\approx 0$) (Fig. 5). At ammonia load of 250 mg $N\text{-}NH_4 \times d^{-1}$ the number of different *amoA* gene forms averaged 2.68±0.54, while at ammonia load of 130 mg $N\text{-}NH_4 \times d^{-1}$ it averaged 3.04±0.6. After the stabilization of ammonia-oxidizing bacteria community in autotrophic phases, in R1 there were 4 different forms of *amoA* gene in activated sludge, while in reactor R2 only 3 different forms of the gene.

The hypothesis that organic carbon load influenced the genetic diversity of ammonia-oxidizing bacteria in activated sludge was also tested. The statistical analysis revealed that in both reactors the diversity of AOB was higher in phases when only inorganic carbon was introduced with wastewater in comparison with phase II (Fig. 6). The Kruskal-Wallis test results were $H_{2,\,82} = 18.41$ ($p \approx 0$) and $H_{2,\,82} = 25.86$ ($p \approx 0$) for R1 and R2, respectively. In reactor R1 the number of different *amoA* gene forms in phases with only inorganic carbon introduction averaged 3.4 and dropped to about 2.8 after organic carbon addition to wastewater, while in reactor R2 the presence of organic carbon in the influent caused that the number of different *amoA* gene forms decreased from about 3.1 to 2.5.

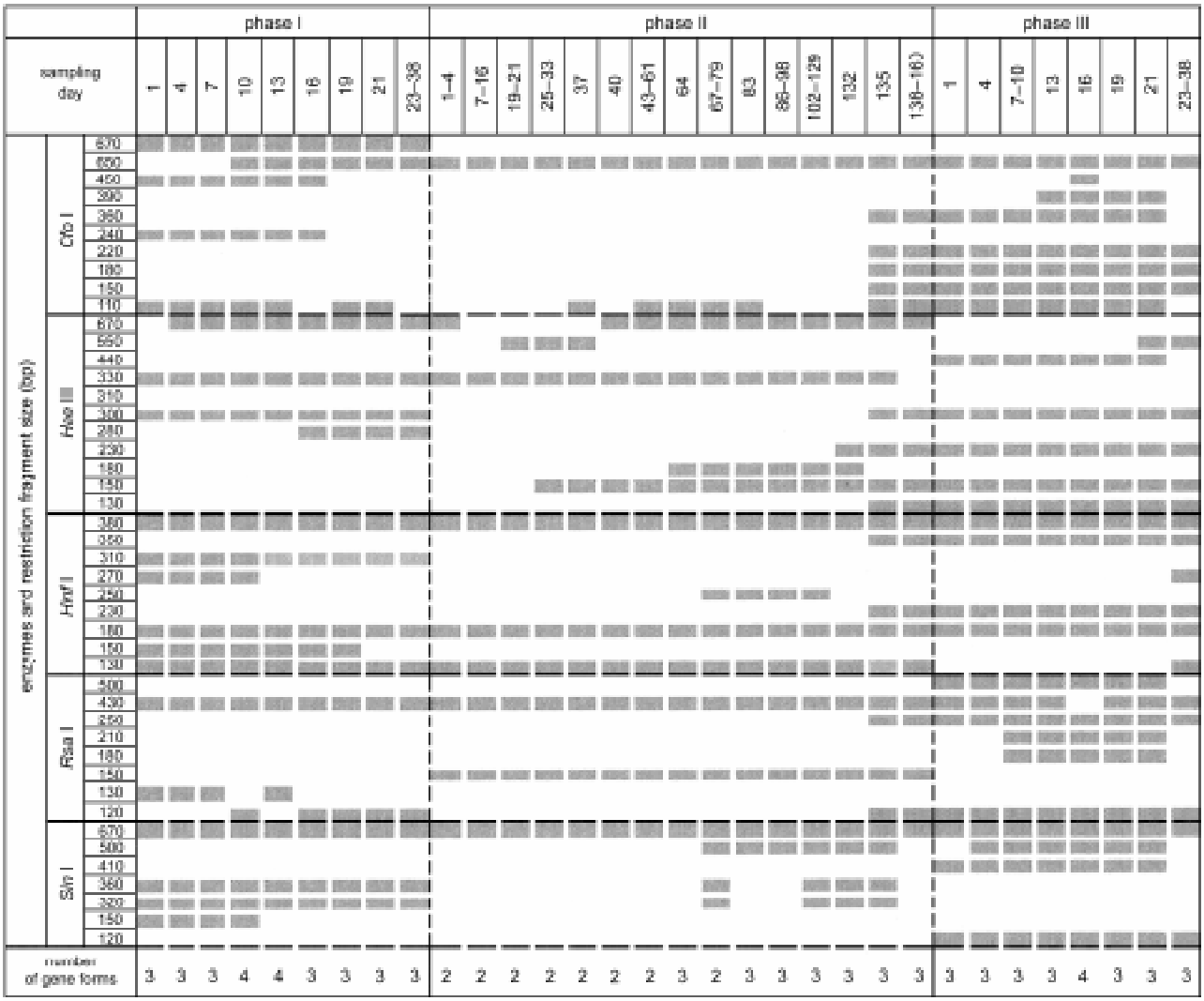

Fig. 3. RFLP patterns of AOB community from the reactor operating at ammonia load of 250 mg $N\text{-}NH_4 \times d^{-1}$ obtained from the digestion of amplified *amoA* gene fragments by five restriction enzymes. Periods of time between days in experimental phases in which restriction pattern remained unchanged are spaced with dashes.

Discussion

The objective of the study was to determine the impact of organic carbon and ammonia load in wastewater on ammonia-oxidizing bacteria community formation and diversity in activated sludge.

In molecular analyses *amoA* primers 301F and 302R (Norton *et al.*, 2002) were employed instead of Rotthauwe primers (Rotthauwe *et al.*, 1997), which are more widely used in AOB diversity surveys. A 301F/302R primer set allows getting about 200 bp longer PCR product, which is important in case of using PCR-RFLP technique. Horz *et al.* (2000) proved that restriction analysis based on *amoA* gene allows reliable description of differences in species composition of ammonia-oxidizing bacteria communities. According to Hughes *et al.* (2001), it is possible to compare relative diversity between communities from successive days or among sites or treatments as long as the measurement unit is defined and held constant and in presented research this condition was fulfilled. Mills *et al.* (2003) points out that bacterial diversity evaluation using restriction enzymes can be hampered by not full DNA fragments digestion and a difficulty with separation of low molecular weight DNA fragments. In presented research, in order to minimize above-mentioned biases, 5 different restriction enzymes and high resolution agarose gels were used.

Our research showed that after technological parameters change a period of time is necessary in order to allow activated sludge adaptation. At the beginning of phase I, ammonia nitrogen cumulated in the effluent from both reactors and nitrification efficiency was low. Molecular analyses revealed that lack of ammonia oxidizing activity resulted from insufficiently developed AOB consortia (seeding sludge was taken from a wastewater treatment plant with high C/N ratio in the influent). An increase of ammonia-oxidizing

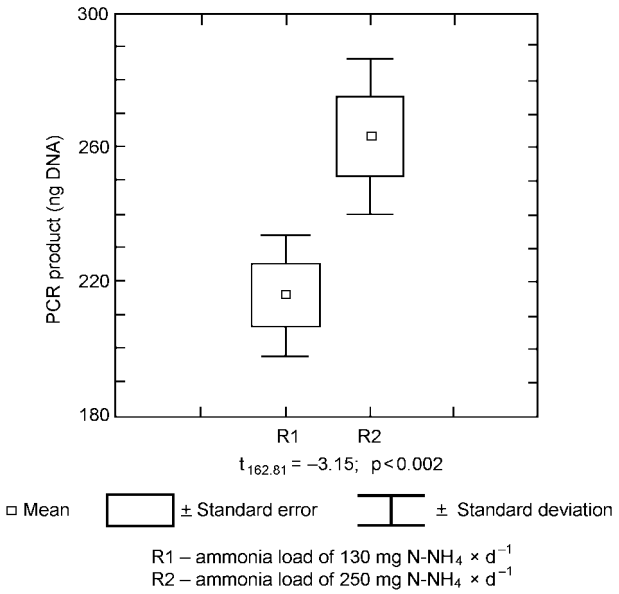

Fig. 4. Dependence between ammonia load in wastewater and AOB participation in activated sludge, estimated on the base of *amoA* gene amplification product (t tests); R1, R2 – reactors 1 and 2, respectively.

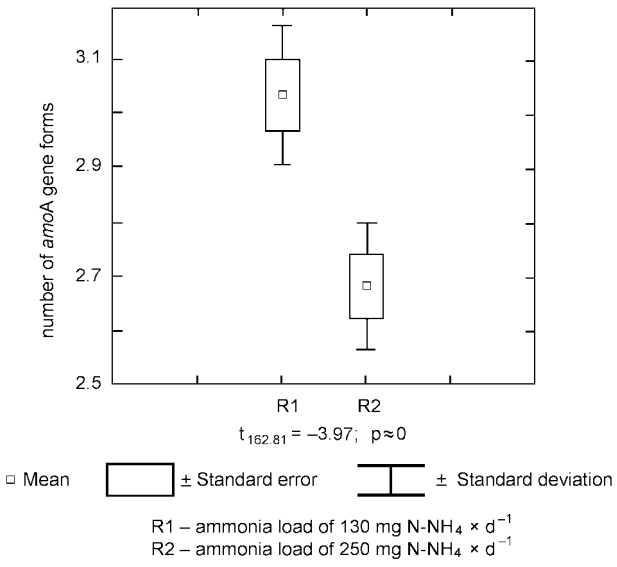

Fig. 5. Dependence between ammonia load in wastewater and the number of different *amoA* gene forms in activated sludge during the experiment (t tests); R1, R2 – reactors 1 and 2, respectively.

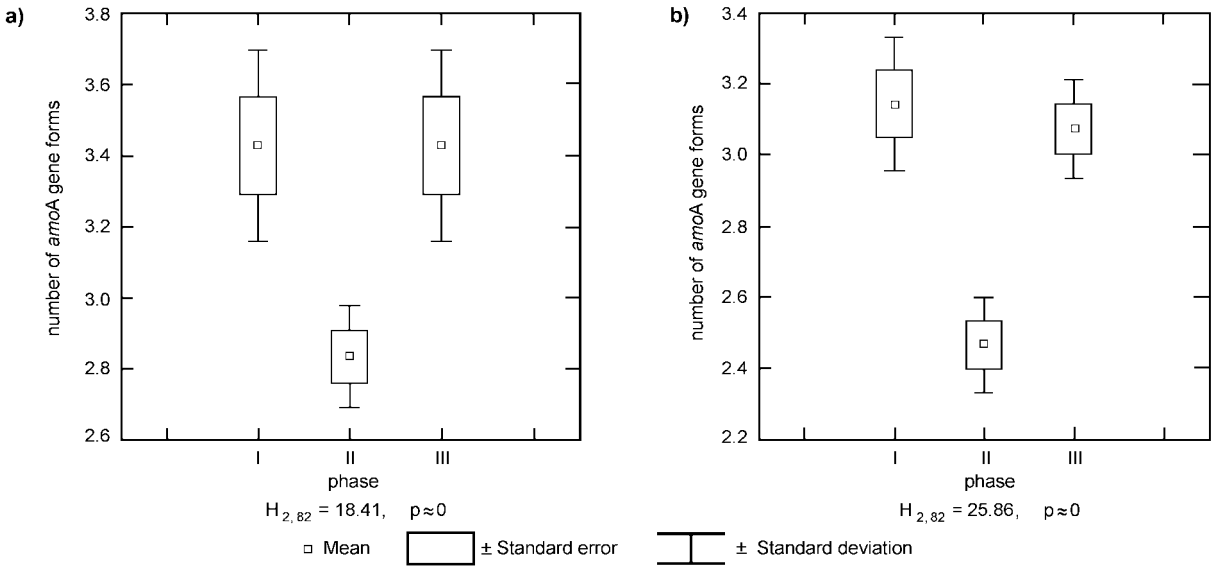

Fig. 6. Dependence between the phase of the experiment and the number of different *amoA* gene forms (Kruskal-Wallis test); a) reactor R1, ammonia load of 130 mg N-NH_4×d^{-1}, b) reactor R2, ammonia load of 250 mg N-NH_4×d^{-1}; phase I and phase III – only inorganic carbon introduced with wastewater, phase II – inorganic and organic carbon introduced with wastewater.

bacteria participation in activated sludge improved ammonia oxidation and from day 25 of phase I nitrification efficiency was about 95 and 70% in reactors R1 and R2, respectively. High nitrification efficiency in both reactors in phase III resulted from the presence in activated sludge a well shaped ammonia-oxidizing bacteria community formed in previous phases.

The time necessary to stabilize species composition of AOB in phases when only inorganic carbon was introduced with wastewater depended on ammonia load. Restriction pattern established about day 23 and 28 of phases in reactors R1 and R2, respectively. Stabilisation of species composition was accompanied by settling of parameters in the effluents from both reactors (data not shown). Similar results were obtained by Egli *et al.* (2003). Authors using FISH and T-RFLP technique analyzed changes in number and composition of ammonia-oxidizing bacteria during startup of two nitrifying reactors fed with synthetic wastewater containing only inorganic carbon. The

changes in AOB community correlated with nitrogen forms concentrations changes in the effluent and stable ammonia oxidation was obtained about 20 days after inoculation of the reactor with activated sludge. It is possible that in our experiment a faster establishment of species composition in R2 was caused by the fact that higher ammonia load that promoted growth of nitrifiers with a high specific growth rate.

The participation of ammonia-oxidizing bacteria in activated sludge was higher in the reactor with higher ammonia load. It was observed, however, that despite higher AOB participation in biomass in reactor R2 their genetic diversity was lower in comparison with that at ammonia load of 130 mg $N\text{-}NH_4 \times d^{-1}$. It can be concluded that a lower ammonia load enables adaptation to feeding conditions for more diverse group of nitrifiers and promotes growth of species with low specific growth rate (Atlas, 1997). Also according to Wijeyekoon *et al.* (2004) such a selection of species based on the substrate concentration is thought to be related to the growth rates of the different strains.

After the introduction of organic carbon in phase II, no stable nitrification in activated sludge was obtained for a long time. Lack of system stability was confirmed by the effluent parameters fluctuations and changes in AOB community composition displayed by PCR-RFLP technique. It is worth stressing that in both reactors the lowest nitrification efficiency was observed in periods when only 2 different forms of *amoA* gene were present in activated sludge. This fact may indicate that the genetic diversity of AOB consortia positively influences the efficiency of ammonia oxidation in activated sludge.

A decrease of AOB population with increasing organic carbon to nitrogen ratio is discussed in literature. Nogueira *et al.* (2002) investigated two biofilm reactors performance during a shift in process operation from pure nitrification to combined nitrification and organic carbon removal. Using FISH technique authors proved that sodium acetate introduction to the biofilm reactor ($0.68\ kg\ COD \times m^{-3} \times d^{-1}$) resulted in reduction of AOB population and subsequent breakdown of nitrification process. In presented research, however, it was proved that the presence of organic carbon in wastewater not only influences ammonia-oxidizing bacteria number but also their diversity in activated sludge. Irrespective of the ammonia load in wastewater, introduction of sodium acetate in amount of 660 mg $COD \times d^{-1}$ to the reactors caused the decrease in the average number of different *amoA* gene forms in biomass. In practice the decrease in AOB diversity after increasing organic carbon load in wastewater can be reduced by *e.g.* periodic inoculation with biomass from nitrifying reactors.

Presented research proved that:

(i) when only carbonates are introduced with wastewater the time necessary to stabilize species composition of AOB depends on ammonia load in wastewater; AOB community established about day 23 and 28 at ammonia load of 250 and 130 mg $N\text{-}NH_4 \times d^{-1}$, respectively,

(ii) AOB participation in activated sludge is higher at higher ammonia load but their genetic diversity is lower in comparison to this observed at lower ammonia load,

(iii) the introduction of organic carbon with wastewater results in lower ammonia-oxidizing bacteria diversity in activated sludge.

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Literature

APHA 1992. Standard Methods for the Examination of Water and Wastewater, 20th edition. American Public Health Association, Washington, DC.

Atlas R.M. 1997. Biodiversity and microbial interactions in the biodegradation of organic compounds. In: T.E. Cloete (ed), *Microbial Community Analysis*, IAWQ, New York, p. 203.

Briones A. and L. Raskin. 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr. Opin. Biotech.* 14: 270–276.

Coelho M.A.Z., C. Russo and O.Q.F. Araujo. 2000. Optimization of sequencing batch reactor for biological nitrogen removal. *Water Res.* 34: 2809–2817.

Daims H., U. Purkhold, L. Bjerrum, E. Arnold, P.A. Wilderer and M. Wagner. 2001. Nitrification in sequencing biofilm batch reactors: lessons from molecular approaches. *Water Sci. Tech.* 43: 9–18.

Egli K., C. Langer, H.-R. Siegrist, A.J.B. Zehnder, M. Wagner and J.R. van der Meer. 2003. Community analysis of ammonia and nitrite oxidizers during start-up of nitritation reactors. *Appl. Environ. Microbiol.* 69: 3213–3222.

Gieseke A., U. Purkhold, M. Wagner, R. Amann and A. Schramm. 2001. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. *Appl. Environ. Microbiol.* 67: 1351–1362.

Horz H., J. Rotthauwe, T. Lukow and W. Liesack. 2000. Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. *J. Microbiol. Meth.* 39: 197–204.

Hughes J.B., J.J. Hellmann, T.H. Ricketts and B.J.M. Bohannan. 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl. Environ. Microbiol.* 67: 4399–4406.

LaPara T.M., C.H. Nakatsu, L.M. Pantea and J.E. Alleman. 2002. Stability of the bacterial communities supported by a seven-stage biological process treating pharmaceutical wastewater as revealed by PCR-DGGE. *Water Res.* 36: 638–646.

McTavish H., J.A. Fuchs and A.B. Hooper. 1993. Sequence of the gene encoding for ammonia monooxygenase in *Nitrosomonas europaea*. *J. Bacteriol.* 175: 2436–2444.

Mills D.K., K. Fitzgerald, C.D. Litchfield and P.M. Gillevet. 2003. A comparison of DNA profiling techniques for monitoring nutrient impact on microbial community composition during bioremediation of petroleum-contaminated soils. *J. Microbiol. Meth.* 54: 57–74.

Nogueira R., L.F. Melo, U. Purkhold, S. Wuertz and M. Wagner. 2002. Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon. *Water Res.* 36: 469–481.

Norton J.M., J.J. Alzerreca, J. Suwa and M.G. Klotz. 2002. Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Arch. Microbiol.* 177: 139–149.

Rotthauwe J.H., K.P. Witzel and W. Liesack. 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63: 4704–4712.

Rowan A.K., J.R. Snape, D. Fearnside, A.R. Barer, T. Curtis and I.M. Head. 2003. Composition and diversity of ammonia-oxidising bacterial communities in wastewater treatment reactors of different design treating identical wastewater. *FEMS Microbiol. Ecol.* 43: 195–206.

Villaverde P., P.A. Garcia-Encina and F. Fdz-Polanco. 1997. Influence of pH over nitrifying biofilm activity in submerged biofilters. *Water Res.* 31: 1180–1186.

Wijeyekoon S., T. Mino, H. Satoh and T. Matsuo. 2004. Effects of substrate loading rate on biofilm structure. *Water Res.* 38: 2479–2488.