MOLECULAR ANALYSIS OF MICROORGANISMS RESPONSIBLE FOR THE FIRST PHASE OF NITRIFICATION IN AN ANOXIC ENVIRONMENT

ALEKSANDRA ZIEMBIŃSKA[1]*, SŁAWOMIR CIESIELSKI[2], ANNA RASZKA[1], KORNELIUSZ MIKSCH[1]

[1] The Silesian University of Technology, Faculty of Power and Environmental Engineering, Environmental Biotechnology Department
[2] University of Warmia and Mazury in Olsztyn, Faculty of Environmental Protection and Fisheries, Department of Environmental Biotechnology
*Corresponding author e-mail: aleksandra.ziembinska@polsl.pl

Abstract: Ammonia-oxidizing bacteria communities were evaluated in a completely mixed, laboratory scale membrane reactor (MBR) working under anoxic conditions for 5 months. The microorganisms in activated sludge were fed a synthetic medium containing 66–150 mg NH_4^+-N/l. The age of the activated sludge in MBR was 50 days and the hydraulic retention time (HRT) was 3.3 days. The estimation of the diversity and complexity of the AOB community together with the identification of the dominant bacteria in the activated sludge under anoxic conditions were performed using denaturing gradient gel electrophoresis (DGGE) and DNA sequencing. Molecular analysis of the microbial community carried out with two microbial molecular markers, 16S rRNA gene and *amoA* gene, suggested that nitrification was led by a *Nitrosomonas*-like species. In the biocenosis of the investigated bioreactor, oxygen was the crucial selective parameter. The results obtained in this work showed that a*moA* gene research is more suitable to study the stability and effectiveness of ammonia oxidation. This information emphasizes the necessity of the usage of molecular markers based on functional genes instead of ribosomal ones in order to present the actual state of the process performed in bioreactors. It was also stated that *Nitrosomonas* - like bacteria are able to perform nitritation even in anoxic environment, that is probably the reason why these bacteria are the most common AOB in different bioreactors.

INTRODUCTION

Nitrogen concentrations over the prescribed levels in wastewater treatment effluent can cause a serious problem to receiving watercourses leading to eutrophication and being toxic to water environment [3]. It is widely known that the removal of nitrogen from wastewater is achieved mainly through a combination of nitrification and denitrification. From the aspect of the efficiency of wastewater treatment, the crucial process responsible for ammonia oxidation is nitrification. This process is led by two phylogenetically distinct groups of microorganisms: aerobic chemoautotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB).

Nitrification is an oxygen-demanding process and low oxygen concentration is the major environmental factor controlling it. It is considered that oxygen levels below 1.5 mg/l should drastically decrease the effectiveness of nitrification [22], but it was proved that wastewater treatment processes led by simultaneous nitrification/denitrification can achieve up to 80% of the total nitrification under minimal aeration conditions where no detectable oxygen concentration was observed [1].

The most abundant bacteria expected to be found in activated sludge under anoxic condition belong to the *Nitrosomonas* sp. [9]. *Nitrosomonas* sp. is known to be obligatory chemolithotrophic, ammonia-oxidizing bacteria, which is able to lead nitrification and denitrification simultaneously under limited oxygen conditions. Under anoxic conditions, nitrite is an electron acceptor for ammonia oxidation without any observed cell growth [20].

Recently molecular analyses of bacteria have provided information about bacterial physiology and ecology, which may be helpful in the improvement of wastewater technology. One of the most useful methods for bacterial diversity and community composition estimation is denaturing gradient gel electrophoresis (DGGE) [25]. Two molecular markers are used in the DGGE analysis of ammonia-oxidizing bacteria: a 16S rRNA gene and an a*moA* gene. The 16S rRNA gene is known to be a good phylogenetic marker as well as a bacterial identification molecule [10]. The a*moA* gene codes the active site subunit of the ammonia monooxygenase − enzyme responsible for ammonia oxidation, and has recently been used as an alternative AOB identification and phylogeny marker [17]. Although the use of the *amoA* gene as a molecular marker is increasing, in comparison with 16S RNA gene, this gene sequence's length and database are still not sufficient enough for AOB analysis.

The main aim of this study was a molecular analysis of *β*-proteobacterial ammonia oxidizers derived from activated sludge in order to estimate the diversity and complexity of an AOB community under anoxic conditions. Furthermore, an attempt to identify the dominant bacteria was undertaken.

MATERIALS AND METHODS

Reactor details and analytical procedure

Activated sludge from a completely mixed, laboratory-scale membrane bioreactor, with a volume of 36 l, was used in this study. The reactor was operated anoxically (oxygen level below 0.5%) with nitrification and fed with a synthetic medium containing 66−150 mg NH_4^+-N/l. The medium consisted of NH_4Cl, $NaNO_2$, KH_2PO_4 and $NaHCO_3$ and it was enriched with landfill leachete from Gliwice, Poland, at the range of 10−50%. The membrane (A4 Size Mat Sheet Membrane, Kubota System) with a pore size of 0.4 μm was submerged in the reactor. Activated sludge from a municipal wastewater treatment plant performing nutrient removal was used for seeding. The age of the sludge was 50 days and the hydraulic retention time (HRT) was 3.3 days in the experimental bioreactor. The level of mixed liquor suspended solids was changing at the range of 11.3–32.2 g/l during the experiment. Nitrogen compound concentrations analysis was performed as described previously [24].

Activated sludge samples preparation and DNA extraction

The experiment was carried out for 5 months. Activated sludge samples (volume of 10 ml) were collected at 4-week intervals from the membrane bioreactor and numbered 1 to 5 respectively. Samples were pelleted by centrifugation (5 000 × g, 10 min, 4°C) and stored at -20°C.

Total genomic DNA was extracted from 0.2 g of the activated sludge samples using a Fast DNA Spin Kit for Soil (MP Biomedicals LLC) according to the manufacturer's instructions. The amount of DNA was measured spectrophotometrically using a Biophotometer (Eppendorf) and stored at -20°C until PCR amplification.

PCR amplification conditions

The nested-PCR procedure was used in the study for 16S rRNA gene amplification. For this purpose two sets of primers were used (Table 1).

The first round of PCR was performed with CTO primers, enabling partial PCR amplification of 16S rRNA bacterial gene belonging to ammonia-oxidizing β-Proteobacteria. The PCR was carried out in a total volume of 30 µl and the reaction mixture. The PCR product was used as a template in the second cycle of nested-PCR as described previously [7, 24].

Table 1. Primers used in the study

Target gene	Primer	Sequence	Reference
β-proteobacterial AOB 16S rRNA	CTO189f – ABC – GC	5' CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG GGA CMA AAG YAG GGG ATC G 3'*	7
	CTO 654r	5' CTA GCY TTG TAG TTT CAA ACG C 3'*	7
Bacterial 16S rRNA	338F-GC	5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3'	12
	518r	5' ATT ACC GCG GCT GCT GG 3'	12
amoA	Amo1-F - GC	5' CGC CGC GCG GCG GGC GGG GCG GGG GCG GGG TTT CTA CTG GTG GT 3'	18, 21, 24
	Amo2R-TC	5' CCC CTC TGC AAA GCC TTC TTC 3'	18, 21, 24

*Degeneracies shown in bold; Y = T/C, M = A/C

Subunit A of ammonia monooxygenase gene (*amoA*) amplification was performed using primers Amo1-F- GC and Amo2R-TC, as described previously [24]. The PCR was carried out in a total volume of 30 µl and the amplification was performed using an Eppendorf thermal cycler. The PCR products were evaluated in agarose gel (0.8% w/v agarose, Promega) in 1×TBE buffer (Tris, boric acid, EDTA, pH = 8.3), stained with ethidium bromide (0.5 µg/l) in MiliQ water and photographed under UV light.

Denaturing gradient gel electrophoresis conditions and DNA bands extraction

DGGE was performed using the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (8% w/v for 16S rRNA gene, 6% w/v for a*moA* gene, 37:1 acrylamide-bisacrylamide, Fluka) with a gradient of 30–60% denaturant was prepared according to the manufacturer's guidelines.

The gel was run for 15 h at 55 V in a 1×TAE buffer (Tris, acetic acid, EDTA, pH = 8.0) at a constant temperature of 60°C. The gel was stained with SYBR Gold (1:10 000, Invitrogen) in MiliQ water for 30 min and destained in MiliQ water for 40 min, then visualized under UV light and photographed using a Kodak 1D.

PCR products purification and sequencing

To identify the most abundant AOB in the activated sludge samples dominant bands from DGGE fingerprint were cut off, extracted from the gel and purified using a GenElute PCR Clean-up Kit (Sigma) according to the manufacturer's instructions and sequenced. DNA sequences of the bacterial 16S rRNA gene obtained in the procedure were identified by comparison with the NCBI GenBank (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool).

Nucleotide sequence accession numbers

The GenBank accession numbers for the partial sequences determined in this study are: FJ907322-FJ907326 (16S rRNA gene sequences) and FJ907200-FJ907202 (amoA gene sequences). Table 2 presents sequence characteristics of DNA derived from electrophoresis gel in this study and their similarities to the closest relatives.

Table 2. Sequence characteristics of DNA derived from electrophoresis gel and their similarities to the closest relatives

Band	Gene	Length (bp)	Accession no.	Closest relatives (accession no.)	coverage/ identity (%)
OZ1	amoA	432	FJ907200	Uncultured bacterium clone amoA SBR JJY 63 (FJ577881)	96/99
OZ2	amoA	432	FJ907201	Uncultured bacterium clone amoA SBR JJY 63 (FJ577881)	96/99
OZ6	amoA	432	FJ907202	Uncultured bacterium clone amoA SBR JJY 63 (FJ577881)	96/99
OZR1	16S rRNA	195	FJ907322	Uncultured Nitrosomonas sp. isolate DGGE gel band B1 (FJ654650)	100/100
OZR2	16S rRNA	195	FJ907323	Uncultured Nitrosomonas sp. isolate DGGE gel band B1 (FJ654650)	100/100
OZR3	16S rRNA	195	FJ907324	Uncultured Nitrosomonas sp. clone LEQUIA R0CTO47 (FM997806)	100/98
OZR4	16S rRNA	195	FJ907325	Uncultured Nitrosomonas sp. isolate DGGE gel band B1 (FJ654650)	100/98
OZR5	16S rRNA	195	FJ907326	Uncultured Nitrosomonas sp. clone LEQUIA R0CTO47 (FM997806)	100/100

Numerical analysis of the DGGE fingerprints

The numerical analysis of DGGE fingerprints was performed using a Kodak 1D. Bacterial biodiversity was estimated on the basis of densitometric measurements and the Shannon diversity index for the samples was calculated [13].

RESULTS

In order to promote the AOB responsible for nitrification in anoxic condition, the oxygen level was kept below 0.5%. The activated sludge was fed with synthetic medium containing 66–150 mg NH_4^+-N/l. The nitrogen species concentrations are presented in Fig 1. At the beginning of the experiment the effectiveness of ammonia nitrogen oxidation was at the level of 80%. From the 12th week of the process the effectiveness started to decrease and reached 10% at the end of the experiment.
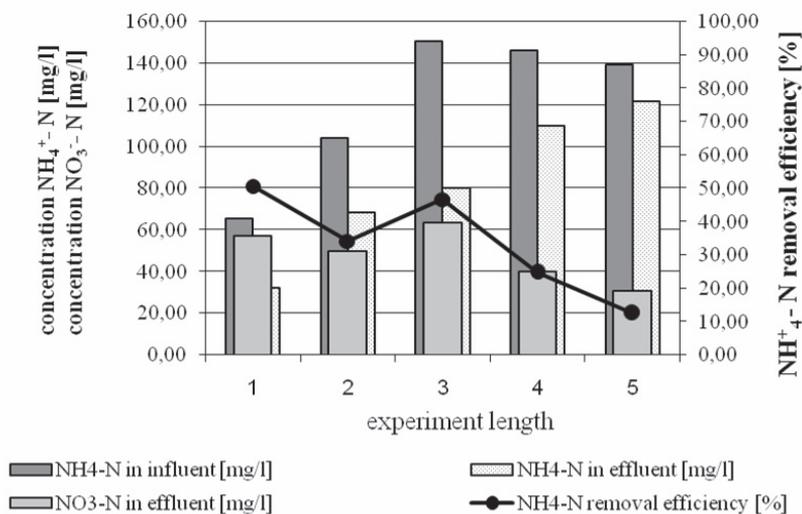

Fig. 1. Nitrogen compounds concentration and ammonia oxidation effectiveness measured for MBR bioreactor during the experiment; NO_2^- concentrations in the effluent not shown due to a very low values obtained

To evaluate the effect of the treatment on the diversity of ammonia oxidizing bacteria, the total DNA obtained at each sampling time (at 4 week intervals) during the 5 months of the experiment was analyzed by PCR-DGGE. The applied approach allowed us to obtain the clear picture of bacterial structure using both primer pairs (Fig. 2). The fingerprint obtained by amplification of ammonia monooxygenase gene fragment (Fig. 2b) possessed more bands but samples were less varied than fingerprint created by resolution of 16S rRNA gene fragment (Fig. 2a).

The dominant bacteria DNA bands were extracted from the gel and underwent purification and sequencing. The analysis revealed that among all obtained sequences eight were unique and each of them corresponds to expected gene. Four sequences of 16S rRNA gene (195 bp) showed the highest similarity to DNA sequences of various uncultured *Nitrosomonas* species. The sequenced DNA of *amoA* gene fragment bands (495 bp) was similar to DNA sequences of undefined and uncultured microorganisms possessing ability of ammonia oxidation (Tab. 2).

In order to compare the diversity of the samples, the fingerprints obtained from DGGE separation of partial 16S rRNA gene fragments of ammonia oxidizers were ana-
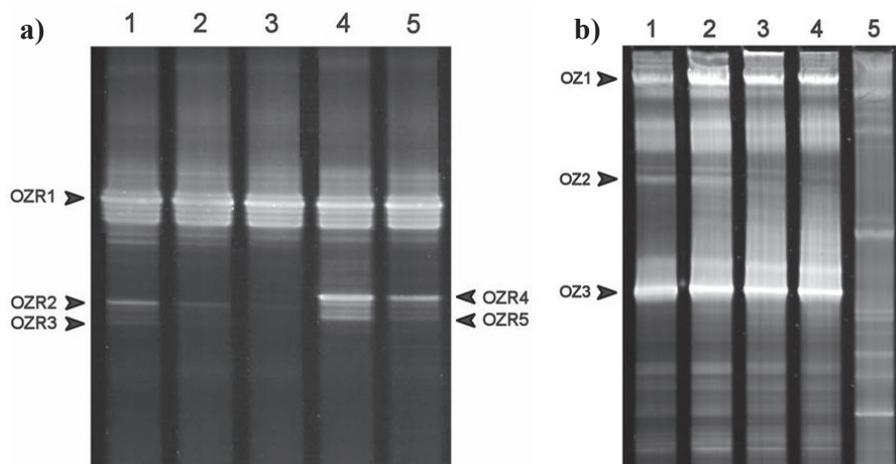
Fig. 2. DGGE gel with MBR activated sludge samples under anoxic conditions photographed in UV light for (a) 16S rRNA gene PCR products (b) *amoA* gene PCR product. Characteristics of species corresponding to labeled bands are given in Table 2

lyzed. Bacterial diversity was estimated on the basis of fingerprint optical density measurements and the Shannon diversity index for the samples was calculated. Figure 3 shows the changes of biodiversity throughout the process, the estimated values of the Shannon index obtained for samples 1 to 5 were: 2.04, 1.74, 1.84, 2.24, and 2.03 respectively.
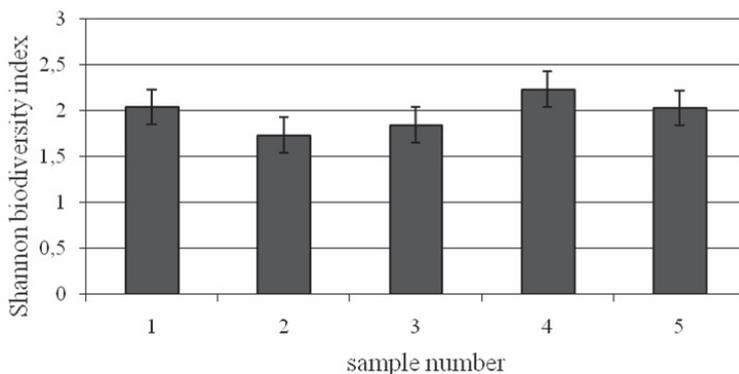

Fig. 3. Biodiversity of *β*-proteobacterial ammonia-oxidizing bacteria obtained in the study

DISCUSSION

From the earliest stage of the research into ammonia oxidizers, chemoautotrophic nitrifying bacteria were considered to be strictly aerobic and the oxidation of ammonia led by them was thought to require dissolved oxygen [15]. Nowadays it has been demonstrated that the complete anoxic conversion of ammonia by *Nitrosomonas europaea* and *Nitroso-*

monas eutropha with nitrite as electron acceptor [19] is possible. Autotrophic AOB have also been found in temporarily and permanently anoxic rhizosphere habitats [5]. In the bioreactor's biocenosis investigated in this research, oxygen was the crucial selective parameter. It seems probable that oxygen stress limited the biocenosis diversity only in relation to the representatives of the genus *Nitrosomonas*. These bacteria are considered to be not only the most abundant, but also the most effective ammonia oxidizers. In this experiment the effectiveness of ammonia oxidation was low, ca. 30%. This could suggest that an uncultured group of *Nitrosomonas* sp. in the anoxic environment identified in this study was not able to lead the nitrification process at the level equal to that in aerobic conditions. Diab *et al.* [4] suggested that nitryfiers can survive oxygen limitation by changing their metabolism to a very low rate, comparable to a state of resting cells. It is highly likely that AOB cells under anoxic conditions change their physiology in such a way, however, this statement needs further research.

It cannot be excluded that other than *Nitrosomonas* sp. ammonia-oxidizing bacteria can be responsible for ammonia oxidation in anoxic conditions, but such situation is difficult to state due to the fact of the very specific primers usage. The relatively short length of the gene sequences identified in the study could also be the reason for the misidentification of bacteria. The method of bacteria identification based on gene sequences comparison may be also deficient. Yu *et al.* [23] noted previously that species affinity in the case of gene sequence based identification in the gene databases is stated when the similarity of the sequences is over 95%. In the case of bacteria, this level of differences can be significant enough for its misidentification.

The clone 16S rRNA gene – OZR1 was the only dominant present in the biocenosis during the entire length of the experiment (Fig. 2a). Clones OZR2 together with OZR4 and OZR3 together with OZR5 were identified as the most similar to the same clone sequence. Interestingly, clones OZR2 and OZR3 disappeared gradually between the first and the twelfth week of the experiment (Fig. 1 and Fig. 2a, 1st to 3rd sampling time). These clones reappeared (as OZR4 and OZR5) in the sixteenth week of the experiment (Fig. 1 and Fig. 2a, sampling time 4th). This situation could be linked to the ammonia concentration, rising gradually to 150 mg/l (to) in the twelfth week (3rd sampling time), and stabilized at the level of 140-150 mg/l till the end of the experiment. It is worth noting that the effectiveness of the process decreased drastically from the twelfth week (3rd sampling time). Such a situation could be caused by the rebuilding of the biomass.

The changeability of the *amoA* gene sequences (Fig. 2b) obtained in the study appears to be more constant when compared with the 16S rRNA gene sequences (Fig. 2a). Despite the fact that each of the 16S rRNA gene sequences identified in the study belonged to uncultured *Nitrosomonas* sp., the a*moA* gene sequences belonged to uncultured and unidentified bacteria. This situation emphasizes the fact that anoxic nitrification can also be led by bacteria not belonging to *β-Proteobacteria* ammonia oxidizers, such as *Planctomycetes,* responsible for the Anammox process or other subclasses of *Proteobacteria*. The comparison of the DNA bands changeability in DGGE gels for the identification of genes (16S rRNA) and functional genes (a*moA* gene) showed that new sequences of *amoA* gene appeared 4 weeks after the beginning of the decline of the process's effectiveness. This could suggest that the bacteria belonging to *Nitrosomonas* sp. identified in the system were not responsible for ammonia oxidation. Such a situation stresses the fact that ammonia oxidation research should be concentrated on studies of functional genes

rather than constitutive ones, because the former are more important to the effectiveness of the process.

It has previously been stated that *Nitrosospira* sp. representatives co-exist in anoxic marine sediments with *Nitrosomonas* sp. [11] and other natural environments, especially in soil [2], sediments [8] and freshwater [6]. *Nitrosospira* sp. is also known to be present in nitrifying activated sludge, but is not commonly reported as relevant AOB in bioreactors [16]. The results obtained by Park *et al.* [14] suggest that a low dissolved oxygen level would be more favorable for *Nitrosospira* sp. In the studies presented no representatives of this genus were present in the system. These results underline the fact that the oxygen level is not the only limiting factor for AOB growth in a lab-scale anoxic bioreactor.

The biodiversity of *β*-proteobacterial ammonia oxidizers was relatively steady during the entire length of the experiment. The changes in the diversity range of 0.5 are not relevant to the stability of the process. It is necessary to mention that the biodiversity of *β*-proteobacterial AOB is *de facto* the diversity of *Nitrosomonas*-like clones.

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ANALIZA MOLEKULARNA MIKROORGANIZMÓW ODPOWIEDZIALNYCH ZA PROWADZENIE PIERWSZEJ FAZY NITRYFIKACJI W ŚRODOWISKU ANOKSYCZNYM

W eksperymencie badano grupę bakterii utleniających amoniak w bioreaktorze membranowym całkowitego wymieszania (MBR), pracującym w warunkach anoksycznych przez 5 miesięcy. Osad czynny zasilano pożywką syntetyczną, zawierającą 66–150 mg N-NH$_4^+$/l. Wiek osadu wynosił 50 dni, a hydrauliczny czas zatrzymania – 3,3 dnia. Oszacowanie różnorodności i złożoności zbiorowiska bakterii utleniających amoniak oraz identyfikacja mikroorganizmów dominujących w badanym osadzie czynnym w warunkach anoksycznych została przeprowadzona metodą elektroforezy w gradiencie denaturacji (DGGE) i sekwencjonowania DNA. Analiza molekularna biocenozy, przeprowadzona z użyciem dwóch markerów molekularnych: genu kodującego 16S rRNA i genu kodującego monooksygenazę amonową (*amoA*), wykazała, że proces nitritacji był prowadzony przez gatunki bakterii z rodzaju *Nitrosomonas*. W biocenozie badanego bioreaktora stężenie tlenu było głównym parametrem selekcyjnym dla nitritatorów. W badaniach wykazano, że markerem molekularnym sprawdzającym się lepiej w monitoringu efektywności procesu nitryfikacji pierwszej fazy jest gen a*moA*. Te dane podkreślają, że w badaniach efektywności prowadzonego procesu zachodzącego w bioreaktorach, informacje uzyskiwane