Comparison of Ammonia-Oxidizing Bacterial Community Structure in Membrane-Assisted Bioreactors Using PCR-DGGE and FISH

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The ammonia-oxidizing bacterial (AOB) communities in three membrane bioreactors (MBRs) were monitored for 2 months after an acclimation period in order to investigate the influence of sludge age and medium type on AOB changeability and its connection with nitrification effectiveness. One MBR with a sludge age of 4 days was fed with a synthetic medium, whereas the other two with sludge ages of 8 and 32 days were fed with landfill leachate. The research revealed that landfill leachate can be effectively treated in an MBR with a higher sludge age for longer periods of time and that this improvement in performance was correlated with an increase in AOB biodiversity. Interestingly, the medium type has a stronger influence on AOB biocenosis formation than the sludge age.

Keywords: Activated sludge, ammonia-oxidizing bacteria, PCR-DGGE, FISH, MBR, 16S rRNA gene

Conventional activated sludge (CAS) systems have been widely used for many years in both industrial and municipal wastewater treatments. Activated sludge is a mixture of organisms, including Bacteria, Protozoa and Metazoa, which are highly efficient in wastewater treatment processes. The composition of and relationships in this biocenosis are important to engineers for estimating its performance effectiveness, as well as to microbiologists for studying the complexity of this artificial system [19]. Nowadays, membrane bioreactors (MBRs) have become an alternative to conventional activated sludge systems, using membrane ultra- and micro-filtration instead of sedimentation [8]. Membrane separation technology can improve liquid/biosolid separation [9], as well as the quality of the effluent in terms of organic matter, suspended solids, and nutrients [2].

The physicochemical parameters of wastewater treatment, such as influent wastewater composition, substrate concentration, temperature, pH level, sludge age, sludge recycling ratio, hydraulic retention time (HRT), and oxygen concentration, affect the diversity of microbial species in activated sludge [3]. One of the crucial factors in the formation of a bacterial activated sludge biocenosis is medium composition, especially when the bioreactor is fed with real wastewater, owing to the variability of bacterial sensitivity to different components. A particular example of a real medium for bioreactor feeding is landfill leachate, which can be defined as a liquid originating from waste decomposition and rainwater infiltration in landfills. This mixture contains organic matter and nitrogen compounds. Its composition fluctuates as a result of the age of the landfill, waste composition, climate, and hydrogeological conditions [20]. The level of ammonia nitrogen in leachates can reach several thousand milligrams per liter [4].

Activated sludge, as well as other complex microbial biocenoses, has recently become a field of great interest in microbial ecology research. The information obtained from activated sludge ecological studies helps in understanding the relationships among different groups of bacteria in industrial ecosystems. One of the most significant microbial groups in activated sludge is ammonia-oxidizing bacteria (AOB), which performs the first step of nitrification, the ammonia-oxidizing process. This step of nitrification is often limiting, not only for the nitrification itself, but also for the total nitrogen cycle in a wastewater treatment plant [5]. Phylogenetically, ammonia oxidizers belong to Betaproteobacteria subdivided into two monophyletic groups, one with Nitrosomonas ssp. and Nitrosococcus mobilis, and the other with Nitrosospira ssp. [16].
AOB analysis is usually performed with the 16S rRNA gene as a universal molecular marker. There is a large sequence database for this molecule and it is still found to be the most suitable marker for the analysis of bacterial diversity and identification. AOB monitoring, especially in bioreactors with real wastewater, enables ecological characterization of an activated sludge biocenosis. From the technological point of view, it is important to estimate the level of AOB diversity and its linkage with nitrification effectiveness in order to facilitate the improvement of wastewater treatment.

Research on nitrifier composition and performance in activated sludge biocenoses has been undertaken widely and for a long time. Microbial communities in environmental samples have usually been described by traditional microbiological methods. It is now known that the earlier picture of microbial diversity was incomplete owing to the fact that only 0.1–10% of the bacteria derived from environmental samples are cultivable [19]. For the last few decades, the molecular approach in microbial research has brought insight into bacterial richness. Useful in such research are methods that do not require previous microbial cultivation. The most popular molecular techniques used in activated sludge bacteria research are denaturing gradient gel electrophoresis (DGGE) and fluorescent in situ hybridization (FISH). DGGE is based on the separation of DNA according to its sequence, not the size of the particle. The DGGE advantage lies in the possibility of excising a single DNA band from the gel and directly sequencing it to identify the dominant genotype. This means of analysis is important because the dominant bacteria are regarded as being primarily responsible for the effectiveness of wastewater treatment [21]. FISH enables the marking of a particular microbial group in a mixture with a fluorescently labeled oligonucleotide probe and visualizing it under the microscope [1].

The aim of this study was to estimate the influence of different sludge ages on AOB community changeability in MBRs using PCR-DGGE and FISH. The influence of landfill leachate medium on the AOB community was also analyzed.

### Materials and Methods

#### Bioreactor Characteristics and Analytical Procedure

The experiment was carried out over a period of 2 months after the acclimation period. Three lab-scale MBRs (volume of 36 L) inoculated with activated sludge from a municipal wastewater treatment plant in Zabrze (Poland) were subjected to the experiment. MBRs were equipped with a submerged membrane (Kubota System, A4) with a nominal pore size of 0.4 µm. Filtration was carried out in the constant flow of operation mode. The pH and dissolved oxygen concentration were maintained at 8.0–8.5 and 2.0–3.0 mg O₂/L, respectively. Hydraulic retention time was maintained at 3.2–3.8 days. Nitrogen compound concentrations were determined colorimetrically according to standard procedure [10].

Bioreactor A (MBRA), with a sludge age of 4 days, was fed with synthetic wastewater because of the fact that such a low sludge age combined with the presence of landfill leachate medium would disturb proper nitrification performance. Bioreactor B (MBRB), with a sludge age of 32 days, and bioreactor C (MBRC), with a sludge age of 8 days, were fed with landfill leachate medium from Gliwice landfill (Poland) containing a high concentration of ammonia nitrogen (>500 mg/L). Details regarding the membrane bioreactor operation in the experiment are presented in Table 1.

#### Activated Sludge Sample Preparation, DNA Extraction, and PCR Conditions

Activated sludge samples (volume of 10 ml) from the MBRs described above were collected at 15-day intervals, pelletted by centrifugation (5,000 × g, 10 min, 4°C), and stored at −20°C. Samples numbered 1–5 from the three MBRs were collected on days 1, 15, 30, 45, and 60 of the experiment.

Total genomic DNA was extracted from 0.2 g of the activated sludge samples using a Fast DNA Spin Kit for Soil (MP Biomedicals) 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.

In this study, nested PCR for 16S rRNA gene amplification was used. The first round of PCR was performed with CTO primers, which enabled a partial PCR amplification of the 16S rRNA bacterial gene belonging to ammonia-oxidizing β-Proteobacteria [11]. The PCR product was used as a template in the second cycle of nested-PCR, performed with 338F with a GC clamp and 518R, which amplified a partial (~180 bp) 16S rRNA gene fragment of all of the bacteria [15]. The PCR procedure was done as described previously [22].

#### Table 1. Characteristics of the membrane bioreactors used in the experiment.

<table>
<thead>
<tr>
<th>Technological parameter</th>
<th>MBRA</th>
<th>MBRB</th>
<th>MBRC</th>
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<tr>
<td>Feeding medium</td>
<td>Synthetic medium*</td>
<td>Landfill leachate</td>
<td>Landfill leachate</td>
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<tr>
<td>Sludge age [d]</td>
<td>4</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>Hydraulic retention time [d]</td>
<td>3.8</td>
<td>3.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Sludge concentration [g/L]</td>
<td>1.6</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.5–8.5</td>
<td>7.8</td>
<td>7.5–8.5</td>
</tr>
<tr>
<td>Ammonia nitrogen concentration [mg NH₄-N/L]</td>
<td>201.7–960.6</td>
<td>694.6–1,070.9</td>
<td>583.7–973.0</td>
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*Synthetic medium consisted of NH₄Cl, KH₂PO₄, and CH₃COONa.
DGGF Conditions and DNA Band Extraction
The PCR products obtained with primers 338F-GC and 518R were separated by DGGE using the Decode Universal Mutation Detection System (BioRad). A polyacrylamide gel (8% for 16S rRNA gene, 37:1 acrylamide–bisacrylamide; Fluka) with a gradient of 30–60% denaturant was prepared according to the manufacturer’s instructions. The gel was run for 15 h at 55 V in 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 Milli-Q water for 30 min and destained in Milli-Q water for 40 min, and then visualized under UV light and photographed using a Kodak 1D imaging system.

DNA bands that were well separated in a fingerprint were excised from the gel using a sterile blade and incubated for 3 h in Milli-Q water. The samples were centrifuged (12,000 × g, 1 min), the supernatant was removed and DNA bands were incubated with 40 μl of Milli-Q water at room temperature overnight.

Cloning and Sequencing of PCR Products and Dendrogram Construction
Before cloning, AT endings were added to the PCR products by incubating a 25 μl reaction mixture with 1.5 U GoTaq Flexi Polymerase (Promega) for 15 min at 72°C. PCR products were cloned using a TOPO Cloning Kit for Sequencing (Invitrogen) into the PCR 4-TOPO vector according to the manufacturer’s instructions and transformed into E. coli TOP10 (Invitrogen). Ampicillin-resistant positive clones carrying PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit ver. 1.1 (Applied Biosystems). Electrophoresis was performed in an ABI Prism 377 (Applied Bioscience).

The sequences obtained in the experiment were identified by comparison with sequences in GenBank of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). The GenBank accession numbers for the sequences determined in this study are GU380345–48 and GU380350–53.

Phylogenetic analysis and dendrogram construction were performed using Clustal X (Multiple Sequence Alignment ver. 2.0.5) on the basis of the neighbor-joining method [18].

Numerical Analysis of DGGE Fingerprints
The analysis of DGGE fingerprints was performed using a Kodak 1D image analysis system. Bacterial biodiversity was estimated on the basis of densitometric measurements, and the Shannon diversity index for the samples was calculated according to the equation

\[ H' = - \sum P_i \ln P_i \]

with

\[ P_i = \frac{n_i}{N} \]

where

\[ P_i \] is the relative probability of DNA band appearance in the fingerprint,
\[ n_i \] is the densitometrically measured intensity of the DNA band, and
\[ N \] is the amount of DNA bands in the fingerprint [14].

Fluorescent in Situ Hybridization (FISH)
Activated sludge samples no. 1–5 described above were fixed with paraformaldehyde solution (4% paraformaldehyde in phosphate buffered saline, 1× PBS, pH = 7.2) at 4°C for 3 h and washed subsequently with PBS. Fixed samples were kept in a PBS:ethanol (1:1) solution at −20°C. In situ hybridization was performed as previously described [6]. Fluorescently labeled oligonucleotides for the detection of the Nitrosomonas oligotropha lineage (Chasteria192), halophilic and halotolerant Nitrosomonas sp. (NEU), Nitrososarcina mobilis (Ncmob), and Nitrosospira sp. (Nsv443) were used in this study. The EUB338 (I, II, III) probe mix was used to detect eubacterial 16S rRNA [7].

RESULTS
Ammonia Nitrogen Removal Efficiency
In this experiment, three MBRs were operated aerobically for 2 months after the acclimation period. MBRA was treated as a control and was fed with synthetic medium containing 201.7–960.6 mg NH$_4^+$-N/L. During the entire length of the experiment, ammonia nitrogen removal was 99.9–100% (Fig. 1A). In this case, neither the high ammonia concentration in the influent, nor the low age of the activated sludge caused nitrification disturbance. MBBR was fed with landfill leachate medium containing 694.6–1,070.9 mg NH$_4^+$-N/L. The sludge age in MBBR was 32 days. Ammonia nitrogen removal was 96–98% (Fig. 1B) and only the second phase of nitrification was slightly disturbed. MBRC was also fed with landfill leachate medium containing 583.7–973.0 mg NH$_4^+$-N/L. The sludge age in MBRC was 8 days. Ammonia nitrogen removal was 40–50% and both nitrification stages were disturbed (Fig. 1C).

DGGE Fingerprinting of Activated Sludge Samples
The changeability of AOB in MBRA was estimated on the basis of fingerprint patterns obtained by nested PCR-DGGE. For PCR amplification of ~180 bp of the 16S rRNA gene, the universal primers 338F-GC and 518R were used. The results of the DGGE separation are shown at Fig. 2. DNA bands marked on the fingerprints, numbered 44, L50, and 55, were excised from the gel and their respective fragments were cloned and sequenced. For all of the MBRs in this experiment, only 9 of 20 excised and cloned fragments appeared to possess a unique DNA sequence. For MBRA, this type of sequence was represented by bands 55 and L50 (GU380345 and GU380346). DNA band L50 seems to have been dominant during the experiment. Interestingly, this sequence was also identified at two other levels of the gel. The same situation occurred with two other bands, designated 44 and 55. This discrepancy could be explained by problems with unambiguous identification of short (< 200 bp) sequences. AOBs present in MBRA also appeared in the experimental bioreactors: band L50 in MBRB and 44 in MBRC (Fig. 3 and 4).

To present the relationships among the AOB sequences obtained in this study and the other representative sequences
Fig. 1. Ammonia nitrogen removal in MBRA (A), MBRB (B), and MBRC (C), performed during entire length of the experiment.

Fig. 2. DGGE fingerprint of activated samples collected from MBRA. DNA bands excised from the gel and sequenced are indicated by the numbers 44, L50, and 55 (samples no. 1–5 taken at 15-day intervals from days 1–60 of the experiment).

Fig. 3. DGGE fingerprint of activated samples collected from MBRB. DNA bands excised from the gel and sequenced are indicated by the numbers L50, L52, and L61 (samples no. 1–5 taken at 15-day intervals from days 1–60 of the experiment).
of this group obtained from NCBI, a phylogenetic tree was created (Fig. 5). On the dendrogram, sequences from MBRA designated 55 and L50 are the closest relatives of uncultured Nitrosomonas ssp. and the uncultured ammonia-oxidizing bacterium AF510864, respectively. Interestingly, in MBRB, there seemed to be a correlation between decreasing ammonia oxidation effectiveness (Fig. 1B) and the fading and eventual disappearance in sample 5 (day 60) of the bacterial genotypes marked L50 and L52 (Fig. 3). In MBRB, three unique sequences were identified. Sequences L50 and L52 (GU380346 and GU380347) were located at the same level of the gel. In the dendrogram, they are clustered together as the most similar strains to the uncultured ammonia-oxidizing bacterium AF510864. Thus, the most possible explanation of such a situation is that the melting temperatures (or sequences) of the two DNA fragments are very similar, so that two bands are visible as one. The organism represented by DNA band L61 (GU380348) was a dominant AOB in MBRB. Its sequence is the most similar to that of the uncultured Nitrosomonas ssp.; they are located on the same branch of the dendrogram and are close together on the DGGE gel (Fig. 5).

Fig. 5 presents the dendrogram created with the 169 bp 16S rRNA gene sequences of the AOBs determined in this study and shows the relationships between the strains detected in the three MRBs and their closest relatives derived from GenBank. Four sequences, GU380346, GU380347, GU380350, and GU380353, are clustered together with that of the uncultured ammonia-oxidizing bacterium AF510864. This cluster is the closest to the N. mobilis branch. Three sequences, GU380345, GU380351, and GU380352, are clustered with the branch containing Nitrosomonas sp. AJ621032/uncultured Nitrosomonas sp. FN552770 and uncultured bacterium AB239544/uncultured bacterium AB264578. These sequences are probably the most similar to N. europaea. On the other hand, only one sequence, GU380348, belongs to the separate branch together with uncultured bacterium FM201188, and its sequence seems to resemble the Nitrosospira sp. AJ298729 partial sequence.

On the basis of the densitometric data obtained for all three MBRs, the Shannon biodiversity index was calculated. The calculations revealed that the highest level of ammonia oxidizer biodiversity was in MBRA, which was fed with synthetic medium and had the highest sludge concentration. In MBRB, which was fed with landfill leachate medium and activated sludge at an age of 32 days, biodiversity increased at the beginning of the experiment but decreased at the end, with no significant correlation with AOB diversity. In MBRC, with real medium and sludge at an age of 8 days, diversity increased more drastically than the increase in efficiency of ammonia nitrogen removal. It should be noted that, although the average effectiveness of ammonia oxidation was higher in MBRB than in MBRC, the slight improvement in MBRC performance at the end of the experiment was correlated with the increase in AOB biodiversity. Temporal changes in the diversity index were greater in the MBRs with landfill leachate than in the MBR with synthetic medium. A comparison of the Shannon biodiversity index for the three MBRs over the course of the experiment is shown in Fig. 6.

**FISH Analysis of Activated Sludge Samples**

FISH analysis of activated sludge samples was performed during the entire length of the experiment. No
representatives of *N. mobilis*-like species or *Nitrosospira* ssp. were present in the bioreactors during the experiment. Table 2 presents the results of the AOB monitoring performed during this experiment.

**DISCUSSION**

Activated sludge age is one of the crucial parameters in wastewater treatment and can be easily controlled. The results of sewage purification depend heavily on this parameter, so it is wise to find the most suitable sludge age in order to obtain the best quality of effluent. MBRs are found to be even more effective than CAS systems, not only in lab-scale experiments, owing to their small size and because of improved liquid/biosolid separation for better wastewater purification. The results obtained in this study confirm the previous research of Laitinen *et al.* [12] and Sadri *et al.* [17], which showed that MBRs are also suitable for landfill leachate treatment.

This medium contains a high concentration of ammonia nitrogen, causing problems in wastewater treatment plants during its treatment. In order to find the most suitable sludge age for effective landfill leachate treatment, we designed an experiment...
where two MBRs, MBRB and MBRC, were operated under different sludge ages (32 and 8 days, respectively) with landfill leachate. To analyze the influence of real and synthetic media on the activated sludge AOB community, the third bioreactor, MBRA, was operated with synthetic medium. On this basis, we could also compare the bacterial community changeability in bioreactors dealing with different wastewaters. MBRA, with a high ammonia nitrogen content and a sludge age of 4 days, was used to determine whether it is possible to obtain full nitrification under such conditions.

The effectiveness of ammonia nitrogen removal was lower in MBRC than in MBRB (Fig. 1B and 1C). These data suggest that a higher sludge age is more suitable for long-term removal of high concentrations of ammonia nitrogen. It is worth mentioning that ammonia removal, with its slight increase in MBR at the end of the experiment, was correlated with AOB biodiversity (Fig. 6). The higher total biodiversity in MBRB, with a sludge age of 32 days, can be explained by a higher possibility for slower growing bacteria to be maintained in the system and proliferate, and by the removal of a smaller volume of sludge from the bioreactor in order to maintain a constant sludge age. In both MBRB and MBRC, the bacterial community was exposed to a high concentration of ammonia nitrogen for a longer time, which leads to the separation of a highly specialized community. Moreover, exposure to other compounds in landfill leachate, such as heavy metals or organic/inorganic matter, can be detrimental to particular members of the biocenosis that are more sensitive to these compounds than the other bacteria. However, the HRT in the experiment was approx. 4 days and, as has been proved in previous research, an HRT of 3.5 days or longer is totally sufficient for toxin removal [17]; thus, the influence of toxic compounds on the activated sludge biocenosis should have been eliminated. There was no such influence of harmful substances in the synthetic medium in MBRA, so the Shannon biodiversity index in this bioreactor was the highest. The higher biodiversity could also depend on the sludge concentration in MBRA, which was higher than in the other MBRs in this experiment. Nevertheless, the experiment showed that medium content has a higher influence on the formation of the biocenosis than sludge age.

It is also worth mentioning that the appearance and disappearance of particular bands in the DGGE patterns were correlated with the efficiency of ammonia nitrogen removal. In the case of MBRB, bands L50 and L52 faded and gradually disappeared in correlation with a decrease in the efficiency of ammonia nitrogen removal (Fig. 1B and 3). In MBRC, the opposite situation occurred, in which the intensity of bands 79 and 80 became stronger while the efficiency of ammonia nitrogen removal increased (Fig. 1C and 4). These results are concordant with those of Li et al. [14] where, during steady-state operation of bioreactors, there was little change in the abundance of bacteria, although some bands showed differences in density.

Bacterial diversity research, especially that which is focused on ammonia oxidizers and is based on molecular analysis using 16S rRNA gene sequences, can have a significant impact on the technological and ecological understanding of the relationships between microorganisms in activated sludge [4]. In the case of AOBs, the level of ammonia nitrogen in the feeding medium is crucial for the formation of community structure and generation of diversity. As was stated previously, a high ammonia nitrogen concentration can limit the growth of bacteria that are sensitive to this factor [22], whereas the growth of

### Table 2. Presence of particular AOB species in activated sludge samples during the experiment (samples no. 1–5 taken at 15-day intervals from days 1–60 of the experiment).

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<td>MBRA</td>
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<td>2</td>
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<td>5</td>
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<tr>
<td>MBRB</td>
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<td>0</td>
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<td>2</td>
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<td>5</td>
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<td>MBRC</td>
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some other bacteria is enhanced by a high concentration of this compound [5]. In such an environment, *N. europaea*, *N. eutropha*, and *N. mobilis* are the most commonly present microorganisms, especially in engineered environments such as wastewater treatment systems. This is probably the reason why *Nitrosomonas*-like sequences appeared in all three MBRs. Interestingly, despite the high concentration of ammonia nitrogen in the bioreactors, no representatives of *N. mobilis*-like species were detected by FISH analysis, whereas the sequences obtained in the study with PCR-DGGE, namely GU380346, GU380347, GU380350, and GU380353, are clustered together near the *N. mobilis* AF037105 sequence.

According to the FISH results obtained in this study, *Nitrosospira* ssp. bacteria were also absent from the experimental MBRs. However, as in the case of *Nitrosomonas*-like sequences, the sequence of clone GU380348 seems to resemble that of *Nitrosospira* sp. AJ298729. This situation can be explained by the short sequence analysis (169 bp) used in the dendrogram construction, as well as the identification process; with such a short fragment of DNA, there is a higher probability of organism misidentification. However, DGGE separation of fragments larger than 300 bp is more difficult and requires the optimization of nearly every separation. Although DGGE seems to be more suitable for monitoring bacterial community changeability, FISH seems to be a more reliable technique for the identification of particular bacteria, owing to the use of an exact genus/species-targeted oligonucleotide probe. Nevertheless, the absence of some DNA sequences usually found in technological installations can also be caused by selective PCR amplification, because PCR primers are designed on the basis of cultivable bacterial DNA sequences [4, 19].

FISH analysis revealed that in the beginning of the experiment, *N. oligotropha* lineage bacteria were present in all MBRs. In MBRA and MBRB, such bacteria seemed to coexist with halophilic and halotolerant *Nitrosoomonas* ssp., whereas in MBRC we observed a change from *N. oligotropha* to halophilic and halotolerant *Nitrosoomonas* ssp. This switch could be correlated with the increase in the ammonia nitrogen concentration, but also with a lower sludge age, which supports fast growing and more resistant bacteria. The efficiency of ammonia nitrogen removal in this MBR increased, so probably a diverse group of *Nitrosoomonas*-like bacteria could have been responsible for this state.

The research performed on the three MBRs in this study revealed that landfill leachates are more effectively treated in MBRs with a higher sludge age (32 days) than with a lower one (8 days). The efficiency of this process was correlated with changes in the diversity of ammonia-oxidizing bacteria, but it did not depend on the total diversity of AOBs. The higher biodiversity occurred in the MBR with a sludge age of 32 days, but temporal changes in both MBRs fed with real wastewater were higher than in the MBR with synthetic medium, which was probably caused by the inflow of harmful compounds to the system. This observation leads to the conclusion that the biodiversity of activated sludge bacteria is generated much more strongly by the type of medium than by the sludge age.

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