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DIVERSITY AND CHANGEABILITY OF ACTIVATED SLUDGE BACTERIA IN TWO STAGE NITRITATION-ANAMMOX MEMBRANE BIOREACTOR TREATING COKE WASTEWATER

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Abstract

Coke is a highly combustible fuel derived from coal distillation, during which a large amount of wastewater, toxic to the environment, is produced. Except harmful compounds, the wastewater contains also high load of nitrogen so it seems to be interesting type of wastewater for Anammox (Anaerobic Ammonia Oxidation) – an ammonia nitrogen removal process cheaper and more effective than traditional combination of nitrification and denitrification. Anammox bacteria are present in technological systems and they are linked ecologically with the other prokaryotes, so the analysis on the bacterial communities performed in technological systems dedicated for Anammox bacteria is relevant. In this experiment we used two stage nitritation-Anammox MBRs treating synthetic coke wastewater. Thus, it is known that the wastewater treatment effectiveness depends on biodiversity level and activated sludge composition the aim of the work was to monitor the changeability and diversity of activated sludge biocenoses in both MBRs. The research revealed that the lab-scale activated sludge community composition differ from technological scale, which is linked with the bioreactors volume and type of feeding medium. GC-rich genotypes dominate in the system during adaptation phase in oxic MBR, while anoxic MBR biocenosis seemed to be less variable. Biodiversity index is slightly lower in oxic MBRA, probably due to its buffering role in the oxic-anoxic system, but it increased in the end of the experiment, most likely because of the nitrogen removal effectiveness increase. DNA fragment of 16S rRNA gene for precise identifications need to be at least 300 bp long.

Streszczenie

Koks jest wysokoenergetycznym paliwem, powstającym w procesach destylacji węgla kamiennego, podczas których produkowane są znaczne ilości wysoce niebezpiecznych dla środowiska ścieków. Poza substancjami niebezpiecznymi zawierają one również wysoki ładunek azotu, co powoduje, że są typem ścieków, w którym można wykorzystać proces Anammox (Anaerobic Ammonia Oxidation) – beztlenowe utlenianie amoniaku, tańsze i efektywniejsze od tradycyjnej kombinacji nitryfikacji i denitryfikacji. Proces ten prowadzony jest przez bakterie obecne w systemach oczyszczania ścieków w ścisłym powiązaniu z innymi bakteriami osad czynnego, stąd analizy prowadzone na biocenozach bakteryjnych w układach technologicznych, w których wypracowuje się Anammox wydają się być słuszne. W tym eksperymencie wykorzystano dwustopniowy układ membranowy, pracujący w systemie skróconej nitryfikacji-Anammox, oczyszczający syntetyczne ścieki koksownicze. Ponieważ wiadomo, że efektywność oczyszczania ścieków w dużej mierze zależy od różnorodności biocenozy osadu czynnego, celem niniejszej pracy był monitoring zmienności i różnorodności zbiorowisk bakteryjnych dwóch reaktorów membranowych w tym układzie. Stwierdzono, że skład jakościowy biocenozy reaktorów laboratoryjnych różni się znacznie od składu biocenozy technologicznej, co zależy od objętości reaktorów i składu pożywki zasilającej. Bogate w pary GC genotypy dominują w reaktorze tlenowym w fazie adaptacji, a sama biocenoza jest bardziej zmienna, niż ta w reaktorze anoksycznym. Poziom bioróżnorodności jest nieco niższy w warunkach tlenowych, prawdopodobnie ze względu na zastosowanie reaktora tlenowego jako swoistego buforu chroniącego reaktor anoksyczny, w którym docelowo miała być wpracowana biocenoza Anammox. Wzrost bioróżnorodności w reaktorze tlenowym na końcu eksperymentu jest związany w głównej mierze ze wzrostem efektywności jego pracy. Wykazano, że do identyfikacji genotypów dominujących niezbędne są fragmenty genu kodującego 16S rRNA o długości przynajmniej 300 pz.

Keywords: **Anammox bacteria; Bacterial diversity; MBRs; Nitritation-Anammox; PCR-DGGE.**

1. INTRODUCTION

Coke is the solid carbonaceous material derived from destructive distillation of coal. It possesses more combustible energy than coal and it is more often used in heavy industry production. During the coal distillation a large amount of wastewater, toxic to the environment, is produced. Coke wastewater contains high load of nitrogen, thus it seems to be interesting material for Anammox (Anaerobic Ammonia Oxidation) usage. Anammox is cheaper and more effective than traditional combination of nitrification and denitrification [1]. Anammox process is based on the oxidation of ammonia to gaseous nitrogen. It was predicted in thermodynamic calculations for 1970s [2], but the research in this field have been developed since 1990s, when Anammox bacteria were discovered. This group of *Procaryota* is impossible to analyze with traditional cultivation methods that is why only culture-independent methods, such as molecular biology tools, enables wide Anammox bacteria research. Although Anammox process is economically beneficial, it has also disadvantages. Anammox bacteria grow slowly (doubling time is ca. 11 days [3]) and they are very sensitive towards unfavorable external conditions. Thus, Anammox is a biological process in which coke wastewater cannot be treated directly, because coke wastewater, except a large amount of nitrogen and barely no phosphorus, also contains phenols, cyanates, thiocyanates, suphates, PAHs and heavy metals. Such composition made them harmful to activated sludge biocenosis, especially to the Anammox bacteria [4]. Table 1 presents short coke wastewater characteristics.

Table 1.
Coke wastewater characteristics [5]

measurement	Unit	value
temperature	[°C]	36
pH	-	7.5-9.1
volatile phenols	[g/m^3]	260-3000
volatile ammonia calculated as NH_4^+	[g/m^3]	110-900
total ammonia calculated as NH_4^+	[g/m^3]	980-6500
thiocyanides	g/m^3]	100-1500
cyanides	[g/m^3]	10-100
calculated as H_2S	[g/m^3]	10-600
chlorides	[g/m^3]	1640
sulphates	[g/m^3]	1480
thiosulphates	[g/m^3]	290
oils and pitch	[g/m^3]	100-240
oxidizability	[g/m^3]	2500-10 000

To prevent activated sludge bacteria from harmful conditions of coke wastewater two-stage MBR system with nitritation-Anammox was constructed. In the first part of the experiment, described in this article, artificial coke wastewater was used. In the second part of the experiment real coke wastewater is planned to be used.

To monitor the changes in activated sludge community one of the most useful molecular methods – PCR-DGGE (Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis) – was used. This tool is a combination of PCR amplification on total DNA isolated from the sample and the electrophoretic separation of PCR products in polyacrylamide gel, containing an increasing gradient in denaturant (urea). The PCR amplicons separation is based not on the molecule size, but on DNA melting temperature (depending on GC content). Thus the PCR products, the same in size, but different on sequence, can be separated into a fingerprint, comparable with the others, presenting the genetic community structure. Moreover, the dominant DNA bands can be also excised from the gel and identified with sequencing.

The aim of this study was to estimate the diversity and changeability of activated sludge bacteria in two stage MBR system with nitritation-Anammox, treating synthetic coke wastewater. The bacterial community monitoring was performed with PCR-DGGE and the bacteria dominant in the system were tried to be identify with DNA sequencing.

2. MATERIALS AND METHODS

2.1. Operational data

For purpose of the study, two membrane bioreactors (MBRA and MBRAB) were started up. Both bioreactors were equipped with flat sheet submerged membrane units (Kubota) of nominal pore size 0.4 μm and membrane areas of 0.1 m^2. They were fed with synthetic wastewater, which was designed to achieve a realistic COD:TN:TP ratio as coke wastewater. Active volume of the bioreactors was equal to 35 L (MBRA) and 45 L (MBRB). MBRA was operated as an aerobic completely mixed reactor at an organic loading rate of 0.07 g COD/g/day and with hydraulic retention time of 72 hours, while MBR B was operated as an anaerobic completely mixed reactor at an organic loading rate of 0.97 g COD/g/day, and a hydraulic retention time of 96 hours. The MBRs system scheme is presented at the Figure 1 and its parameters in Table 2.

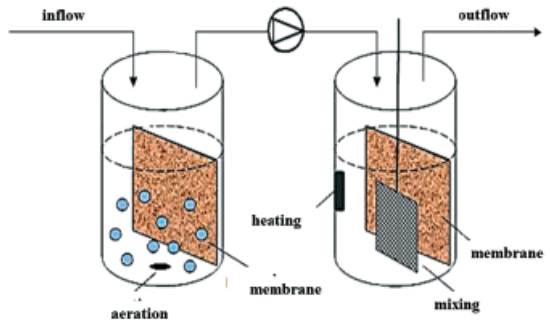

Figure 1.
Two stage oxic – anoxic MBR system scheme

Table 2.
Technological parameters of two stage oxic-anoxic MBR system used in the experiment

parameter	oxic MBRA	anoxic MBRB
volume [m^3]	0.035	0.045
Retention time [d]	3.0	4.0
flow [m^3/d]	0.011	0.01
Membrane working surface [m^3]	0.1	0.1
Oxygen concetration [g/m^3]	0.5-2.5	0
temperature [°C]	15-20	30-36
pH	8.7	8.1

2.2. Activated sludge sampling, DNA isolation and PCR conditions

Activated sludge samples (volume of 10 ml) were collected from both MBRs at 4-week interval, pelleted by centrifugation (5 000 × g, 10 min, 4°C) and stored at -45°C until DNA isolation. Total genomic DNA was extracted from 0.2 g of the activated sludge samples using mechanical method. The samples were washed three times with 1 × PBS buffer (Sigma) and disintegrated with bead beating (Roth, Germany) in lysis buffer (Tris-HCl 100 mM, EDTA 100 mM, NaCl 1.5 M; pH = 8.0). The samples were incubated 20 minutes in 1400 rpm and 200 µl 10% SDS was added. After 30 minutes of incubation at 65°C samples were centrifuged twice at 13 000 rpm and placed on spin filters (A&A Biotechnology). DNA attached to the filter was washed twice with A1 solution (A&A Biotechnology). The amount of DNA was measured spectrophotometrically using Qubit (Invitrogen) and stored at -20°C until PCR amplification.

Partial 16S rRNA gene amplification of all the bacteria was performed with a set of primers: 338f with a GC clamp and 518r, which amplified a partial (ca. 180 bp) 16S rRNA gene fragment [6]. PCR procedure was described previously [7].

2.3. Denaturing gradient gel electrophoresis conditions and results analysis

The DGGE of the PCR products obtained in reaction with 338F-GC/518R primers underwent electrophoretic separation in the Dcode Universal Mutation Detection System (BioRad). 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 instruction. The gel was run for 10 h at 70 V. DGGE tank was filled with 1 × TAE buffer 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 distained in MiliQ water for 40 min, then visualized under UV light and photographed using Quantity One 1D (BioRad).

The analysis of DGGE fingerprints was performed using a Quantity One 1D software (BioRad). Bacterial biodiversity was estimated on the basis of densitometric measurements and Shannon diversity index for the samples was calculated, according to the equation (1):

$$H' = -\sum P_i \ln P_i \qquad (1)$$

with:

$P_i = n_i / N_i$

where: P_i = relative probability of DNA band appearance in the fingerprint, N_i = amount of DNA bands in the fingerprint, n_i = densitometrically measured intensity of DNA band.

2.4. DNA sequencing and bacterial identification

Well separated and strong DNA bands were excised from DGGE gel with sterile blade. Sterile MiliQ water in volume of 200 µl was added to the bands and the probes were incubated for 30 minutes at room temperature. After the incubation the water was poured out and 30 µl of sterile MiliQ water was added again to the probes. The excised bands were crushed and frozen in -20°C until reamplification.

The 1 µl DNA eluted from the DNA bands was used as a template for PCR reamplification. The PCR program consisted of the same steps as the previous one but the number of the cycles was lower (25 cycles). The PCR products were purified from the PCR reaction residues with Clean-Up Kit (A&A Biotechnology) and underwent DNA sequencing using ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

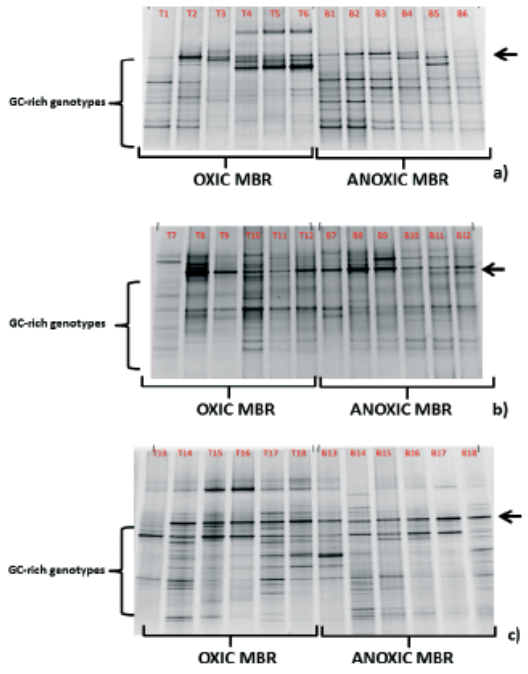

Figure 2.
DGGE fingerprints of the activated sludge biocenoses in oxic-anoxic MBR system monitored during 18 month experiment; samples T – oxic MBR, B – anoxic MBR; a-c – consecutive parts of the monitoring

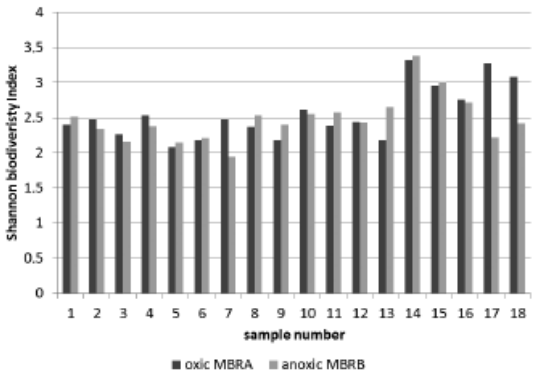

Figure 3.
Shannon biodiversity index changes calculated on DGGE fingerprints of the activated sludge biocenoses in oxic-anoxic MBR system during 18 month experiment for 18 months of the experiment

3. RESULTS

The experiment was performed for 18 months on synthetic coke wastewater in two stage nitritation-Anammox MBR system. Activated sludge samples were collected monthly from both bioreactors and the constant monitoring of activated sludge biocenoses was performed with PCR-DGGE.

Several dominant DNA bands were excised from the gel and underwent reamplification and sequencing (data not shown). The results were compared with National Center for Biotechnology Information (NCBI) Genbank with BLAST tool. None of the sequenced DNA fragments was possible to be identified, probably because the database resource increased heavily during last few years and the sequences length of 180 bp are too short to be properly aligned.

As it can be seen in Figure 2 a-c the samples from oxic MBRA (samples 1-18 T) present higher changeability in the fingerprint pattern mainly in the first 13 sampling times (13 months of the experiment). This changeability has a reflection in biodiversity index (Figure 3) which is fluctuating for samples 1-13 with a major increase for sample 14. The biodiversity increased from 2.18 (for sample T13) to 3.32 (for sample T14). Such an increase is relatively high in ecological standards. Biodiversity changes in anoxic MBRB are lower in comparison with MBRA, with a major rise also in samples B13/B14. Interestingly, the biodiversity in MBRB decreased after 14th sampling time, while in MBRA the increase was maintained.

4. DISCUSSION

The MBR system was inoculated with activated sludge from "Jadwiga" Coke Plant in Zabrze, Poland and it was adapted to the lab-scale MBR system feed with synthetic medium. The bacterial monitoring was performed constantly for 18 months of the experiment. It was observed that in the beginning of the experiment biocenoses of both bioreactors were highly variable (with MBRA more changeable, than MBRB), but after the period of 17 months communities structure become similar, but not identical, in both MBRs to the structure from the beginning of the experiment. Such results suggest that for the lab-scale activated sludge different genotypes are dominant than for the technological scale. The qualitative content of activated sludge is modified by the volume of the bioreactors and the type of the feeding medium. It is worth mentioning, that in the beginning of the adaptation phase GC-low genotypes (upper parts of the gels, Figure 2 a-c) were dominant in the system in oxic MBRA, while in anoxic MBRB the genotype structure of the biocenosis was similar to inoculum, with most of the genotypes in the lower part of the gel (GC-rich). It was probably caused by the sort of the system used, while for oxic MBRA was fed with synthetic medium with phenol, which is more harmful

for the activated sludge bacteria and causes higher selective pressure towards bacterial community, than the outflow from MBRA which was partially treated and directed to anoxic MBRB. The oxic MBRA was a particular type of buffer for MBRB's activated sludge. It is also interesting that in both MBRs several genotypes (Fig. 2, arrow) are located at the same level in the gel, thus probably some bacteria in activated sludge can be present in both bioreactors, regardless of the sort of feeding medium or oxic conditions. As it can be seen in Figure 2a some of the genotypes insensitive towards the oxic conditions were present in the inoculum and became stronger during the experiment probably because of the preferable conditions in the MBRs.

To identify the dominant genotypes the DNA bands were excised from the gel and underwent sequencing, but it appeared that 180 pb long sequence is too short for precise identification. NCBI GenBank data supplies increased in the last few years, moreover the sequence between primers 338f and 518r is highly variable, and thus it is clear that for the identification of bacterial 16S rRNA gene fragments longer than 200 bp are required.

Shannon biodiversity index was lower for oxic MBRA than in anoxic MBRB, and the fluctuations of the biodiversity are more visible for first 13 sampling times (Figure 3). This supports the thesis of oxic MBRA usage as a buffer for this technological system. Biodiversity increases in the end of the experiment in both MBRs but in MBRA the level of biodiversity was higher and this level was relatively constant, probably because of the increase of nitrogen removal effectiveness. As it was previously stated [8] those well performing bioreactors present high bacterial biodiversity. In anoxic MBRB biodiversity after its increase in 13th month of the experiment started to decrease, probably due to the fact that MBRB performance was far lower (data not shown). It should be stated that although the biodiversity level seems to be relatively constant for both MBRs (Figure 3), the biocenoses remodeled during the experiment, as it can be seen in Figure 2a-c. This underlines the necessity of both, biodiversity measurements together with fingerprints changeability monitoring.

5. CONCLUSION

It can be stated that in the lab-scale MBR system different bacterial genotypes are dominant in activated sludge, than in technological scale, which is related to the bioreactors volume and type of feeding medium.

GC-rich genotypes dominate in the system during adaptation phase in oxic MBRA, while in anoxic MBRB biocenosis seemed to be similar in its structure to inoculum. There are dominant genotypes at the same level in the gel for both MBRs which suggest the presence of bacteria insensitive towards medium type and oxic conditions, probably present in the inoculum. It was impossible to identify them with DNA sequencing because of too short and too variable DNA fragment of 16S rRNA gene used. DNA sequences for precise identifications need to be at least 300 bp long.

Biodiversity index is a bit lower in oxic MBRA, probably due to its buffering role in the oxic-anoxic system but community diversity increased in the end of the experiment probably because of the nitrogen removal effectiveness increase related to the increase of the level of more active genotypes.

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