DGGE-BASED MONITORING OF BACTERIAL DIVERSITY IN ACTIVATED SLUDGE DEALING WITH WASTEWATER CONTAMINATED BY ORGANIC PETROLEUM COMPOUNDS

ALEKSANDRA ZIEMBIŃSKA*[1], SŁAWOMIR CIESIELSKI[2], JAROSŁAW WISZNIOWSKI[1]

[1] The Silesian University of Technology, Environmental Biotechnology Department, Akademicka str. 2, 44-100, Gliwice, Poland
[2] University of Warmia and Mazury in Olsztyn, Department of Environmental Biotechnology, Słoneczna str. 45G, 10-719, Olsztyn, Poland
* Corresponding author e-mail: aleksandra.ziembinska@polsl.pl

Abstract: Polycyclic aromatic hydrocarbons (PAHs) belong to the group of recalcitrants that on reaching wastewater can irreversibly inhibit some sensitive biological processes in activated sludge such as nitrification. This situation leads to wastewater treatment failure due to the influence of these substances on bacteria responsible for important biochemical processes. Observation of the changes in bacterial diversity using molecular tools, such as denaturing gradient gel electrophoresis (DGGE), could be the first step in finding a way of preventing wastewater treatment failure. The aim of this experiment was to monitor bacterial biodiversity in a membrane bioreactor (MBR) dealing with synthetic wastewater contaminated with high concentration of petroleum organic compounds (POCs) and to study the influence of POCs contamination on bacterial changeability in activated sludge. COD removal in investigated membrane bioreactors was at a level of 93%. The organics removal efficiency was not affected by the maximal tested dose of petroleum contamination (1000 μl POCs/l of wastewater) and the MBRs wastewater treatment performance was undisturbed. DGGE analysis revealed that the biodiversity fluctuated slightly in control MBR, while in experimental MBR the biodiversity index decreased drastically after adding the highest experimental concentration of POCs. These results suggest that concentrations of POCs at levels from 50 μl/l to 500 μl/l stimulate biodiversity growth, while the concentration 1000 μl POCs/l of wastewater seems to inhibit the most sensitive processes in wastewater treatment by influencing the bacterial biocenosis.

INTRODUCTION

It is common knowledge that the increasing environmental pollution caused by the inappropriate treatment of some industrial wastewater has a dramatic influence on wastewater composition. There is a wide group of chemicals which on reaching a wastewater treatment plant may inhibit sensitive biological processes such as nitrification. The consequence of such a situation is the treatment failure. Polycyclic aromatic hydrocarbons (PAHs) belong to this group. Some of them can be mutagenic or potentially carcinogenic, while all of them can be dangerous to human health and aquatic life [1, 7,

14]. PAHs are present in petroleum products as petroleum organic compounds (POCs) and this is the reason why the dramatic development of industries such as petrochemistry, transportation and car exploitation in recent years may be one of the main sources of these substances in WWTPs (wastewater treatment plants).

For the past several years membrane bioreactors (MBRs) come to be considered an excellent solution for wastewater treatment for several reasons: their smaller size, which is also suitable for lab-scale experiments, and in most cases an effluent of much better quality than conventional systems in terms of organic matter, suspended solids, and nutrients [4, 8].

The classic microbiological approach fails in the studies of activated sludge microbial diversity mainly because more than 90% of microorganisms present in bioreactors are uncultivable [11]. The only solutions for such experiments are molecular biology tools. One of the most useful methods to study bacterial diversity in complex communities is denaturing gradient gel electrophoresis (DGGE). DGGE allows for the electrophoretic separation of PCR products amplified on DNA derived from a microbial mixture (e.g. activated sludge) [2, 16]. In this study the 16S rRNA coding gene was used due to the fact that it is known to be universal bacterial molecular marker [9]. The diversity of bacteria in complex microbial communities can be estimated on the basis of DNA fingerprints obtained in DGGE, where the Shannon diversity index [H] as an estimation of species richness is calculated [13].

The aim of the study was to monitor the bacterial diversity changes in lab-scale membrane bioreactor (MBR) dealing with wastewater containing a high level of petroleum organic compounds (POCs) using denaturing gradient gel electrophoresis. An investigation of the influence of the POCs on bacterial changeability in activated sludge in an MBR dealing with artificial municipal wastewater was also undertaken.

MATERIALS AND METHODS

Experiment conditions
The experiment was carried out for 150 days. It was performed in two lab-scale membrane bioreactors each with a volume of 10.5 l supplied with a Kubota A-4 size membrane. The bioreactors were operated aerobically and inoculated with activated sludge

Table 1. The composition of the synthetic medium for MBRs

Component	MBR A	MBR B
Dry meat extract (mg/l)	80	80
CH_3COONa (mg/l)	700–1300	700–1300
Yeast extract (mg/l)	10	10
NH_4Cl (mg/l)	200–250	200–250
K_2HPO_4 (mg/l)	27	27
KH_2PO_4 (mg/l)	10	10
$MgSO_4 \times 7H_2O$ (mg/l)	15	15
Tween 80 (µl/l)	5-15	5-15
P-30 fraction (µl/l)	0	0–1000

derived from a municipal WWTP in Zabrze (Poland). Bioreactor A (control) and B (containing an increasing concentration of POCs) were fed with a synthetic medium (Tab. 1).

The concentration of petroleum organic compounds in the form of a P-30 distillate of crude oil (Oil Refinery, Poland) gradually increased during the experiment in bioreactor B at a rate of 50–1000 µl POCs/l of wastewater. POCs were added to the feeding medium of bioreactor B at 4-week intervals as a mixture emulsified with Tween-80 after the 60 day acclimation period. Nitrogen compound concentrations were determined colorimetrically: ammonia with a Nessler reagent, nitrite with an alfanaftyloamine reagent and nitrate with a dimethylphenol reagent; COD was measured as described previously [15].

Activated sludge samples analysis
Activated sludge samples (volume of 50 ml) were collected at 2-week intervals and stored at -20°C until the DNA extraction. Total bacterial DNA was isolated using a Fast DNA Spin Kit for Soil (MP Biomedicals) according to manufacturer's instructions. PCR reaction was performed using standard primers: 338f-GC (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3') and 518r (5' ATT ACC GCG GCT GCT GG 3') amplifying 180 bp of bacterial 16S rRNA gene [12] as described previously [18].

PCR products were separated using a Dcode Universal Mutation Detection System (BioRad) in polyacrylamide gel (8%, 37:1 acrylamide-bisacrylamide, Fluka) with a 30-60% gradient of denaturant (urea) prepared according to the manufacturer's instructions. The gel was run for 9 h at 60 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 distained in MiliQ water for 40 min, and then visualized under UV light and photographed using the Gel Doc System (BioRad).

Densitometric analysis of DGGE fingerprints was performed using Quantity One 1-D Software (BioRad) and the Shannon diversity index [H] was calculated for all of the samples [13].

RESULTS AND DISCUSSION

COD removal in bioreactors A and B (Fig. 1A, C) during the total length of experiment was at a level of 93% and 99%, respectively.

The organics removal efficiency was not affected by the maximal tested dose of petroleum contamination reaching 1000 µl POCs/l of wastewater and both the control and experimental MBRs wastewater treatment performance was undisturbed. The ammonia removal in control bioreactor A revealed no disturbances during the experiment (Fig. 1B), while bioreactor B was influenced by petroleum contaminants at the end of the experiment (Fig. 1D). An inhibition of nitrification was observed when contamination was at a level reaching 1000 µl POCs/l of wastewater. The results of COD and NH_4^+ removal in the activated sludge sampling times are shown in Fig. 1A, B for bioreactor A and in Fig. 1C, D for bioreactor B.

The results of the Shannon biodiversity index measurements are shown in Fig. 2A for bioreactor A, and in Fig. 2B for bioreactor B.
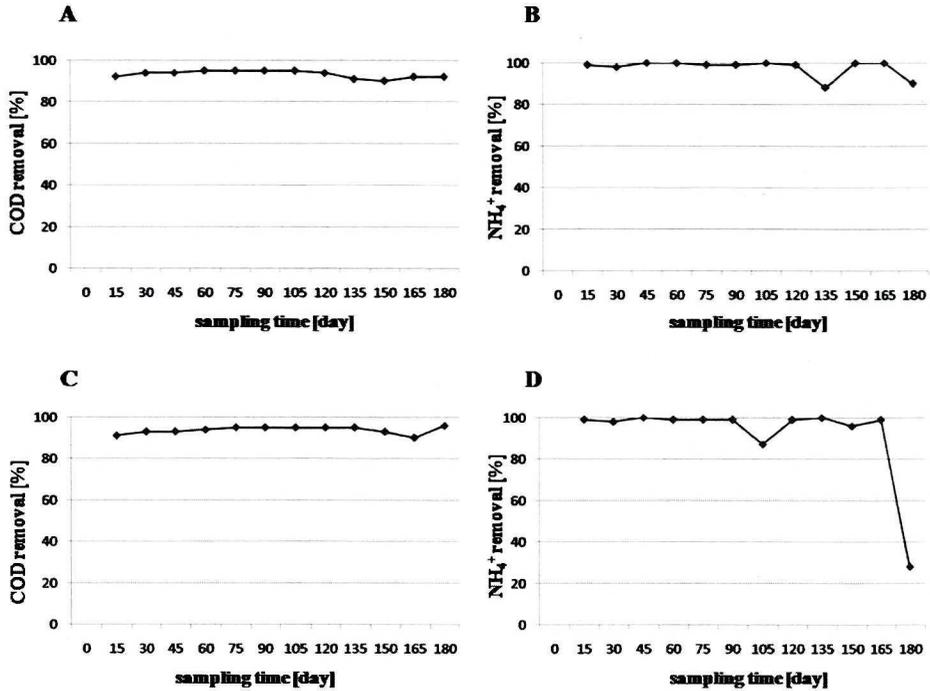
Fig. 1. COD and NH_4^+ removal for bioreactor MBRA (A, B) and MBRB (C, D); 0 day sampling time refers to inoculum activated sludge.
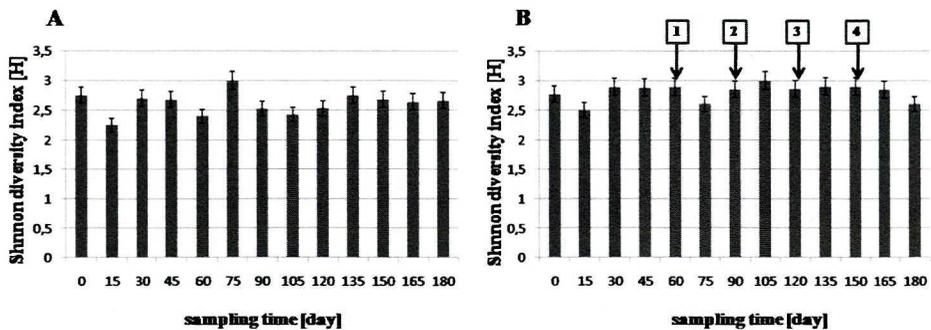

Fig. 2. Shannon diversity index changes [H] for bacteria in MBRA (A) and MBRB (B) during the total length of the experiment; 0 day sampling time refers to inoculum activated sludge; numbers 1-4 indicate increasing doses of POC contamination in wastewater: 50 μl/l, 200 μl/l, 500 μl/l and 1000 μl/l, respectively.

DGGE analysis revealed that the biodiversity of the total number of bacteria in the seeding activated sludge samples (inoculum) for both bioreactors was at the same level. During the total length of the experiment the biodiversity fluctuated slightly in bioreactor A, while in bioreactor B the biodiversity index decreased drastically at the end of the

experiment after adding the highest experimental concentration of POCs (1000 µl POCs/l of wastewater). DGGE fingerprint analysis showed that the addition of increasing doses of POCs to wastewater did not affect the total bacterial diversity of the activated sludge until the addition of the highest concentration of POCs. The biodiversity decreased within the first two weeks of the acclimation period in both MBRs – 0.5 for bioreactor A and 0.3 for bioreactor B (Fig. 2, sampling time 0–15th days). Such a situation is natural in the case of adaptation to the new environment. A drastic change in the bioreactor's volume from a high volume of the WWTP bioreactor to a smaller one – 10.5 l resulted in lowering the level of biodiversity. A smaller bioreactor possesses fewer ecological niches for potential inhabitants than a bigger one. Moreover, the activated sludge in the MBR system was only operated under aerated conditions (dissolved oxygen level above 2.0 mg/l), while in the WWTP bioreactors underwent variable: anaerobic-anoxic and aerobic conditions. For MBR B a slight decrease in biodiversity appeared after adding the first dose of POCs – 50 µl/l in the 60th day of the experiment. The POCs dosage was the stress factor in the system, so the decrease in biodiversity was also caused by the adaptation to the new conditions. For the period when increasing doses of POCs were added to MBR B (60th–150th days of the experiment) no significant changes in the level of biodiversity were observed. This situation can be explained in two ways. Firstly, the level of POCs harmfulness for the activated sludge in this system was at the point of 1000 µl/l of wastewater so the microorganisms did not respond to lower doses of the chemicals until reaching the threshold of the negative influence of the POCs. Secondly, there is also possibility that the high concentrations of colloidal and soluble organic particles, such as extracellular polymeric substances (EPS) [3] and surfactants [5], in the activated sludge flocs protect bacteria against the harmful influence of POCs by forming soluble complexes [17] or storing POCs until the level of recalcitrants reached the threshold that was damaging for activated sludge microorganisms. It cannot be excluded that the POC contaminants could have a stronger influence on the biocenosis' changeability, as well as wastewater treatment performance, that may appear after a longer time of exposition exceeding the length of this experiment.

From the results obtained in this study, it can be concluded that concentrations of POCs at levels from 50 µl/l to 500 µl/l seem to stimulate biodiversity growth. It had been proven before that aromatic compounds can serve as a carbon source for several bacterial strains in soil [6] where the degradation of POCs is connected with solubility in water. In WWTP the fraction that it is possible for bacteria to use seems to be higher. So it cannot be excluded that petroleum organic compounds could be an additional carbon source for activated sludge bacteria in this range of concentrations, while a dose of 1000 µl/l of wastewater seems to be the concentration that inhibits the most sensitive processes in wastewater treatment. The results mentioned above can suggest that nitrification may be the first process to be disturbed by a high level of POCs in wastewater treatment. It is possible that nitrifiers would be the first group of microorganisms removed from the system, which would cause a decrease in the biodiversity. This statement requires further research because it has been stated that nitrification is more sensitive to temperature fluctuation than to the influence of recalcitrants due to the presence of flocculating compounds [10].

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MONITORING RÓŻNORODNOŚCI BAKTERYJNEJ W OSADZIE CZYNNYM OCZYSZCZAJĄCYM ŚCIEKI ZANIECZYSZCZONE ORGANICZNYMI ZWIĄZKAMI ROPOPOCHODNYMI Z UŻYCIEM DGGE

Substancje ropopochodne należą do grupy zanieczyszczeń, które, docierając do oczyszczalni ścieków, mogą zaburzać wrażliwe procesy biochemiczne, takie jak nitryfikacja. Taka sytuacja prowadzi do problemów z oczyszczaniem ścieków, ponieważ substancje te mogą wpływać na bakterie odpowiedzialne za podstawowe procesy biochemiczne. Obserwacja zmian różnorodności bakteryjnej w osadzie czynnym za pomocą narzędzi biologii molekularnej, takich jak elektroforeza w gradiencie denaturacji (DGGE), może być pierwszym krokiem w opracowaniu metody zapobiegania zaburzeniom procesu oczyszczania. Celem tej pracy był monitoring różnorodności bakteryjnej w osadzie czynnym bioreaktora membranowego usuwającego ścieki zanieczyszczone związkami ropopochodnymi i określenie wpływu tych związków na zmienność bakteryjną badanej biocenozy. Usunięcie związków organicznych wyrażonych wskaźnikiem zanieczyszczeń ChZT zarówno w kontrolnym, jak i eksperymentalnym bioreaktorze membranowym, wynosiło powyżej 93%. Najwyższe zastosowane stężenie związku zanieczyszczającego (frakcja P-30, próżniowy destylat ropy naftowej) wynosiło 1000 µl/l ścieków i nie miało wpływu na efektywność oczyszczania ścieków. Analiza wzorów prążkowych DGGE wykazała, że bioróżnorodność bakteryjna w bioreaktorze kontrolnym zmieniała się nieznacznie w trakcie trwania eksperymentu, podczas gdy w bioreaktorze eksperymentalnym spadła drastycznie po dodaniu dawki zanieczyszczającej o najwyższym stężeniu. Wyniki te sugerują, że stężenia modelowych związków ropopochodnych w zakresie 50 µl/l to 500 µl/l stymulują wzrost bioróżnorodności, podczas gdy zastosowanie zanieczyszczenia w stężeniu 1000 µl/l ścieków prawdopodobnie hamuje najbardziej wrażliwe procesy oczyszczania ścieków poprzez wpływ na biocenozę bakteryjną.