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MONITORING OF BACTERIAL BIODIVERSITY IN ANAEROBIC MEMBRANE BIOREACTORS (AnMBRs) DEALING WITH WASTEWATER CONTAINING X-RAY CONTRAST MEDIA COMPOUNDS

Iodinated contrast media compounds (ICM) have been identified in wastewater within the last 20 years. In this study, the biodiversity of activated sludge in anaerobic membrane bioreactors dealing with synthetic hospital wastewater with addition of ICM was investigated, using fluorescent *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE). During the adaptation of microorganisms to anaerobic growth conditions and to ICM presence, differences in the content of *Alpha*- and *Betaproteobacteria* were noted and bioreactors showed higher biological diversity (H = 2.9), suggesting that ICM were not toxic to the bacteria. The long sludge age had the strongest influence on the composition of activated sludge biocenosis.

1. INTRODUCTION

Many pharmaceuticals such as iodinated contrast media compounds (ICM), lipid regulators, antiphlogistics, β-blockers, antiepileptics, antibiotics, and illicit drugs have been detected in the aquatic environment within the last 20 years [1]. Studies have shown that unmetabolised drugs (and the products of their metabolic transformations) enter the municipal sewage system and then are directed to the wastewater treatment plant. Treatment processes in the plant fail to completely eliminate drugs from wastewater. In consequence, drugs, together with treated wastewater, are released into surface waters [2].

Although the concentration of pharmaceutical compounds encountered in most aquatic environments currently are at very low levels (from ng/dm^3 to µg/dm^3), their

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continuous input may constitute a long-term potential risk for aquatic and terrestrial organisms, including human beings [3].

Iodinated X-ray contrast media are the most widely used pharmaceutical compounds for imaging soft tissues, internal organs, and blood vessels and can be administered to humans at doses up to 300 g per diagnostic session [4]. The annual worldwide consumption of ICM is approximately 3.5×10^6 kg [5]. They are of low human toxicity and are hardly metabolized. ICM are excreted mostly unchanged within 24 h (>95%) [6]. Most of ICM are derivatives of 2,4,6-triiodobenzoic acid with polar carboxyl and hydroxyl moieties in their chains. Some are ionic – have one or several free carboxyl groups, others are amide derivatives and, as such, are neutral compounds.

Based on our research, we can state that specific contrast agents can be found in wastewater in Poland in the concentrations of up to 30 $\mu g/dm^3$ [7]. A higher concentration (1 mg/dm^3) was determined for hospital wastewater [8] and Gartiser et al. [9] reported that X-ray contrast media and their metabolites contain organic bound halogens and therefore contribute to adsorbable organic halides (AOX).

The elimination of ICM from wastewater using biological processes of activated sludge proved to be a major challenge regardless of the used technology [10]. In conventional activated sludge processes (CASP), ICM removal efficiency was estimated to 40%. The highest removal efficiency (60%) was observed for membrane bioreactors [10]. These reactors have become very commonly used due to their advantages over settler-operated systems. The membranes are able to retain particles of different sizes, depending on the membrane type within the reactor [11]. Application of membranes in activated sludge systems enables a much more effective separation of solids from wastewater. It should be noted that elimination of contrast media in anaerobic conditions is more economically attractive than the aerobic processes, mainly due to the cost of aeration and disposal of excess sludge.

Transformation of ICM has been investigated in sludge systems, as well as in sediment-water systems; however the results have been inconsistent [4, 6, 12, 13]. In addition to the lack of consensus concerning the biotransformation of iodinated X-ray contrast media and characterization of microbial transformation pathways, there is also a scarcity of information about changes of a bacterial community structure in activated sludge induced by synthetic hospital wastewater containing ICM.

The aim of the study was to estimate biodiversity of activated sludge in anaerobic membrane bioreactors dealing with synthetic hospital wastewater containing iodinated X-ray contrast media (diatrizoate, iodipamide and iohexol). In the first stage of the experiment, the adaptation of microorganisms to anaerobic growth conditions and to ICM presence was observed. In the second stage, the adaptation to different sludge ages (40, 70 and 100 days) was investigated. Temporal changes in the bacterial communities were investigated by culture-independent methods – fluorescent *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE).

2. MATERIALS AND METHODS

Reactor details and operational data. Bacteriological material from three completely mixed, laboratory-scale anaerobic membrane bioreactors was used in this study. The membrane (flat-sheet Kubota, pore size of 0.4 μm) was submerged in the bioreactor (Fig. 1). Diffusion of oxygen was limited by a polystyrene layer. Activated sludge from a municipal wastewater treatment plant (Gliwice) was used as a seed.

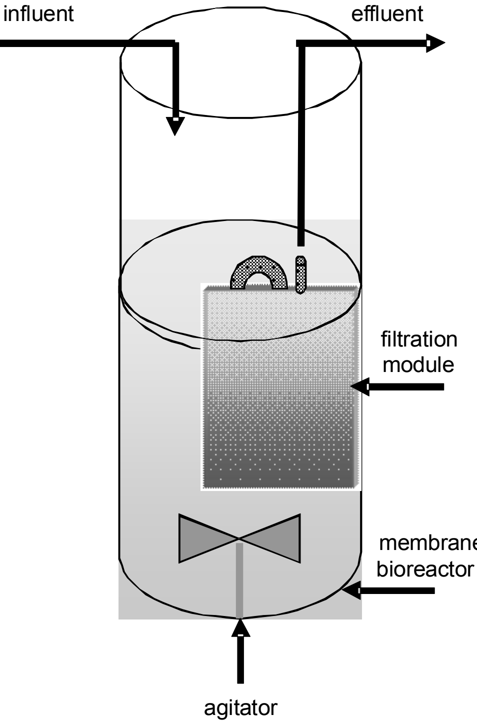

Fig. 1. Scheme of the membrane bioreactor

The study was conducted in two stages: in the first, the adaptation of microorganisms to anaerobic growth conditions and to ICM presence was observed (0–6 months) and in the second, when sludge age was established (7–8 months).

Sludge age was calculated as follows:

$$SRT = \frac{V_r X}{Q_e X} \tag{1}$$

where: V_r is the reactor volume, dm^3, X – concentration of biomass within the reactor, g SS/dm^3, Q_e – amount of excess sludge, dm^3/d.

Since in the MBR reactor *X* – numerator is the same as the denominator, then

$$SRT = \frac{V_r}{Q_e} \tag{1a}$$

In order to obtain a particular sludge age, a constant and appropriate volume of activated sludge ($Q_e = V_r/SRT$) was removed daily from each reactor. Technical parameters of bioreactors used in the study, in the stage of age regulation, are shown in Table 1.

Table 1

Technical parameters of the bioreactors used in the study in the stage of age sludge regulation

Technical parameter	Bioreactor A	Bioreactor B	Bioreactor C
Bioreactor volume, dm^3	45	45	45
SRT, d	40	70	100
Oxygen concentration, mg O_2/dm^3	0–1	0–1	0–0.76
HRT, h	67.2	67.2	67.2
Flow speed, dm^3/d	9.2–20.1	11.2–19.4	12.2–18.7
Volatile suspended solids, g SS/dm^3	1.88–5.15	3.06–5.41	4.05–5.62
Amount of excess sludge, dm^3/d	1.125	0.64	0.45
Organic loading rate, mg COD/(dm^3·d)	0.19–0.55	0.22–0.52	0.24–0.51
Organic sludge loading rate, mg COD/(g SS·d)	0.04–0.25	0.04–0.15	0.04–0.11
Efficiency of COD removal, %	37–80	43–94	68–95
Temperature, °C	19.5–26.1	19.6–26	19.7–25.9
pH	8–9	8–9	8–9

The reactors were fed with a synthetic hospital wastewater containing the following: CH_3COONa (1.670 g/dm^3) used as a source of carbon and energy, KH_2PO_4 (0.044 g/dm^3), and NH_4Cl (0.250 g/dm^3) used as a source of nitrogen and phosphorus, respectively; diatrizoate (0.001 g/dm^3); iohexol (0.001 g/dm^3) and iodipamid (0.0015 g/dm^3). Properties of ICM used in the studies are shown in Table 2. The concentration of biomass in the bioreactors was determined by dry weight method (PN-72/C-04559/02). Concentration of ICM in the samples was determined by reversce phase high performance liquid chromatography (RP HPLC), UltiMate 3000, Dionex.

Fluorescent in situ hybridization. Samples were fixed with a paraformaldehyde solution (4% paraformaldehyde in phosphate buffered saline, PBS, pH 7.2) at 4 °C for 3 h and subsequently washed in PBS. Fixed samples were stored in PBS and ethanol (1:1 v/v) solution at –20 °C. *In situ* hybridization was performed as described previously by Daims [14]. 16S rRNA targeted fluorescently labeled oligonucleotide probes and the sequences are listed in Table 3. The probes EUB338, EUB338 II and EUB338 III were mixed together (EUB338 mix) in the proportion 1:1:1 in order to detect all bacteria. Details on the chosen oligonucleotide probes are available at probeBase [15]. The probes were 5′-labeled

with FLUOS (5(6)-carboxy-fluorescein- N-hydroxysuccinimide ester) or Cy3. Both the probes and unlabeled competitor oligonucleotides were obtained from Bionovo, Poland.

Table 2

Properties of ICM used in the studies

Formula	Diatrizoate	Iodipamid	Iohexol
IUPAC name	3,5-diacetamido-2,4,6-triiodobenzoic acid	3-{5-[(3-carboxy-2,4,6-triiodo-phenyl)carbamoyl]pentanamido}-2,4,6-triiodobenzoic acid	1-N,3-N-bis(2,3-dihydroxy-propyl)-5-[N-(2,3 –dihydroxy propyl)acetamido]-2,4,6-triiodo benzene-1,3-dicarboxamide
Molecular	$C_{11}H_9I_3N_2O_4$	$C_{20}H_{14}I_6N_2O_6$	$C_{19}H_{26}I_3N_3O_9$
Skeletal			
CAS number	117-96-4	606-17-7	66108-95-0
Molecular weight, Da	613.92	1139.29	821.14
Osmolality, mOsm/kg H_2O	1500 –2000	664	322–844
Application	angiography, urography, computed tomography	cholangiography, cholecystography	myelography, arthrography

Table 3

rRNA-targeted oligonucleotide probes used in the study [19]

Probe	Target organisms	Sequence (5′–3′)	Formamide [%]
EUB 338	Most bacteria	5′- GCT GCC TCC CGT AGG AGT -3′	0–50%
EUB 338 II	*Planctomycetales*	5′- GCA GCC ACC CGT AGG TGT -3′	
EUB 338 III	*Verrucomicrobiales*	5′- GCT GCC ACC CGT AGG TGT -3′	
ALF968	*Alphaproteobacteria*, except of *Rickettsiales*	5′- GGT AAG GTT CTG CGC GTT -3′	20%
BET42a	*Betaproteobacteria*	5′- GCC TTC CCA CTT CGT TT -3′	35%
Comp-BET42a	a	5′- GCC TTC CCA CAT CGT TT -3′	a
GAM42a	*Gammaproteobacteria*	5′- GCC TTC CCA CAT CGT TT -3′	35%
Comp-GAM42a	b	5′- GCC TTC CCA CTT CGT TT -3′	b

a – used as unlabeled competitor together with probe BET42a.
b – used as unlabeled competitor together with probe GAM42a.

Prior to microscope observations, samples were embedded in Citifluor (Citifluor, Ltd, UK) to reduce fluorochrome fading. A fluorescence microscope (MOTIC BA400T) was used to examine the microbial community. Image processing was performed using Motic Images Plus 2.0 software package. For cell quantification, ImageJ software was used.

Activated sludge samples for DGGE, DNA extraction and PCR conditions. Activated sludge samples (volume of 50 cm^3) were collected from bioreactors (A, B, C) at 2 month intervals and stored at –20 °C. Total genomic DNA was extracted from 0.2 g of the activated sludge samples using FastDNA® spin kit for soil (MP Biomedicals, USA) according to manufacturer's instructions and stored at –20 °C until PCR amplification. Primers [16]: 968F with a GC clamp (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAAGAA CCT TAC 3′) and 1401R (5′ CGG TGT GTA CAA GGC CC 3′) were used for partial 16S rRNA bacterial gene PCR amplification. PCR was carried out in a 30 μl (total volume) reaction mixture containing 18.9 μl of sterile MiliQ water, 6 μl of PCR buffer (GoFlexi TAQ, Promega), 2.4 μl of $MgCl_2$ (2 mM), 0.3 μl of both primers (5 pmol/μl), 1.3 μl dNTPs (20 pmol/μl), 0.5 μl of genomic DNA and 0.3 μl Taq DNA polymerase (1.5 U).

PCR amplification was performed using an Eppendorf thermal cycler and the following steps: (1) the initial denaturation (5 min at 94 °C); (2) 30 cycles, each single cycle consisting of denaturation (1 min at 94 °C), annealing (1 min at 53 °C), and elongation (1 min at 72 °C); and (3) the final extension step (10 min at 72 °C). Products were evaluated in agarose gel (0.8% w/vol agarose, 1× TBE buffer), stained with ethidium bromide (1% w/vol.) in MiliQ water and photographed under UV light.

DGGE denaturing gradient gel electrophoresis. The DGGE of PCR products obtained in reaction with 8F and 536R primers were performed using the Dcode Universal Mutation Detection System (BioRad). The polyacrylamide gel (8% v/v with a gradient of 30–60% denaturant) was run for 9 h at 55 V in a 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 20 min and washed with MiliQ water twice for 15 min, then visualized under UV light and photographed.

Numerical analysis of the DGGE fingerprints. The DGGE banding patterns with 16S rDNA PCR products were analyzed using the Quantity One 1D Software (BioRad). The structural diversity of the bacterial community was estimated based on the Shannon–Weaver diversity index, H [17] estimated from the relative band intensities obtained from the DGGE fingerprints.

3. RESULTS AND DISCUSSION

3.1. CHANGES OF PROTEOBACTERIA CLASSES ANALYZED BY FISH

Initial analyses were carried out basing on the activated sludge originating from the wastewater treatment plant. At the beginning of the experiment, the percentage of various Proteobacteria classes was characteristic of that of the activated sludge from a municipal wastewater treatment plant. The community structure profile indicated the *Betaproteobacteria* to be the numerically dominant group of bacteria, i.e. 47% of bacterial cells were *Betaproteobacteria* (BET42a⁄EUBmix), followed by *Alphaproteobacteria* at 31% (ALF968⁄EUBmix) and *Gammaproteobacteria* at 19% (GAM42a ⁄EUBmix) (Fig. 2). In probing COD removing activated sludges from various municipal plants with oligonucleotide probes specific for Proteobacteria, Wagner et al. [18] demonstrated the dominance of Proteobacteria, which together comprised 60–75% microbial cells. Wagner and Amann [19] reported members of the *Betaproteobacteria* as playing a major role in the microbial consortia of activated sludge plants and alpha- and gammaproteobacterial classes being less abundant.

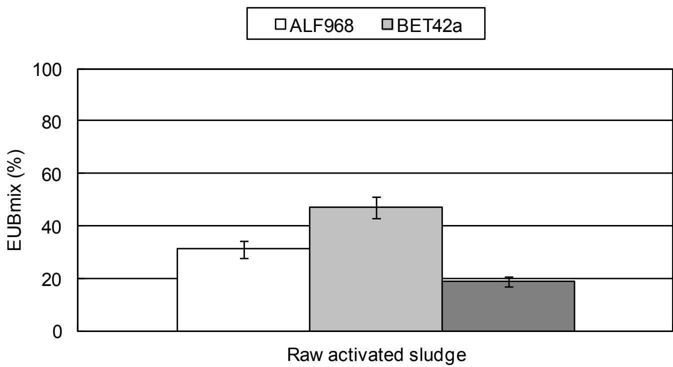

Fig. 2. Percentages of group-specific probes relative to EUBmix counts in sample from WWTP

Degenaar et al. [20] showed similar community structure profile during domestic wastewater treatment process. The numerically dominant group was *Betaproteobacteria* (33%), followed by *Alphaproteobacteria* at 26% and *Gammaproteobacteria* at 15%. Mudaly et al. [21] showed a similar trend of bacterial predominance, with the *Betaproteobacteria* comprising 22% of total cells, followed by *Alphaproteobacteria* (19%) and *Gammaproteobacteria* (17%) in a laboratory scale EBPR sludge-treating domestic wastewater. These results reflect a similar scenario in full scale processes treating domestic wastewater. Wagner et al. [18] identified members of the *Betaproteobacteria* predominating in aerated activated sludge systems with 42% of

cells binding to BET42a with respect to probe EUB. Wong et al. [22] surveyed wastewater treatment plants in Japan and suggest the *Betaproteobacteria* to be the most abundant group in two treatment plants treating domestic wastewater and accounted for 20% and 30% of EUBmix-stained cells, respectively.

In the fourth month of the experiment, during the adaptation of microorganisms to anaerobic growth conditions and to high ICM concentrations, differences in the content of *Alpha*- and *Betaproteobacteria* were noted (Fig. 3).

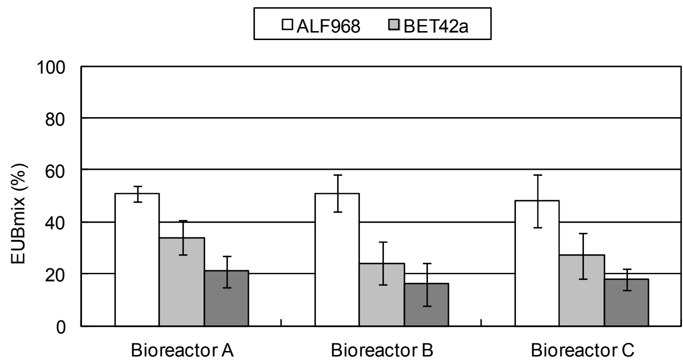

Fig. 3. Percentages of group-specific probes relative to EUBmix, counts in samples from bioreactor A, B and C in the fourth month of the experiment

The community structure profile indicated the *Alphaproteobacteria* to be the numerically dominant group of Bacteria, i.e. 48–51% of bacterial cells were *Alphaproteobacteria* (ALF968/EUBmix), followed by *Betaproteobacteria* at 24–34% (BET42a/EUBmix) and *Gammaproteobacteria* at 17–21% (GAM42a/ EUBmix).

In the fourth month of the experiment, the content of *Alphaproteobacteria* was higher by 19% and that of *Betaproteobacteria* was lower by 19% as related to the initial analyses. The content of *Gammaproteobacteria* has not changed significantly in relation to the beginning of the experiment. The *Alphaproteobacteria* include most of the oligotrophic Proteobacteria (those capable of growing at low nutrient levels). However, the *Betaproteobacteria* overlap the *Gammaproteobacteria* metabolically but tend to use substances that diffuse from organic decomposition in the anaerobic zone of habitats. Some of these bacteria use hydrogen, ammonia, methane, volatile fatty acids, and other substances [23].

After adaptation of microorganisms to anaerobic growth conditions and to ICM presence, in the eight month of the experiment, the content of *Betaproteobacteria* increased in bioreactors (Fig. 4).

The community structure profile indicated the *Betaproteobacteria* to be the numerically dominant group of bacteria, i.e. 44–49% of bacterial cells were *Betaproteobacteria* (BET42a/EUBmix), followed by *Alphaproteobacteria* at 36–37% (ALF968-/EUBmix) and *Gammaproteobacteria* at 14–17% (GAM42a/ EUBmix).

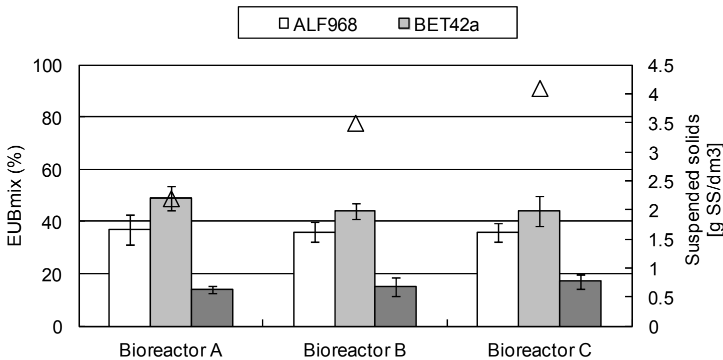

Fig. 4. Percentages of group-specific probes relative to EUBmix, counts in samples from bioreactor A, B and C and biomass concentration in eighth month of the experiment

In the eighth month of the experiment, the content of *Betaproteobacteria* was higher by 17% and that of *Alphaproteobacteria* was lower by 14% as related to the previous analyses in the fourth month. The content of *Gammaproteobacteria* has not changed significantly (decrease by 3%).

3.2. THE EFFICIENCY OF ICM REMOVAL

In eighth month of the study, differences in the biomass concentrations were noted (Fig. 4). The biomass concentration in the bioreactor A was about 2.2 g/dm^3 and in the bioreactors B and C – 3.5 g/dm^3 and 4.1 g/dm^3, respectively. With comparable technological parameters and ICM removal efficiency, it can be assumed, that the lower biomass content should be related with higher microbial activity in the elimination of contaminants. The efficiency of ICM removal in the bioreactors is shown in Table 4.

Table 4

The efficiency of ICM removal in the eighth month of the experiment

Bioreactor [SRT, days]	Efficiency of ICM removal [%]		
	Iohexol	Iodipamid	Diatrizoate
A (40)	17	49	85
B (70)	19	53	86
C (100)	12	57	90

Biotransformation of ICM has been investigated in sludge systems, as well as in sediment–water systems and in all studies the mineralization of ICM was incomplete. However, mineralization was observed for a precursor of ICM, 5-amino-2,4,6-triiodophthalic acid, in a two-stage anaerobic-aerobic reactor [24]. The formation of most ICM biotransformation products in the aerobic conditions can be explained by

succession of one, two or three of the following biochemical reaction types: oxidation of the primary or secondary alcohol groups, decarboxylation and cleavage of N–C bond [25]. If an anaerobic digester is present in a sewage treatment plant or in anaerobic parts of sediments, the deiodination process of ICM was observed [24, 26].

Kalsch [13] and Haiss and Kümmerer [4] suggest that partial degradation of diatrizoate in aerobic and anaerobic conditions is possible. In this study, the ionic diatrizoate removal efficiency was high in all three bioreactors (85–90%). However, HPLC analysis indicated that triiodinated aromatic ring was not modified completely. Despite the high removal efficiency of diatrizoate, its metabolite was detected. It can be assumed that the change in diatrizoate structure was due to removal of one or two atoms of iodine (not published data). The non-ionic iodipamid removal efficiency was lower but acceptable (49–57%) and highest removal efficiency was noted for longest sludge age. The non-ionic iohexol removal efficiency was low (12–19%). There is few data on the removal of iodipamid and iohexol from wastewater. Busetti et al. [27] showed microfiltration and reverse osmosis were effective treatment for removal of iodpipamid and iohexol. Jeong et al. [3] demonstrated higher removal efficiency of iohexol by ozonation (35–55%) and it appears that loss of iodine from the ICM should result in more biodegradable products during treatment.

3.3. DGGE ANALYSIS OF THE BACTERIAL COMMUNITY

The fingerprints obtained from DGGE separation of 16S rRNA gene fragments are shown in Fig. 5. The DNA-based DGGE pattern had changed in all three bioreactors during the experiment.

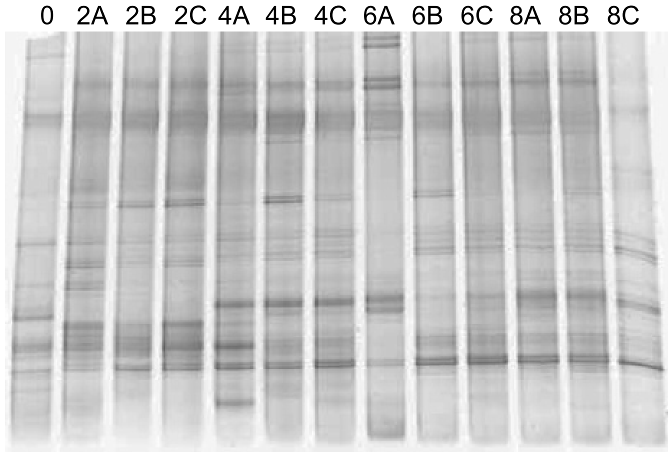

Fig. 5. DGGE pattern of fragments of 16S rRNA genes of the size of ca. 500 bp amplified using DNA obtained from activated sludge samples;
A, B, C – bioreactors; 0, 3, 4, 6, 8 – month of the experiment

The dendrogram (Fig. 6) obtained from DGGE fingerprint analysis presents the value of similarity among activated sludge samples. This could be calculated by summarizing the length of branches connecting particular samples of the dendrogram. The lower this value, the higher similarity the samples present. After two months of the experiment, samples collected from bioreactors revealed high similarity values, which suggests similar changes in microbial communities. The reason for such situation could be activated sludge volume reduction, adaptation of microorganisms to anaerobic growth conditions and to ICM presence. For the next six months, the sample from bioreactor C seems to be less congruent to the bioreactors A and B. There is a possibility that long sludge age had strongly influenced the activated sludge biocenosis composition.

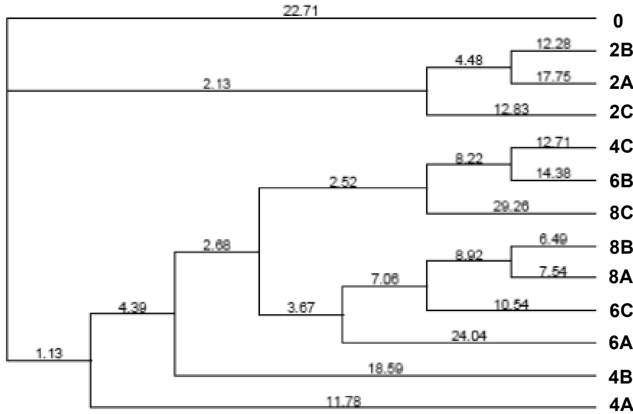

Fig. 6. DGGE tree based on neighbor-joining clustering method;
A, B, C – bioreactors, 0, 3, 4, 6, 8 – month of the experiment

Biodiversity, that is biological diversity in an ecosystem, is expressed in statistical terms by means of the Shannon biodiversity index [17]. Temporal dynamics of the Shannon–Weaver diversity values (H) for activated sludge samples are shown in Fig. 7.

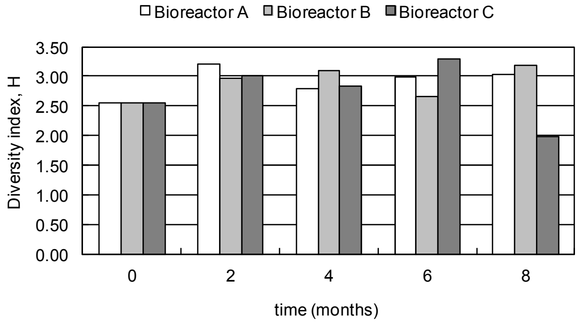

Fig. 7. Temporal dynamics of the Shannon–Weaver diversity values (H)
for activated sludge samples from bioreactor A, B and C

In the second month of the experiment, the biological diversity increased in all three bioreactors to H_A = 3.19, H_B = 2.96 and H_C = 3.01, respectively. The reason for such situation could be the composition of synthetic wastewater, containing easy available source of carbon and energy. Furthermore, studies have shown that in short-term tests with bacteria (*Vibrio fischeri*, *Pseudomonas putida*) no toxic effects were detected at concentrations of ICM as high as 10 g/dm^3 [6]. In the bioreactor A, in the fourth month of the experiment, the diversity decreased to 2.78 and in sixth and eighth month slightly increased (3.0). In the bioreactor B, the decrease in biodiversity was observed (2.65) in the sixth month of the experiment, but in the eighth month an increase was noted (3.19). The variability of the bacterial community according to the results obtained in the Shannon–Weaver index estimations was the highest in the bioreactor C. The diversity increased in the sixth month of the experiment (3.29) but in the eighth month a noticeable decrease was observed (1.97). The reason for such situation could be long sludge age in bioreactor C and development of a specialized bacterial community capable of transforming ICM. To verify these assumptions it would be necessary to carry out more detailed molecular investigations.

4. CONCLUSIONS

Fluorescence *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) allowed us to estimate differences in biodiversity of activated sludge, in anaerobic membrane bioreactors dealing with synthetic hospital wastewater with addition of ICM. Initial analysis indicated that the percentage of the *Alpha*-, *Beta*- and *Gammaproteobacteria* classes was characteristic of that of an activated sludge from a municipal wastewater treatment plant. In the fourth month of the experiment, during the adaptation of microorganisms to anaerobic growth conditions and to ICM presence, the content of *Alphaproteobacteria* increased in bioreactors. However, in the eighth month of the experiment, after SRT adaptation, the *Betaproteobacteria* was the numerically dominant class of Proteobacteria. The reason for such situation could be the ability of *Betaproteobacteria* to use substances that diffuse from organic decomposition in the anaerobic conditions. In quantitative analysis of Proteobacteria in activated sludge, the biomass concentration should be taken into consideration. The major differences in biodiversity was noted in a bioreactor with activated sludge of the age of 100 days. The reason for that situation could be formation of a specialized microbial community but more detailed molecular investigations are required.

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