COMPETITIVE INTERACTIONS AMONG BACTERIA IN BIOAUGMENTED ACTIVATED SLUDGE DURING OIL-CONTAINING WASTEWATER DEGRADATION

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ABSTRACT
Fats, oils and greases (FOGs) are important and burdensome organic constituents of most wastewater. The amount of lipids in municipal wastewater is approximately 30 – 40% of the total organic matter, measured as chemical oxygen demand (COD). FOGs have been discharged from food industries, restaurants, slaughterhouse, household *etc*. High concentration of these compounds in wastewater often causes a major problem in biological wastewater treatment processes. Bioaugmentation is a method for enhancing biodegradation of lipids by addition of microorganisms (indigenous or genetically modified) or enzyme supplements for treatment of wastewater.
The aim of the study was the experimental verification of microbial supplement usage in long-term wastewater treatment process. Efficiency of COD removal, lipolytic activity, the composition of *Proteobacteria* community and biodiversity of activated sludge in two bioreactors: without and with microbial supplement, were taken into consideration.
The methods denaturing gradient gel electrophoresis (DGGE) based on 16S rRNA gene PCR products and fluorescent *in situ* hybridization (FISH) with 16S rRNA gene probes revealed differences in the microbial community structure in the two bioreactors. According to the results obtained in this study, a bioreactor with microbial supplement is characterized by higher microbial community diversity than non-bioaugmented bioreactor and there was a significant difference among the beta and gamma-proteobacteria content in the reactor with microbial supplement.

INTRODUCTION

Fats, oils and greases (FOGs) are important components in domestic and some industrial wastewater. The amount of lipids in municipal wastewater is approximately 30 – 40% of the total organic matter, measured as chemical oxygen demand (COD) (Dueholm *et al.*, 2001). Wastewater produced from edible oil refinery, slaughterhouse and dairy products industry contains a high (> 100 mg/L) concentration of lipids (Saifuddin & Chua, 2006). High concentration of these compounds in wastewater often causes major problem in biological wastewater treatment processes (Chipasa & Mędrzycka, 2006). Lipids can solidify at lower temperatures and cause operational damage, such as clogging and developing unpleasant odours. FOGs interfere with activated sludge oxygen transfer rate. They can form oil films on the surface of activated sludge flocs and hinder the diffusion of oxygen and substrates. Furthermore, lipids promote growth of filamentous microorganisms (*Sphaerotilus natans, Thiothrix* sp., *Beggiatoa* sp., *Nocardia* sp., *Microthrix* sp.), which cause bulking and foaming (Jenkins *et al.*, 1993; Martins *et al.*, 2004; Cammarota *et al.*, 2006).

The amount of wastewater containing high levels of fats, greases and oils increases each year due to urbanization and the development of industrial plants. FOGs can be removed from wastewater by physical or chemical methods (grease traps, DAF systems or coagulation, flocculation and

neutralization by addition of chemical compounds) (Willey, 2001; Tano-Debrah *et al.*, 1999, Konieczny *et al.*, 2005). However, lipids can pass through physicochemical treatment processes and contribute to the levels of BOD and COD (biological/chemical oxygen demand) in the effluents. Therefore, biological treatment process is commonly used to remove FOGs from lipid-rich wastewater.

The use of mixed microbial cultures such as activated sludge is a good option for the treatment of wastewater containing FOGs. However, sometimes it requires improvement. The current practice to make biodegradation of lipids more effective is to apply bioaugmentation (Brooksband *et al.*, 2007; Chipasa & Mędrzycka, 2006). Bioaugmentation is a method for enhancing biodegradation of lipids by addition of microorganisms (indigenous or genetically modified) or enzyme supplements for the treatment of wastewater. The use of enzyme supplements, mainly lipases, is less attractive than application of viable microorganisms, because it is only used for hydrolysis of lipids, for example fats and oils to fatty acids and glycerol. The application of different species of microorganisms can be a perfect solution of the problem. Microorganisms hydrolyze the lipids as well as biodegrade them further to carbon dioxide and water. Moreover, microorganisms have a greater tolerance to changes in environmental conditions than enzyme supplements.

Numerous microorganisms capable of degrading FOGs have been identified and may be potential candidates for bioaugmentation products (Crine *et al.*, 2006; El-Bestawy *et al.*, 2005; Mongkolthanaruk & Dharmsthiti, 2002; Suzuki *et al.*, 2001; Markossian *et al.*, 2000). *Proteobacteria* are a major group in activated sludge (Wagner *et al.*, 1993) and beta- and gamma-proteobacteria show good potential for use in the treatment of wastewater containing FOGs (Wakelin & Forster, 1997; Fong & Tan, 2000; Suzuki *et al.*, 2001; Sugimori *et al.*, 2002; Mongkolthanaruk & Dharmsthiti, 2002; El-Masry *et al.*, 2004; Hasanuzzaman *et al.*, 2004; El-Bestawy, 2005; Matsumiya *et al.*, 2007). A relatively small number of studies assessing the performance of commercially available FOGs-degrading supplements has been published in professional literature. There is a lack of knowledge concerning the efficiency of commercially available products and changes in microbial composition of activated sludge after bioaugmentation. The use of molecular methods, specifically hybridization with fluorescently labeled oligonucleotides (FISH) and DGGE, which is based on the separation of DNA fragments of the same length, but with different nucleotide sequence, provides novel insights with respect to the structure and dynamics of microbial communities in activated sludge (Bouchez *et al.*, 2000).

The aim of the study was to compare activated sludge communities in two bioreactors containing activated sludge with and without microbial supplement. Efficiency of COD removal, lipolytic activity, composition of *Proteobacteria* community and biodiversity of activated sludge in two bioreactors: bioaugmented and non-bioaugmented, were taken into consideration.

MATERIALS AND METHODS
Reactor details and operational data
The activated sludge used in this investigation was obtained from a wastewater treatment plant (Zabrze Śródmieście). Two bioreactors were used: without (D) and with ($D_{cont.}$) commercial microbial supplement (Fig. 1). Composition of the microbial supplement is shown in Table 1. The microbial supplement contained lipase-producing microorganisms and it was used for bioaugmentation at the beginning of the experiment.
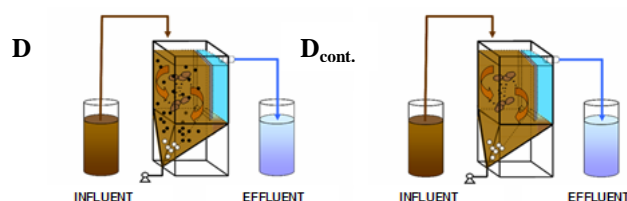
Figure 1. The scheme of aerobic bioreactors used in the experiment. D – without and D$_{cont.}$ – with microbial supplement (●); bioreactors were spatially separated

Table 1. Composition of the commercial microbial supplement used in the experiment

Bacillus subtilis	*Lactobacillus helvetius*	*Deinococcus rediodurans*
Bacillus licheniformis	*Lactococcus lactis*	*Botritis cinerea*
Bacillus megaterium	*Acinetobacbacter* sp.	*Saccharomyces cerevisiae*
Bacillus sp.	*Comamonas* sp.	*Sporotrichum dimorphosporium*
Pseudomonas putida	*Rhizobium* sp.	*Trichoderma roseus*
Pseudomonas fluorescens	*Nitrosomonas* sp.	*Alcaligenes* sp.
Pseudomonas sp.	*Nitrobacter* sp.	*Acetobacter* sp.

The reactors were fed with a synthetic medium containing high FOGs concentrations (mixture of lipids – rapeseed oil, margarine, fish oil; v/v 1:1:1). The synthetic wastewater was composed of the following ingredients dissolved in water: mixture of lipids (250 – 1000 mg/L), Tween®80 – used as an emulsifying agent (25 – 100 mg/L), peptone (200 mg/L), $(NH_4)_2SO_4$ (400 mg/L), KH_2PO_4 (200 mg/L), $CaCl_2$ (50 mg/L), $MgSO_4$ (20 mg/L), $FeSO_4$ (50 mg/L), $ZnSO_4$ (20 mg/L) and $CuSO_4$ (20 mg/L). pH was maintained at a level of 7.0 – 7.2 using $NaHCO_3$ (Hsu *et al.*, 1983; Chipasa & Mędrzycka, 2008).

The performance of the reactors was monitored by analysis of the influent and effluent. The technological parameters of the investigated reactors are shown in Table 2. COD was determined photometrically (Merck Spectroquant® Tests). Lipolytic activity of activated sludge was determined by colorimetric method based on the activity in cleavage of *p*-nitrophenyl laurate (*p*-NPL) (Winkler and Stuckmann 1979, modified).

Table 2. Technical parameters of two bioreactors used in the study

TECHNICAL PARAMETER	REACTORS	TECHNICAL PARAMETER	REACTORS
Bioreactor volume, [L]	8	O_2, [mg/L]	1.9 – 2.8
Sludge age, [days]	18	pH	7.08 – 7.26
Flow speed, [L/d]	2.79 – 2.87	Temperature, [°C]	18 – 24
HRT, [d]	2.84 – 2.87	Organic sludge loading rate, [mg COD/g VSS • d]	104 – 1334

Activated sludge samples for DGGE
Activated sludge samples (volume of 50 ml) were collected from both bioreactors at 15-day intervals and stored at -20°C.

DNA extraction and PCR conditions

Total genomic DNA was extracted from 0.3 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 (Muyzer *et al*., 1993): 338f with GC clamp (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') were used for partial 16S rRNA bacterial gene PCR amplification. PCR was carried out in a 30 µl (total volume) reaction mixture containing 19 µl sterile MiliQ water, 6 µl PCR buffer (GoFlexi TAQ, Promega), 2.4 µl $MgCl_2$ (2 mM), 0.25 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 step (10 min at 95°C); (2) 30 cycles, each single cycle consisting of denaturation (1 min at 95°C), annealing (1 min at 53°C), and elongation (2 min at 72°C); and (3) the final extension step (12 min at 72°C). Products were evaluated in agarose gel (0.8% w/vol agarose, $1 \times$ 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 338f-GC and 518r 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 $1 \times$ 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 in 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 Quantity One 1D Software (BioRad). The structural diversity of the bacterial community was estimated on the basis of the Shannon-Weaver diversity index, H (Eichner *et al*., 1999; Luxmy *et al*., 2000), estimated from the relative band intensities obtained from the DGGE fingerprints.

FISH – Fluorescent *in situ* hybridization
Sample preparation

Samples were fixed with a paraformaldehyde solution (4% paraformaldehyde in phosphate-buffered saline, PBS, pH 7.2) at 4°C for 3 hours and subsequently washed in PBS. Fixed samples were stored in PBS:ethanol (1:1) solution at -20°C.

Oligonucleotide probes

In situ hybridization was performed as described previously by Daims (Daims et al., 2005). 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 (Loy et al., 2003). 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 3. rRNA-targeted oligonucleotide probes used in the study

Probe	Target organisms	Sequence (5' – 3')	FA, %	Reference
EUB 338	most bacteria	5'- GCT GCC TCC CGT AGG AGT -3'	0-50%	Amman *et al.*, 1990
EUB 338 II	*Planctomycetales*	5'- GCA GCC ACC CGT AGG TGT -3'	0-50%	Daims *et al.*, 1999
EUB 338 III	*Verrucomicrobiales*	5'- GCT GCC ACC CGT AGG TGT -3'	0-50%	Daims *et al.*, 1999
ALF968	α-*proteobacteria*, except of *Rickettsiales*	5'- GGT AAG GTT CTG CGC GTT -3'	20%	Neef *et al.*, 1997
BET42a	β-*proteobacteria*	5'- GCC TTC CCA CTT CGT TT -3'	35%	Manz *et al.*, 1992
Comp-BET42a	[a]	5'- GCC TTC CCA CAT CGT TT -3'	-[a]	Manz *et al.*, 1992
GAM42a	γ- *proteobacteria*	5'- GCC TTC CCA CAT CGT TT -3'	35%	Manz *et al.*, 1992
Comp-GAM42a	[b]	5'- GCC TTC CCA CTT CGT TT -3'	-[b]	Manz *et al.*, 1992

[a] – used as unlabeled competitor together with probe BET42a

[b] – used as unlabeled competitor together with probe GAM42a

Fluorescence microscopy and cell estimation
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.

Results and Discussion
Efficiency of lipids mixture removal by activated sludge under bioaugmentation conditions and in a reference system
Changes in the removal of organic compounds from wastewater during the experiment in reactors supplied with the mixture of lipids is presented in Figs. 2 and 3.
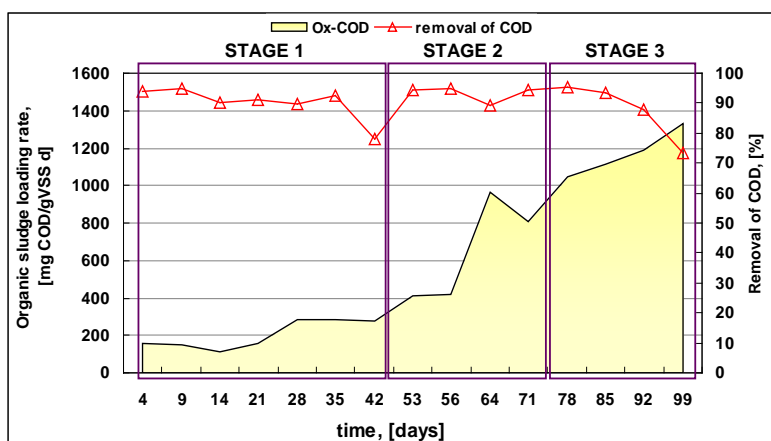

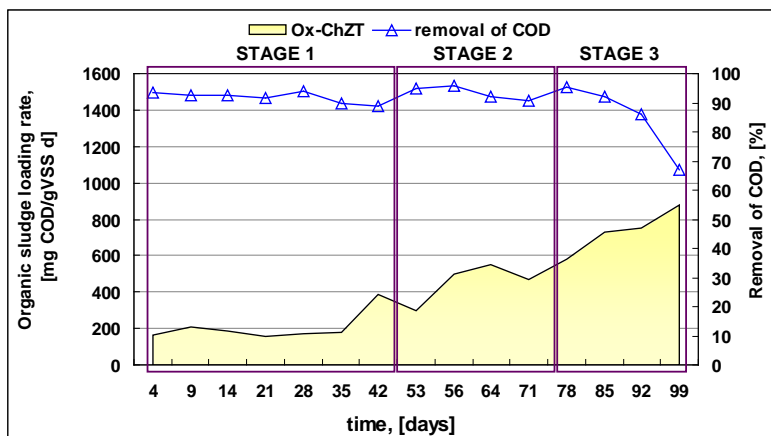**Figure 2.** The mixture of lipids removal efficiency for reactor D (bioaugmented reactor)

Figure 3. The mixture of lipids removal efficiency for reactor $D_{cont.}$ (reference reactor)

The organic sludge loading rate varied during the experiment and increased within the range of from 115 to 1334 mg COD/gVSS d in the bioaugmented reactor and from 153 to 878 mg COD/gVSS d in the reference reactor. During the first two stages of the experiment, when the concentration of the lipids mixture fed was less than 1000 mg/L, the COD removal efficiency was high and amounted to 90 and 93% on the average in the bioaugmented system, and to 91 and 93% in the reference reactor. In the terminal phase of the experiment reduction in COD removal efficiency was observed, particularly in the reference system, where COD removal efficiency during the last days of the experiment decreased to 67%. However, when organic matter in the reactors is taken into consideration, the most prominent differences in COD removal efficiency between system D and system $D_{cont.}$ were observed at the second and third stages of the experiment.

The mixture of lipids fed to the systems contained: rapeseed oil, fluid margarine and fish oil. At the first stage of the experiment, the efficiency of lipids removal was high in both the bioaugmented system and the reference system and amounted to 90 and 91%, respectively. The literature indicates that lipids bioavailability depends on the fatty acids present in the molecules. Biodegradability of long-chain fatty acids increases with decreasing carbon chain length and increasing number of unsaturated bonds in the molecule (Loehr & Roth, 1968). The efficiency of lipids removal might have been affected by the addition of animal (fish) oil, which has a content of unsaturated fatty acids (among them docosahexaenoic acid) of ca. 75%, but which is not usually present in municipal wastewater, and therefore the population of microorganisms able to decompose it may be limited.

Microscopic observations enabled the estimation of the number of filamentous bacteria. It was found that it was much higher in the reference system. The bioaugmented system contained *pinpoint* flocs, wherein low content of filamentous bacteria and high content of free-swimming bacteria resulted in the formation of very fine flocs, leading to deterioration of settling properties of the activated sludge and the carrying away of the suspension from the bioreactor (Traczewska, 1997).

Therefore the question raised was whether a set of microorganisms able to efficiently eliminate lipids under the conditions of high pollutant load has been formed in the bioaugmented system fed with a mixture of lipids.

Lipolytic activity of bioaugmented and reference sludge
Changes in lipolytic activity of bioaugmented activated sludge and reference sludge fed with a mixture of lipids is shown in Fig. 4.
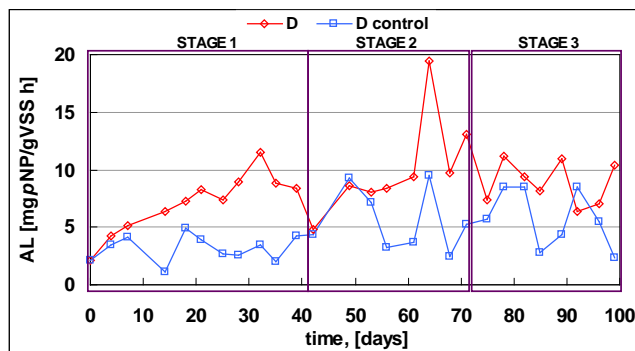
Figure 4. Changes in lipolytic activity of bioaugmented (D) and reference sludge (D_{cont.})

In the bioaugmented system, to which a mixture of lipids was fed, increase in lipolytic activity was observed up to day 32 of the experiment. This was followed by a decrease in activity, whereas the highest activity of these enzymes occurred on day 64 of the experiment. Lipolytic activity in the reference system was lower (except for days 49 and 92). Higher lipolytic activity of the bioaugmented activated sludge fed with a mixture of lipids during the second and third stages of the experiment corresponds with the high pollutant load.

Changes of *Proteobacteria* classes and diversity of the microbial community in bioaugmented and reference activated sludge

Subsequent analyses were carried out to verify whether there was a relationship between lipolytic activity and changes of the biocoenosis of the activated sludge during the treatment of wastewater of high lipids content. The tests performed comprised in particular the monitoring of changes in the composition of activated sludge biocoenosis using fluorescent *in situ* hybridization. The presence of alpha-, beta- and gammaproteobacteria in the activated sludge was determined. Initial analyses were carried out on the activated sludge brought from the wastewater treatment plant before inoculating it with the microbial supplement. The percentage of the various *Proteobacteria* classes was characteristic of that of an activated sludge from a municipal wastewater treatment plant. Percentage of cells labelled with probes specific for the various *Proteobacteria* classes related to those labelled with the EUBmix probe in the system fed with the lipids mixture is shown in Fig. 5.
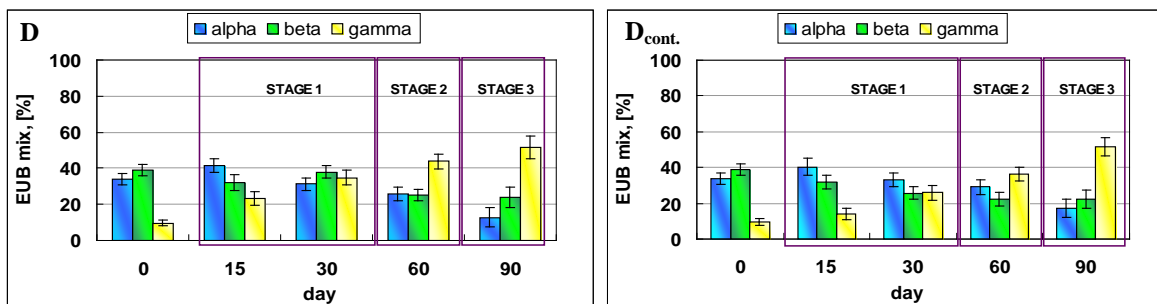

Figure 5. Percentages of group-specific probes relative to EUBmix, counts in samples from reactors D and D_{cont.}

On day 15 of the experiment a difference in the content of gammaproteobacteria was noted in the bioaugmented system fed with the lipids mixture (increase by 9% as compared to the reference system). Subsequent analyses were performed on days 30, 60 and 90 – changes were observed in

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beta- and gammaproteobacteria. On day 30 the content of betaproteobacteria was higher by 12% and that of gammaproteobacteria by 9% as related to the reference system, and on day 60 the content of gammaproteobacteria was higher by 8% in relation to the reference system. In the course of the experiment the content of gammaproteobacteria in the bioaugmented system increased. At the start, before inoculation of the sludge with the microbial supplement, the content of gammaproteobacteria was 10%, and it increased to 23% on day 15 of the experiment. This was in line with increased lipolytic activity and, as there was no change in the reference system, it must have been due to bioaugmentation. The content of gammaproteobacteria continued to increase during the further course of the experiment. On days 30, 60 and 90 it amounted to 35, 44 and 52%, respectively, and it corresponded with lipolytic activity higher than that in the reference system. The main purpose of the analyses was to follow the differences in the composition of the bacterial biocoenosis of the activated sludge caused by inoculation with the microbial supplement.

The microbial supplement consisted of the following genera of alphaproteobacteria: *Rhizobium*, *Nitrobacter, Acetobacter* and betaproteobacteria: *Comamonas*, *Nitrosomonas*, *Alcaligenes*. The microbial supplement used also contained representatives of gammaproteobacteria: the genera *Pseudomonas* and *Acinetobacter*. The percentage share of the various classes of *Proteobacteria* in the microbial supplement was not known. It is, however, known from the literature that many species of gram-negative bacteria present in the supplement have the capability to generate lipolytic enzymes (Mongkolthanaruk & Dharmsthiti, 2002; Matsumiya *et al*., 2007), whereas of special interest is the *Pseudomonas* genus (Fong & Tan, 2000; El-Masry *et al*., 2004), which belongs to the class of gammaproteobacteria. On day 15 of the experiment the composition of the biocoenosis of the activated sludge in the bioaugmented system changed in relation to that of the reference reactor: the content of gammaproteobacteria in the system fed with the lipids mixture had increased by 9%. Increase in the content of gammaproteobacteria corresponded with increased lipolytic activity. This could be due to multiplication of gammaproteobacteria included in the microbial supplement, which contained, *inter alia*, bacteria of the *Pseudomonas fluorescens*, *Pseudomonas putida* species and of the *Acinetobacter* genus, known for their high lipolytic activity. To verify these assumptions it would be necessary to carry out more detailed investigations based on the analysis of specific bacterial species.

The relationship between lipolytic activity and the percentage share of gammaproteobacteria in the bioaugmented and reference activated sludge fed with the lipids mixture is shown in Fig. 6. The high value of R^2 (0.76) for the bioaugmented reactor indicates a high degree of match between the two parameters. For the reference system the correlation between lipolytic activity and the content of gammaproteobacteria was much lower (R^2 = 0.2). The increase in the content of
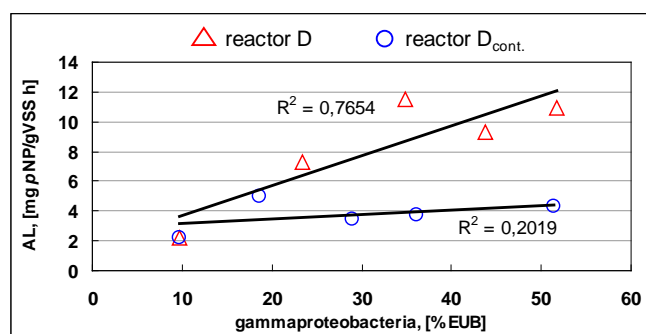

Figure 6. The relationship between lipolytic activity and the percentage share of gammaproteobacteria in the bioaugmented (D) and reference (D$_{cont.}$) activated sludge

gammaproteobacteria was observed in both the bioaugmented and reference systems fed with the lipids mixture. This is due to the fact that the determined class of gammaproteobacteria embraces a number of morphologically and functionally dissimilar groups of microorganisms (Wagner & Loy, 2002), among them some species of filamentous bacteria. Microscopic observations indicated that in the bioaugmented system the probe signal was received from rod-shaped and spherical bacterial cells, whereas in the reference system it originated from filamentous bacteria.

Biodiversity, that is biological diversity in an ecosystem, is expressed in statistical terms by means of Shannon biodiversity index (Shannon & Weaver, 1963). In the reference reactor fed with the lipids mixture, up to day 45 of the experiment the biological diversity decreased to $H_{Dcont.} = 2.46$, as opposed to that of the bioaugmented system ($H_D = 3.14$) (Fig. 7).
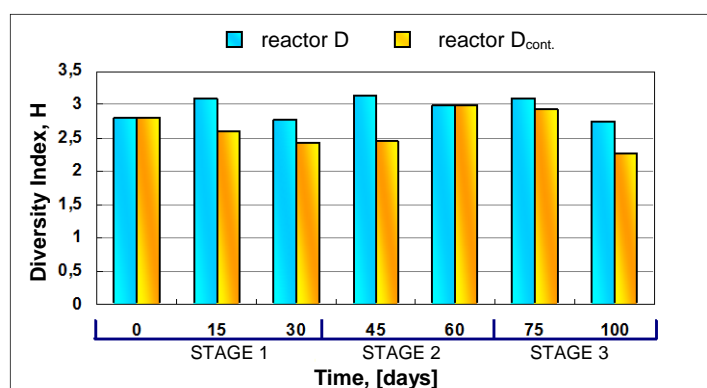

Figure 7. Temporal dynamics of the Shannon-Weaver diversity values (H) for activated sludge samples

Further decrease in biodiversity was observed in the final stage of the experiment ($H_D = 2.75$, $H_{Dcont.} = 2.25$). However, the bioaugmented system still showed higher biological diversity, suggesting that the microbial supplement produced an enriching effect.

CONCLUSIONS

The mixture of lipids removal efficiency in the reactor with microbial supplement was higher than in the reference reactor, particularly when organic sludge loading rate was high (1048 – 1334 mg COD/gVSS d). High lipolytic activity of activated sludge in the reactor with microbial supplement was correlated with the increase of the amount of gamma-proteobacteria (9% increase in the first stage of experiments). There was a difference between the beta- and gamma-proteobacteria content in the reactor with microbial supplement. The microbial supplement enriched the bacterial community which contributed to the maintenance of higher level of diversity.

ACKNOWLEDGEMENTS

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