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Full-scale bioreactor pretreatment of highly toxic wastewater from styrene and propylene oxide production

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ABSTRACT

The wastewater originating from simultaneous production of styrene and propylene oxide (SPO) is classified as highly polluted with chemical oxygen demand level in the range 5965 to 9137 mg L^{-1}—as well as highly toxic. The dilution factor providing for a 10 percent toxic effect of wastewater samples in a test with *Paramecium caudatum* was 8.0–9.5. Biological approach for pretreatment and detoxification of the wastewater under full-scale bioreactor conditions was investigated. The number of suspended microorganisms and the clean up efficiency were increased up to 5.5–6.58 × 10^8 CFU mL^{-1} and 88 percent, respectively during the bioreactor's operation. Isolates in the *Citrobacter*, *Burkholderia*, *Pseudomonas*, and *Paracoccus* genera were dominant in the mature suspended, as well as the immobilized microbial community of the bioreactor. The most dominant representatives were tested for their ability to biodegrade the major components of the SPO wastewater and evidence of their role in the treatment process was demonstrated. The investigated pretreatment process allowed the wastewater to be detoxified for conventional treatment with activated sludge and was closely related to the maturation of the bioreactor's microbial community.

1. Introduction

Currently, the petrochemical industry plays a major role in the economic growth of every nation and, in the specific case of Russia, is the recipient of major government subsidies. Its products are used in almost all industrial sectors. One typical petrochemical intermediate is styrene, which is important in rubber and plastics manufacture. For economic reasons styrene is generally co-produced together with propylene oxide. The significant use of petrochemical products results in the contamination of different ecosystems. During various stages in the production of styrene and propylene oxide (SPO), such as dehydration, dehydrogenation, and oxidation of organic compounds, highly polluted wastewater is formed. This wastewater contains volatile toxic compounds (Shokrollahzadeh et al., 2008) such as acetophenone, 1-phenyl ethanol, benzene, and phenol, as well as nonvolatile compounds such as mono- and dipropylene glycol, propanol, etc. The chemical oxygen demand (COD) of petrochemical wastewaters is typically very high. In multiple studies from different sites it has variously been estimated as 1620–1896 mg L^{-1} (Calheiros et al., 2009), 2500–4100 mg L^{-1} (Wei et al., 2010), and 2200–4700 mg L^{-1} (Chang et al., 2011). Currently, pollution of the environment by hazardous chemical compounds is one of the most important problems (Mantis et al., 2005). Those petrochemical wastewaters need to be treated before discharging into the environment to avoid river, soil, and air pollution (Stromgren et al., 1995; Chen et al., 1998; Jerez et al., 2002; Chen et al., 2003).

Industrial wastewater treatment methods can be divided into two main groups, physicochemical and biological. Most wastewater treatment processes rely on the use of activated sludge (Soddell and Seviour, 1990; Amann et al., 1998; Blackall et al., 1998; Yang et al., 2011), due to its considerably lower cost in comparison with the physicochemical method, and reasonable efficiency (Shokrollahzadeh et al., 2008; Babaee et al., 2010; Chang et al., 2011). However, some organic substances produced during chemical production are toxic or resistant to biological treatment in conventional biological processes (Adams et al., 1996; Pulgarin and Kiwi, 1996; García et al., 2001; Lapertot et al., 2006; Muñoz and Guieysse, 2006). Meanwhile, the use of chemical and physical techniques requires very expensive pretreatment, usually results in the production of secondary effluent (Sangave et al., 2007) and does not always reduce the pollutant concentration to acceptable levels, necessitating further pretreatment before the water is finally treated with conventional activated sludge (CAS) (Babaee et al., 2010).

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The high COD level in wastewater from SPO production makes CAS processes unsuitable for treatment due to its high toxicity to biota, although most components of the wastewater, such as glycols, are known to be biodegradable (Miller, 1979; Van Hamme et al., 2003; Shokrollahzadeh et al., 2008). Therefore, we proposed a pretreatment process of SPO wastewater using a biotechnological approach for further treatment with activated sludge. The use of a bioreactor, containing highly active specialized microorganisms, immobilized on a carrier, has been demonstrated for the removal of toxic substances, such as phenol, heavy metals and pesticides, from wastewater (Erhan et al., 2004; Wasi et al., 2011; Park et al., 2013). This approach is considered the most promising prospective one for pollutant elimination during SPO wastewater pretreatment. The carrier can maintain a high concentration of bacterial biomass, protect microorganisms immobilized on it from being shocked by any sudden change in pH, temperature, or oxygen concentration, which can lead to the reduction of sludge (Ye and Ni, 2002). In contrast to free microorganisms, immobilized microorganisms can biodegrade higher substrate concentrations (Wang et al., 2007). Bioreactor microflora plays an important role in COD load reduction (Wang et al., 2011). However, there was not sufficient data on microbial communities that could sustain extremely high COD levels as in the case of SPO production before, in which the COD level is more than 6000 mg L^{-1}. Furthermore, isolating and identifying microorganisms responsible for hydrocarbon transformation is important both for fundamental knowledge of microbial metabolic pathways and practical applications. Thus, this study aimed to characterize the microbial community cultivated from a full-scale bioreactor for SPO wastewater.

2. Materials and methods

2.1. Wastewater

Wastewater was collected from the SPO wastewater pretreatment plant (WWPTP) at Nizhnekamskneftekhim (NKNH) Enterprise. NKNH Inc. is a large petrochemical company headquartered in Nizhnekamsk city, Russia. The influent wastewater is characterized as highly alkaline (pH 8.0–12.5) with a high organic load (COD 5965–9137 mg L^{-1}). The main components of SPO wastewater determined by GC–MS were propylene glycol: 784.29–2517.78 mg L^{-1}, 1-phenyl ethanol: 113.62–237.46 mg L^{-1}, phenol: 105.93–182.41 mg L^{-1}, toluene: 80.63–85.87 mg L^{-1}, ethanol: 52.30–398.69 mg L^{-1}, propanol: 22.51–641.20 mg L^{-1}, dipropylene glycol: 20.63–86.75 mg L^{-1}, ethoxy propanol: 18.22–46.77 mg L^{-1}, methanol: 17.17–41.10 mg L^{-1}, acetophenone: 11.07–40.13 mg L^{-1}, ethylene glycol: 10.01–19.52 mg L^{-1}, diethylene glycol: 8.81–11.27 mg L^{-1}, allyl alcohol: 6.22–66.39 mg L^{-1}, propanal: 4.71–19.74 mg L^{-1}, benzyl alcohol: 4.04–8.33 mg L^{-1}, hydroxypropanone: 3.48–8.13 mg L^{-1}, benzaldehyde: 2.41–14.13 mg L^{-1}, acetone: 2.01–179.98 mg L^{-1}, *n*-butanol: 1.41–25.04 mg L^{-1}, ethyl butanol: 0.81–10.53 mg L^{-1}, acetaldehyde: 0.47–14.80 mg L^{-1}, isopropanol: 0.44–198.40 mg L^{-1}, styrene: 0.27–1.95 mg L^{-1}, methyl propyl ketone: 0.24–1.43 mg L^{-1}, hexanone: 0.08–0.31 mg L^{-1}, benzene: 0.01–0.02 mg L^{-1}, and ethylbenzene: 0.001–0.002 mg L^{-1}. Preliminary purification of SPO wastewater is performed under full-scale bioreactor conditions with the participation of freely suspended and immobilized microorganisms (Fig. 1).

2.2. Full-scale bioreactor system

The bioreactor system consisted of two blocks: a preparation block (A) and a biological treatment block (B) (Fig. 1). The system operated at a maximum wastewater feed rate of 25 m^3 h^{-1}. The volume of the bioreactor (6) was 1200 m^3. Block (A) contained a heat exchanger (1), acid (2) and biogenic substances (3) feed tank. Passing through a heat exchanger, the influent wastewater (14) was cooled to 25–35 °C. Before entering the bioreactor the alkaline wastewater was neutralized to pH 6.8–7.5 by sulfuric acid and supplied with superphosphate and ammonium sulfate to create favorable living conditions for microorganisms. Block (B) contained a fermenter (4) used once per year to recreate the microflora during annual bioreactor (6) preventive maintenance. The bioreactor was a round bottom tank which was divided into a zone of flotation (10) and a zone of aeration (9), containing carriers for immobilization (11). The influent wastewater, air for aeration (5) and air for flotation (16) came into the bioreactor at its bottom. The zone of flotation consisted of an annular partition (7) that had windows (8) for the fluid inlet, and provided a separation of the effluent wastewater and microbial biomass. In the upper part of the flotation zone the microbial biomass returned to the aeration zone in the foam form. The effluent wastewater (17) after the bioreactor was then sent to the all-factory wastewater CAS treatment plant. Waste air (13) was treated in a biological filter (12).

The microbial community of SPO bioreactor was obtained from enrichment community of the industrial activated sludge and wastewater slim. The studied microbial community was adapted to gradual increase of the COD by diluting the SPO wastewater. Fiberglass “brushes” and polyurethane foam carriers were used in the SPO bioreactor to immobilize the microflora.

2.3. Sampling

The sampling points were the influent wastewater (14), mix liquor from the bioreactor (6) and the effluent wastewater (17). The influent and effluent wastewaters were used for chemical and toxicological analyses. The mix liquor samples from the bioreactor were analyzed for their suspended cultivated microbial community and so were collected aseptically. Mix liquor samples were collected in the first, 16th and 32nd week after initiation of the bioreactor system. For assessment of the immobilized microbial community, we extracted the immobilized material after bioreactor shutdown at the 40th week. Mix liquor and carrier samples were transferred into sterile glass tubes and analyzed by microbiological methods within 6 h after sampling. In accordance with the mode of SPO production the system pauses after the 40th week for preventive maintenance. At the same time the sample of immobilized bacteria was collected aseptically and stored by laboratory BIOSTAT cultivation with diluted SPO wastewater. Before annual launching of the bioreactor, microbial community accumulation was performed in the full-scale fermenter (4) with the presence of diluted SPO wastewater and growth stimulating additives such as peptone and yeast extract. Initiation of the bioreactor system starts with microbial-free carriers. At the same time, SPO wastewater was mixed with microbial biomass from the fermenter (4). The fermenter works until the bioreactor biomass reaches 3×10^7 CFU mL^{-1}, thereafter the reactor performs without replenishment from the fermenter (4).

2.4. Microbiological analysis

Mixed liquor samples were inoculated on various rich and selective media such as meat-extract agar medium, yeast growth medium, N-free medium, Czapek-Dox medium, and King's B medium at different dilutions (10^{-2}–10^{-7} dilutions). For microbial sludge sample we used dilutions series up to 10^{-10}. The composition of these media is described below: meat extract medium—per liter fish meal pancreatic hydrolyzate (18 g), NaCl (2 g), agar (20 g), yeast growth medium—per liter glucose (40 g), peptone (10 g), yeast extract (4 g), agar (20 g), N-free medium—per liter sucrose (20 g), K_2HPO_4 (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.2 g), K_2SO_4 (0.1 g), $CaCO_3$ (5 g), agar (20 g), Czapek-dox medium—per liter sucrose (30 g), $NaNO_3$ (3 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), agar (15 g), and King's B medium—per liter peptone (20 g), K_2HPO_4 (1.5 g), $MgSO_4 \cdot 7H_2O$ (2.8 g), glycerol (8 mL), agar (15 g). The pH was adjusted to 6.8–7.2.

After 3–5 days of incubation at 28 °C, different colonies were selected from the cultivated plates and repeatedly inoculated into agar plates until pure cultures were obtained. Total numbers of counted microorganisms were reported as CFU mL^{-1} for suspended microflora and CFU g^{-1} for immobilized microflora. The ratios of different bacterial types in the cultivated microbial community were estimated by identification of each colony on Petri dishes using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The ability of dominant isolates of the bioreactor's microbial community, which were obtained during the study, to degrade wastewater compounds was assessed by their growth in a liquid medium using one of the main components of SPO wastewater as a single carbon source for 5 days. For this purpose we used a mineral medium (g L^{-1}): $(NH_4)_2SO_4$—1, $MgSO_4$—0.25, KH_2PO_4—3, $Na_2HPO_4 \cdot 12H_2O$—4.5 (pH 6.8–7.2). The main components of SPO wastewater were added to the mineral medium in the following scheme: propylene glycol—10 g L^{-1}, dipropylene glycol—10 g L^{-1}, the remaining components (ethylene glycol, diethylene glycol, styrene, 1-phenyl ethanol, acetophenone, benzene, toluene)—2 g L^{-1}. We also assessed the ability of these strains to biodegrade the components of SPO wastewater by assessing their growth in the influent wastewater. Optical density values at 600 nm were used to monitor growth. We used −, + and ++ for OD_{600} values of less than 0.1, 0.1–1, and more than 1, respectively. All the experiments were performed in triplicate.

2.5. Identification of microorganisms

The dominant isolates chosen from the microbial community and occupying a major fraction of the total in the bioreactor were identified. These isolates were identified using molecular-genetic analysis of 16S rRNA gene sequences. Genomic DNA from pure cultures of microorganisms was isolated using phenol-chloroform extraction (Maloy, 1989). The DNA concentration of the resulting solution was

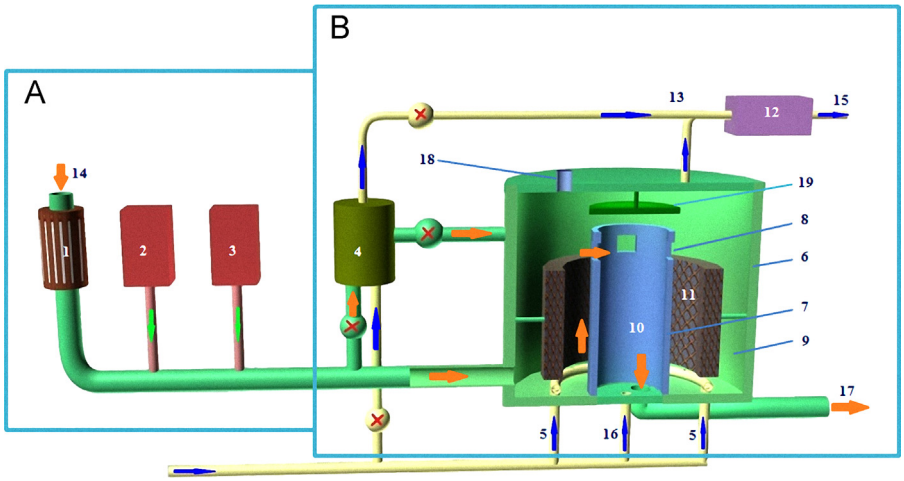

Fig. 1. Scheme of the biological pretreatment system for SPO production wastewater: (A) preparation block; (B) biological treatment block; 1—heat exchanger; 2—acid feed tank; 3—biogenic substances feed tank; 4—fermenter; 5—air for aeration; 6—bioreactor; 7—annular partition; 8—windows; 9—zone of aeration; 10—zone of flotation; 11—immobilized material; 12—biological filter; 13—waste air; 14—influent wastewater; 15—purified air; 16—air for flotation; 17—effluent; 18—window for sampling; 19—defoamer.

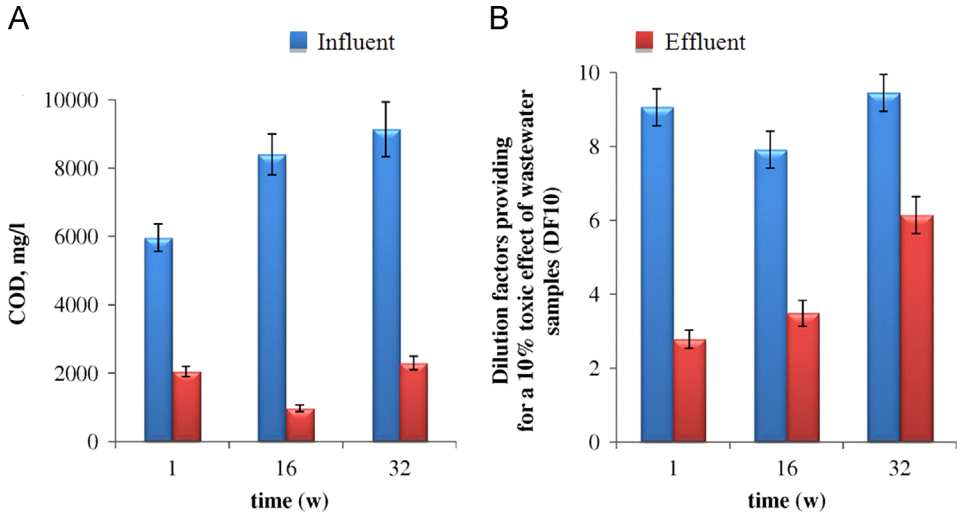

Fig. 2. Evaluation of chemical and toxicological parameters of the plant. (A) Chemical oxygen demand (COD) of the influent and effluent samples. (B) Dilution factors providing for a 10 percent toxic effect of wastewater samples (DF10) in a test with the infusorian *Paramecium caudatum*.

evaluated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The samples were stored at −20 °C. A bacterial 16S rRNA gene fragment was Polymerase Chain Reaction (PCR)-amplified using the universal bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′–ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). PCR was performed using a MJ Mini Gradient Thermal Cycler (Bio-Rad, USA) amplifier. The PCR program was as follows: denaturing step of 95 °C for 2 min, followed by 35 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension for 2 min at 72 °C, followed by a final extension at 72 °C for 7 min. The nucleotide sequences of the 16S rRNA gene fragments from the pure cultures were analyzed by the Synthol Company (Moscow, Russia) using an automatic capillary sequenator. The obtained 16S rRNA gene sequences of the isolates were compared with those in the NCBI database using the BLAST software package (http://blast.ncbi.nlm.nih.gov). The DNA sequences were aligned using the CLUSTAL W function of the BIOEDIT program (version 7.0.4) (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999). A phylogenetic tree was constructed using the maximum likelihood method and the PHYLIP software package (version 3.5) (http://evolution.genetics.washington.edu/phylip/getme.html). The TreeView program was used for visualization (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

MALDI-TOF MS analysis was performed using a Bruker Biotyper system (Bruker Daltonics, Germany), which included a Microflex LT instrument and FlexControl, Biotyper 3.0 program software. Single colonies were identified by direct smearing onto a ground steel target. One microliter of chemical matrix (saturated solution of alpha-cyano-4-hydroxycinnamic acid in 50 percent acetonitrile and 2.5 percent trifluoroacetic acid) was added to each sample and dried at room temperature. Mass spectra were recorded according to the manufacturer's instructions. The obtained spectra were compared with reference spectra in the integrated database. Scores greater than 2.0 were used for identification to the genus and species level.

2.6. Chemical analysis

The COD was determined by the standard dichromate method. The main components of SPO wastewater were determined by GC–MS. The GC/MS analyses were performed using a Fisons MD 800 mass spectrometer operated in EI mode to 70 eV. The source temperature was 240 °C. The gas chromatograph was equipped with a capillary column WAX 52 (30 m × 0.25 mm) with 0.25 μm film thickness. The temperature was programmed from 90 °C (1 min) to 120 °C at 10 °C/min and then to 200 °C at 3.5 °C/min and the final oven temperature of 250 °C (keeping this temperature for 11 min). The efficiency of the bioreactor's operation was estimated by the reduction of COD level and the components concentration (individual peak square was measured by GS–MS) of the effluent in comparison with those in the influent wastewater. All the experiments were performed in triplicate.

2.7. Toxicity tests

Toxicity was estimated by the death of infusoria (*Paramecium caudatum*) upon a 1-h exposure to the samples in comparison with dechlorinated water used as the

control. In this test the dilution factor of a sample giving a 10 percent toxic effect was used. The probit analysis method was used to determine the dilution factor changing the toxicity of a water extract by 10 percent (DF10) (Petrov et al., 1998). All the experiments were performed in triplicate.

3. Results and discussion

3.1. Wastewater chemical characteristics

The measurement of COD values during three occasions in the one year study period significantly exceeded the typical domestic wastewater values and varied from 5965 to 9137 mg L^{-1} (Fig. 2A). According to the literature, for small, medium, and highly polluted domestic wastewaters the COD value is 250, 430 and 800 mg L^{-1}, respectively (Tchobanoglous et al., 2003), while the COD value of low polluted industrial wastewater is less than 1000 mg L^{-1} and the COD value of highly polluted water is over 4000 mg L^{-1} (Chan et al., 2009). Consequently, the SPO wastewater is classified as extremely highly polluted. For petrochemical wastewaters CAS treatment is usually used at COD level of no more than 1500 mg L^{-1} (Chan et al., 2009), so SPO wastewater requires dilution or a pretreatment.

The composition of the studied wastewater is variable depending on the season and the facility's operation. The main components were propylene glycol, dipropylene glycol, 1-phenyl ethanol, benzene, acetophenone, styrene, and phenol. All of these compounds can be microbially biodegraded (Van Hamme et al., 2003). Among them, propylene glycol and ethylene glycol are capable of being degraded by a variety of soil, water, and sewage microorganisms (Miller, 1979). According to Miller's research (1979), under different testing conditions, complete degradation of these glycols usually occurred within 3–20 days. Singh and Celin revealed that benzene, toluene, ethyl benzene, and xylene (BTEX) can be biodegraded at a concentration of 0.5–5.0 percent (Singh and Celin, 2010).

In addition to the bioavailability of wastewater components, the toxicity index is an important and integral indication as well. Our study with the undiluted influent wastewater revealed complete mortality of infusoria after a 1-h exposure, demonstrating very high toxicity (100 percent). Using the probit analysis method we determined that the DF10 values of the influent wastewaters varied from 8.0 to 9.5 (Fig. 2B). Despite the low toxicity of glycols (the main components of the wastewater), SPO wastewater demonstrates a high toxicity due to its highly concentrated composition (Sánchez-Meza et al., 2007) and the presence of other toxic components (methanol, ethanol, propanol, *n*-butanol, benzyl alcohol, phenol, benzaldehyde, benzene, toluene, and acetone) (Katritzky et al., 2010) as well as their synergistic effect. For example, according to Hitchcock, the toxicity of a mixture of low toxic effluent wastewaters was higher than the individual ones (Hitchcock et al., 1997). Consequently, SPO wastewater is not suitable for a CAS process because it causes the death of protozoa.

The clean up efficiency depended on the duration of bioreactor operation after initiation. In general, WWPTP allowed the COD level to be reduced to 2500 mg L^{-1} or even below up to 1000 mg L^{-1} (Fig. 2A). Clean up efficiency may be affected by many factors, such as the wastewater's chemical composition, organic loading, as well as the growth of the microbial community. Thus, clean up efficiency of the bioreactor was 66 percent in the initial period, while it was increased by up to 88 percent in two later periods. This is probably due to growth of the immobilized microflora. Furthermore, the system remained relatively stable even on the background of increased initial loading. Similar results were obtained when using bioreactors with immobilized microflora in the crude oil processing industry (Sekoulov and Brinke-Seiferth, 1999).

The pretreatment system generally allowed the influent wastewater's toxicity to be reduced by 1.5–3 fold. However, the toxicity reduction determined at the three different periods did not correlate with the corresponding COD reduction. This has also been reported before (Barbusinski, 2005). This phenomenon can be related to the instability of the polluted wastewater's composition, different biodegradation, sorption, and volatilization capability of wastewater components. Furthermore, in some cases chemical oxidation may even lead to increased toxicity due to the formation of more toxic oxidation by-products (Bowers et al., 1989; Wang et al., 1989). Therefore, the greatest reduction in COD in the output of the WWPTP processing SPO wastewater, such as in the 16th week, did not bring a correlative reduction in toxicity (Fig. 2A and B). Analysis of the chemical components of the influent and effluent wastewaters in the 16th week showed that the fractions of propylene glycol, ethylene glycol, acetaldehyde, benzaldehyde, methyl propyl ketone, hexanone, hydroxypropanone, *n*-butanol, and benzene were reduced up to 100 percent, while highly toxic components such as propanol, acetone, and ethanol were reduced by 79.54 percent, 79.79 percent, and 99.88 percent, respectively. Reduction of the remaining highly toxic components was around 51–61 percent (benzyl alcohol—51.21 percent, ethoxy propanol—53.44 percent, methanol—53.47 percent, allyl alcohol—56.34 percent, and phenol—61.21 percent).

Generally, preliminary treatment of highly polluted wastewater from SPO production can reduce the toxicity and the COD level of the influent wastewater (the DF10 and COD levels of the effluent wastewaters varied from 2.7 to 6.1 and 969 to 2300 mg L^{-1}, respectively) and make the effluent more available for further treatment in a CAS process. However, the influent wastewater still needs to be diluted to reduce its toxicity level before entering the CAS process.

3.2. Microbial community analysis

The initial numbers of suspended microorganisms in the bioreactor (at the first week after startup) was comparatively high (0.73×10^8 CFU mL^{-1}) in comparison with those in the activated sludge (Sun et al., 2013). Eighteen different culturable strains of aerobic heterotrophs were identified in culture samples from the bioreactor; among them, ten dominant representatives were revealed (Fig. 3A). This parameter showed specific features of the microbial community, while activated sludge is characterized by higher taxonomic ranks (Ibarbalz et al., 2013). The dominant bacterial strains, such as *Citrobacter amalonaticus* DD2, DD5, DD17b, *Burkholderia cepacia* DD68, *Pseudomonas putida* DD1, *Paracoccus versutus* DD4b, *Lysinibacillus fusiformis* DD17, *Raoultella planticola* DD30, *Morganella morganii* DD9, and *Stenotrophomonas maltophilia* DD52 were isolated for subsequent determination of their biodegrading abilities (Table 1). The phylogenetic position of these bacterial strains, as well as *Achromobacter* sp. DD75, *S. maltophilia* DD73, *C. amalonaticus* DD29, DD74, *Brevibacterium* sp. DD14b, and *Kocuria rosea* DD22, were determined (Fig. 4). Besides the major isolates (aerobic heterotrophs), we also isolated different strains such as yeasts, diazotrophs, and fungi. Their quantities in the bioreactor were not so significant and accounted for 10^4–10^5 CFU mL^{-1} in comparison with a total heterotroph population of 10^7–10^8 CFU mL^{-1}. The presence of yeast and fungi in the community, as well as the increase in the number of microorganisms during bioreactor operation indicated a low toxicity of the studied wastewater in relation to microorganisms, which in turn led to an increase in the number of microorganisms during bioreactor operation. Despite the high organic load of 8399–9137 mg L^{-1} in the 16th–32nd weeks, the number of aerobic heterotrophs increased by up to 7.5–9 fold. However, there was a significant change in the community's composition. We

discovered a trend toward a narrow diversity of species and changes in the ratios of the dominant representatives. Such species as *C. amalonaticus*, *B. cepacia*, *P. putida*, *P. versutus*, and *K. rosea* were either not detected or were present in only small numbers in the bioreactor in the first week, but after the 16th and 32nd weeks they became more widespread (Fig. 3). Small changes in the quantities of different groups of microorganisms and the emergence or disappearance of the minor groups during biological wastewater treatment have been reported before. For instance, it has been shown for activated sludge, treating wastewaters with a biochemical oxygen demand (BOD) level of 153–288 mg L^{-1}, that the bacterial community structure changed an average of 20.4 ± 11.2 percent every 15 days (Wang et al., 2011). Another study showed that different loading rates do not cause evident changes in the bacterial community in two-stage constructed wetlands treating industrial wastewaters (COD 1758 ± 138 mg L^{-1}), but the stage positioning (first or second unit in the series) seemed to have a major effect on the dynamics of the bacterial communities (Calheiros et al., 2009).

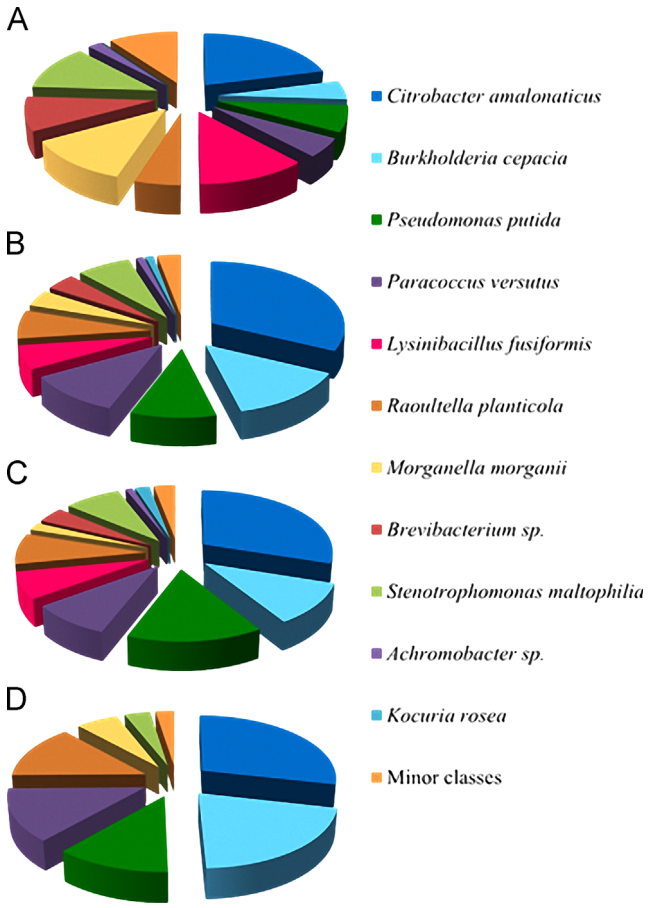

Fig. 3. Ratio of suspended (A–C) and immobilized (D) cultivated heterotrophic bacteria in the bioreactor based on Biotyper data (A) in the first week after startup of the plant (0.73×10^8 CFU mL^{-1}), (B) in the 16th week (5.50×10^8 CFU mL^{-1}), (C) in the 32nd week (6.58×10^8 CFU mL^{-1}), and (D) in the 40th week (4.00×10^{11} CFU g^{-1}). Rare classes with less than 1 percent abundance are grouped as "Minor classes".

In our study the dependence on loading rates seemed to be more significant and the community structure changed in terms of the predominance of certain species (*P. putida*, *P. versutus*, *C. amalonaticus*, and *R. planticola*) in samples with higher COD values (COD 9137 mg L^{-1}), whereas strains of *M. morganii* and *L. fusiformis* were typical for relatively lightly loaded samples. Most probably, these bacteria do not play a key role in the treatment of SPO wastewater.

The bacterial community structure stayed relatively stable after the 16th week, in which the predominant species in the microbial community were: *C. amalonaticus* (30–32 percent), *B. cepacia* (11–14 percent), *P. putida* (10–15 percent), *P. versutus* (9–11 percent), *L. fusiformis* (6–7 percent), *R. planticola* (7–8 percent), *S. maltophilia* (7–8 percent), *M. morganii* (2–4 percent), *Brevibacterium* sp. (4 percent), *K. rosea* (1–2 percent), and *Achromobacter* sp. (1 percent). This stability has been primarily achieved through retention of biomass on the immobilized carrier of the bioreactor (Fig. 3B and C).

The immobilized microbial community contained the same dominant isolates as those of the suspended microbial community, such as *C. amalonaticus* (28.5 percent), *B. cepacia* (21 percent), *R. planticola* (13.5 percent), *P. versutus* (13.25 percent), *P. putida* (12 percent), *M. morganii* (5.75 percent), and *S. maltophilia* (3.5 percent). The number of the immobilized microbial community was 4.00×10^{11} CFU g^{-1}.

Various studies in the literature have noted that activated sludge from wastewater treatment plants is usually characterized by heterotrophic bacteria from the *Pseudomonas*, *Chromobacter*, *Achromobacter*, *Alcaligenes*, and *Flavobacterium* genera (Mara and Horan, 2003). In other work it was found that bacteria of the *Pseudomonas*, *Flavobacterium*, *Comamonas*, *Cytophaga*, *Acidovorax*, *Sphingomonas*, *Bacillus*, and *Acinetobacter* genera are included in the activated sludge from a wastewater treatment plant processing wastewater from a petrochemical plant (COD of 900 mg L^{-1}) containing *n*-alkanes, aromatics, and polycyclic hydrocarbons (Shokrollahzadeh et al., 2008). In the work of LaPara et al. (2000) the same phyla—*Proteobacteria* (α-proteobacteria, β-proteobacteria, γ-proteobacteria, and δ-proteobacteria subdivisions) was dominant in the microbial community, participating in highly toxic pharmaceutical wastewater treatment.

In our study we identified the major culturable isolates and the result of phylogenetic analysis showed the diversity at the phylum

Table 1
Metabolic potential of the dominant isolates referring to the main components of the SPO wastewater (growth in liquid medium by OD_{600}).

The dominant isolates	PG	DG	EG	DG	Styrene	PE	ACP	Benzene	Toluene	Influent wastewater
Citrobacter amalonaticus DD5	+	+	++	−	−	−	−	−	−	−
Citrobacter amalonaticus DD17b	+	+	+	+	−	−	+	−	−	−
Citrobacter amalonaticus DD2	+	+	−	−	+	+	−	−	−	+
Burkholderia cepacia DD68	+	+	+	−	−	−	−	−	−	+
Pseudomonas putida DD1	++	++	++	+	−	−	−	−	−	++
Paracoccus versutus DD4b	++	+	++	+	−	+	−	−	−	+
Lysinibacillus fusiformis DD17	+	+	+	−	−	+	+	−	−	+
Raoultella planticola DD30	+	+	+	−	−	−	+	−	−	+
Morganella morganii DD9	++	+	−	−	−	+	−	−	−	−
Stenotrophomonas maltophilia DD52	+	+	+	−	+	−	−	−	−	−

PG, propylene glycol; DG, dipropylene glycol; EG, ethylene glycol; DG, diethylene glycol; PE, 1-phenylethanol; ACP, acetophenone; −, no growth; +, growth; ++, high growth.

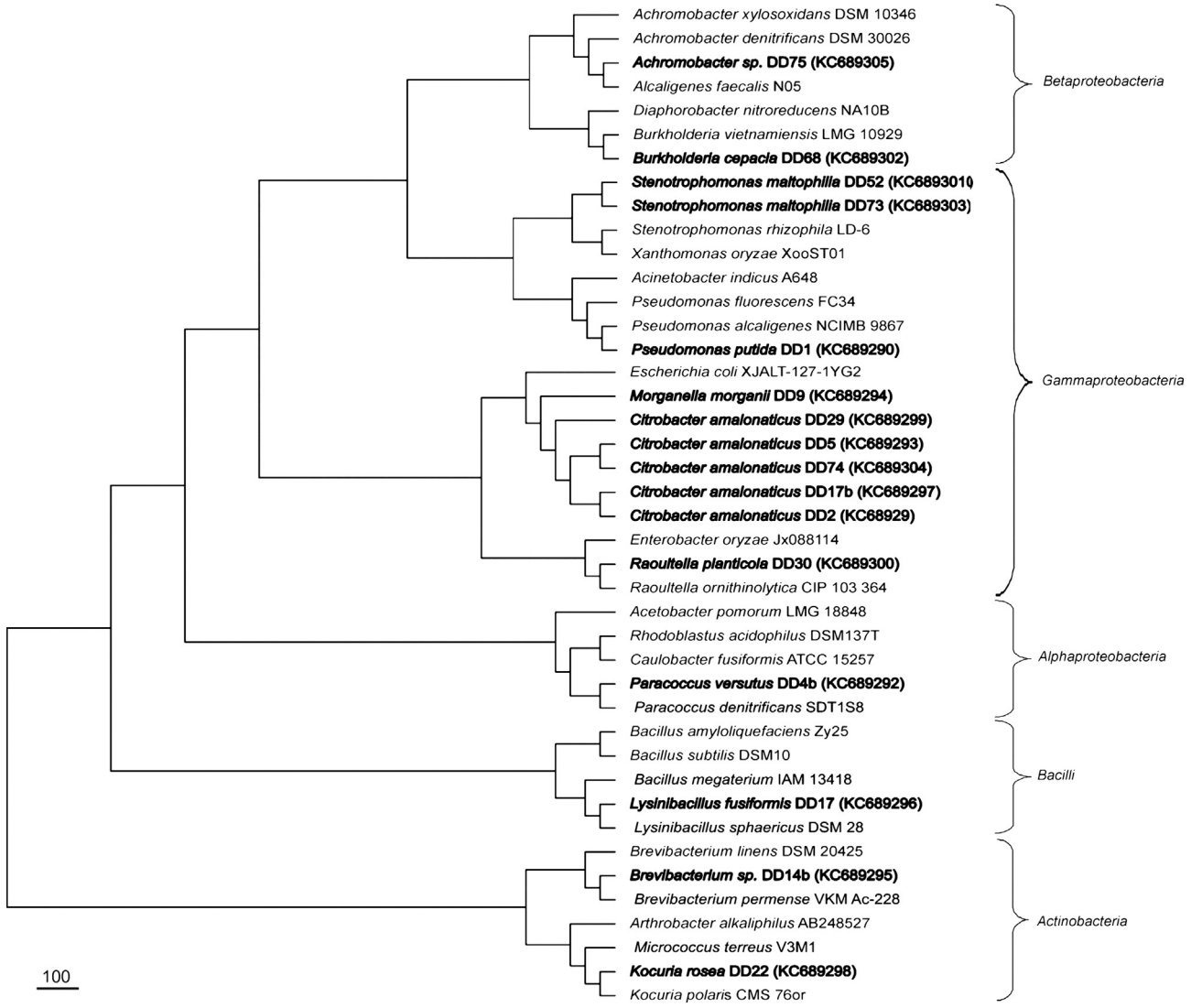

Fig. 4. Phylogenetic analysis of dominant cultivated isolates based on 16S rRNA genes.

level (Fig. 4). The most important isolates were distributed in the *Proteobacteria*, *Bacilli*, and *Actinobacteria* phyla. The actual diversity of the bioreactor's microbial community could be significantly higher due to the presence of uncultivated microorganisms that we have not analyzed. However, since the density of culturable microorganisms was so high (10^8 CFU mL^{-1}), we proposed that they play a major role in the treatment process.

In comparing the community structures of different plants treating municipal or industrial wastewaters, it is obvious that correlation is usually observed on the class taxa, and less often on the family and genus taxons (Mara and Horan, 2003; Shokrollahzadeh et al., 2008; LaPara et al., 2000). Additionally, not only is the species composition important for wastewater treatment but the specific characteristics of individual isolates are as well.

In our study we showed a distribution of metabolic activities of isolated strains in relation to wastewater compounds (Table 1). This is a good sign, i.e. that almost all dominant isolates can grow on the influent wastewater even with small growth. Most studied strains showed higher growth in propylene glycol and dipropylene glycol than in the remaining glycols or aromatic hydrocarbons. Assessment of biodegradability of fuel systems icing inhibitors performed by Meshako et al. (1999) indicated that diethylene glycol derivatives were more toxic than dipropylene glycol for wastewater treatment plant microbial communities. It was shown that more toxic compounds, such as styrene, phenol, phenol-related, and other aromatic compounds, are capable of being degraded by *Pseudomonas* (Margesin et al., 2005). Other research showed that different soil isolates from oil contaminated sites can utilize benzene and toluene and be resistant to high concentration of these compounds up to 5 percent v/v in growth medium (Singh and Celin, 2010). Although none of the studied bacteria showed visible growth (by OD_{600}) in aromatic compounds (Table 1), the chemical analysis of SPO wastewater showed great reductions in these compounds during the pretreatment process. This can be explained by co-metabolic effects between wastewater components, which needs further research. Bacteria in the *Paracoccus*, *Pseudomonas*, *Morganella*, *Citrobacter*, and *Burkholderia* genera showed relatively high growth levels in our study (Table 1). However, there are differences in metabolic capacities within a single species as can be seen in different strains of *C. amalonaticus* (Table 1). Bacteria in the *Citrobacter* genus are typical inhabitants of industrial wastewaters and able to degrade aromatic compounds with different efficiency (Selvakumaran et al., 2008).

The dominant representatives of the bioreactor's microbial community such as *Pseudomonas*, *Burkholderia*, *Citrobacter*, and *Stenotrophomonas* are well known for their high adaptability and wide range of xenobiotic degradation activity (Van Hamme et al., 2003; Fritsche and Hofrichter, 2005; Salmerón-Alcocer et al., 2007).

Thus, not all representatives of the obtained microbial community are capable of biodegrading major wastewater components. However, a good reduction (up to 88 percent) in the COD load, including all organic compounds (both easy and hard to biodegrade) can be achieved under bioreactor treatment of highly polluted petrochemical wastewater.

4. Conclusions

The investigated WWPTP showed a principle applicability of biological approach for detoxification of extremely polluted wastewater and made the effluent wastewater compatible with the protozoa in the activated sludge, and therefore, suitable for further treatment by the CAS process. We established that the isolates of the *Citrobacter*, *Burkholderia*, *Pseudomonas*, and *Paracoccus* genera were dominant in WWPTP from SPO production during 40 weeks of monitoring the process and are capable of biodegrading the main components of the wastewater. The obtained data about microbial community structure and metabolic potential of single isolates is useful for improving wastewater pretreatment procedure by increasing the abundance of the isolates that are capable of degrading toxic and recalcitrant xenobiotics.

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