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ORIGINAL PAPER

Biodegradation of diesel fuel by a microbial consortium in the presence of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chloride homologues

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Abstract Fast development of ionic liquids as gaining more and more attention valuable chemicals will undoubtedly lead to environmental pollution. New formulations and application of ionic liquids may result in contamination in the presence of hydrophobic compounds, such as petroleum mixtures. We hypothesize that in the presence of diesel fuel low-water-soluble ionic liquids may become more toxic to hydrocarbon-degrading microorganisms. In this study the influence of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chloride homologues (side-chain length from C_3 to C_{18}) on biodegradation of diesel fuel by a bacterial consortium was investigated. Whereas test performed for the consortium cultivated on disodium succinate showed that toxicity of the investigated ionic liquids decreased with increase in side-chain length, only higher homologues (C_8–C_{18}) caused a decrease in diesel fuel biodegradation. As a result of exposure to toxic compounds also modification in cell surface hydrophobicity was observed (MATH). Disulphine blue active substances method was employed to determine partitioning index of ionic liquids between water and diesel fuel phase, which varied from 1.1 to 51% for C_3 and C_{18} homologues, respectively. We conclude that in the presence of hydrocarbons acting as a solvent, the increased bioavailability of hydrophobic homologues is responsible for the decrease in biodegradation efficiency of diesel fuel.

Keywords Biodegradation · DBAS · Diesel fuel · Hydrocarbons · Microbial consortium · Ionic liquids · Toxicity

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Introduction

Wide employment of ionic liquids (ILs) as valuable reagents and solvents in organic synthesis, catalysis, in construction of novel materials and other applications (Welton 1999; Gordon 2001; Olivier-Bourbigou and Magna 2002; Vaher et al. 2002; Seddon 2003;

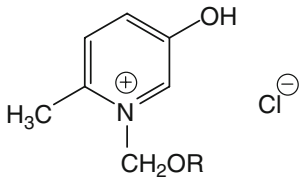
Rogers and Seddon 2002) will undoubtedly lead to environmental contamination. Because of practically no vapor pressure, ILs were often referred to as "green solvents", although their overall environmental impact has been a subject of only recent research (Kralisch et al. 2005; Zhang et al. 2008). Since ILs are relatively "new" chemicals, not much data regarding their influence on environmental processes are available. However, the claim that ILs are less harmful than classical solvents seems to be justified only in terms of air pollution. Over last years interaction of ILs with natural environment components is gaining more and more attention (Stepnowski et al. 2007; Modelli et al. 2008; Studzińska et al. 2008; Matzke et al. 2009) and for the purpose of risk assessment still more studies are required with respect to their ecotoxicological characteristics (Jastorff et al. 2005; Arning et al. 2008).

Low-melting-point salts (<100°C) composed of *N*-alkylpyridinium cation and an organic or inorganic anion belong to an important class of ILs. Theoretically, millions of pyridinium ILs can be synthesized with various combinations of cations and anions, and with various side-chain lengths attached to positively charged ring moiety (Wasserscheid and Welton 2002). Since the beginning of the twentieth century chemists have given considerable attention to design and synthesis of pyridinium chlorides or bromides because of their biocidal properties (Pernak et al. 2001a, b; Pernak and Chwała 2003). Most of performed research on the toxicity of quaternary ILs was conducted with the use of single species (Pernak and Branicka 2004; Pernak et al. 2004; Docherty and Kulpa 2005). This approach is fully justified because allows comparing chemicals, as well as microorganisms, to each other. However, from microbial ecology point of view selection of a single strain represent environmental conditions only in very little extend, as usually microbial communities consisting of various species of different metabolic activities are found in environmental samples (Maila et al. 2006).

In the present research we investigated toxicity of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides of various side-chain lengths (Table 1) to a hydrocarbon-degrading bacterial consortium and examined their influence on biodegradation of diesel fuel. A group of structurally similar ILs (1-alkoxymethyl-3-hydroxypyridinium chlorides) has been previously tested for their biological activity and it was concluded that the presence of hydroxyl group attached to the pyridinium ring contributed to their lower toxicity, as determined at cellular and molecular level (Stasiewicz et al. 2008), and limited their toxicity to various microorganisms (Pernak and Branicka 2003). The significance of side-chain length on toxicity of ILs has been previously indicated and a general trend of increasing toxicity with increasing chain length, and consequently lipophilicity, was observed (Ranke et al. 2004). However, some studies reported limited toxicity for various ILs with long side chains, containing fourteen, sixteen and more carbon atoms (García et al. 2001; Pernak and Branicka 2003; Pernak and Chwała 2003; Pernak et al. 2004; Cieniecka-Rosłonkiewicz et al. 2005). Although not always raised by the authors, this was probably an artifact from limited solubility of these compounds, which were not bioavailable for microorganisms to cause toxic effect.

It is possible that contamination with ILs occurs in the environment with significant fraction of hydrophobic medium constituents, as a result of waste disposal through wastewater streams, leakages of landfill sites or accidental spills. Together with the discoveries of new applications for ILs, contamination may also occur in the presence of petroleum hydrocarbons. ILs were already tested for their ability to upgrade crude oils (Fan et al. 2007; Siskin et al. 2008; Shi et al. 2008) and their use in desulfurization of diesel fuel hydrocarbons has been an area of intensive research (Bösmann et al. 2001; Gao et al. 2008; Liu et al. 2008; Mochizuki and Sugawara 2008). Thus it is important to investigate whether the presence of ILs is likely to affect biodegradation of petroleum hydrocarbons. For that purpose, diesel fuel biodegradation experiments in the presence of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chloride homologues, followed by investigation of the phase partitioning of investigated ILs, were conducted. A working hypothesis is that the biological activity of ILs and their toxicity increases in the presence of petroleum hydrocarbons, most probably as a result of enhanced bioavailability. These types of research are important to understand complex aspects of interaction of native microbial consortia with ILs, and give a new insight into their influence on natural biodegradation processes occurring in the environment.

Table 1 1-Alkoxymethyl-2-methyl-5-hydroxypyridinium chloride homologues used in the experiments

Molecular structure	Chemical name	R
	1-Alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides	$-C_3H_7$
		$-C_4H_9$
		$-C_5H_{11}$
		$-C_6H_{13}$
		$-C_7H_{15}$
		$-C_8H_{17}$
		$-C_9H_{19}$
		$-C_{10}H_{21}$
		$-C_{11}H_{23}$
		$-C_{12}H_{25}$
		$-C_{14}H_{29}$
		$-C_{16}H_{33}$
		$-C_{18}H_{37}$

Materials and methods

Microorganisms

Consortium used for this research was originally isolated from contaminated with crude oil soil, collected from the Polish Carpathian Mountains. In this location petroleum pollutants have been present for more than 100 years as a result of uncontrolled leakage from old oil wells. Isolation was performed using five-step enrichment with diesel fuel as sole carbon and energy source, employing modified procedure as described by Ito et al. (2008). Consortium was identified by restriction fragment length polymorphism of 16S rRNA gene amplicons and sequencing to consist of seven bacterial strains with the closest match to strains such as: *Pseudomonas alcaligenes*, *Ochrobactrum intermedium*, *Klebsiella oxytoca*, *Sphingobacterium multivorum*, *Pseudomonas putida*, *Chryseobacterium massiliensis*, *Stenotrophomonas maltophilia*.

1-Alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

1-Alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides (Table 1) were prepared by nucleophilic substitution of 2-methyl-5-hydroxypyridine (0.02 mol), dissolved in anhydrous acetone (25 ml), and chloromethyl alkyl ethers (0.022 mol). Chloromethyl alkyl ethers were prepared by passing HCl through a mixture of formaldehyde and the desired alkyl alcohols. The mixture was stirred and heated for 5 min, then cooled to 0–5°C, kept at that temperature for 2 h, and filtered. The filtrate was concentrated to dryness, washed with dry *n*-hexane, recrystallized from acetone or a mixture of water/acetone, and dried in a vacuum. The purity was assessed by the means of NMR and elemental analysis, performed at the Adam Mickiewicz University in Poznań (Poland). ^{1}H NMR spectra were recorded with a Varian model XL 300 spectrometer at 300 MHz with tetramethylsilane as the standard and ^{13}C NMR spectra were examined using the same instrument at 75 MHz. Cationic-active matter content was determined by two-phase titration according to ISO 2871-2:1990 (1990).

Calculation of octanol-water partition coefficient (log K_{OW}) for 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

Octanol-water partition coefficients (log K_{OW}) for 1-alkoxymethyl-2-methyl-5-hydroxypyridinium cations were calculated using KOWWIN software (www.vcclab.com). The structure of ILs was represented by pyridinium cation only, neglecting the presence of chloride anion and was entered using SMILES notation. KOWWIN software predicts log K_{OW} using a group contribution method proposed by Meylan and Howard (1995).

Diesel fuel and chemicals

Diesel fuel derived from petroleum was purchased from a petrol station belonging to PKN Orlen,

Poland, and was produced according to EN 590:2004. Before use, it was sterilized by filtration (Millex, pore size of 0.2 μm; Millipore). Diesel fuel contains ~15–22% of mono and two ring aromatic hydrocarbons, and various straight and branch-chain and cyclic alkanes of various chain lengths (Sjögren et al. 1995; Potter and Simmons 1998; Endo and Schmidt 2006). All other chemicals were reagent grade and obtained from commercial sources.

Utilization of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

In order to investigate possible biodegradation of ILs, series of agar plates (diameter 9 cm) covered with each of the ILs and pyridine serving as sole carbon sources were prepared (0.25, 0.5, 1.0, 2.5, 5.0, 10.0 mg per 1 ml of acetone) by spreading 1 ml of acetone solution onto solid agar surface (Kästner et al. 1994). Acetone was let evaporated and 50 μl of cell suspension dilutions pregrown for 24 h on 4 g l^{-1} disodium succinate or for 72 h on diesel fuel (0.5% v/v) were centrifuged, washed twice and resuspended in mineral medium prior spreading on the agar surface. Plates without ILs were serving as controls. Simultaneously, growth on diesel fuel was a reference. Plates were cultivated for 7 days at 30°C and enumerated.

Microbial growth inhibition in the presence of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

For the measurements of the toxic effects and the determination of EC50 values, investigated compounds were added to exponentially growing on disodium succinate cultures, as described by Heipieper et al. (1995). The concentration of disodium succinate was 4 g l^{-1}. We used this water soluble compound as a sole carbon source to observe effect exhibited by ILs on cells grown on water soluble substrate, excluding possible interferences between cells, ILs, and non aqueous phase liquid, which would be the case if diesel fuel had been used in toxicity test. Nevertheless, effect exerted by ILs on cells grown on diesel fuel will be examined later in biodegradation test. Stock solutions in acetone were used for the short-chain homologues. Due to restricted water solubility of ILs with chain length higher than C_7 it was not possible to determine their actual EC50 values. Growth inhibition caused by toxic compounds was measured by comparing the differences in growth rate between cultures with ILs ($\mu_{1,\ tox}$) with that of control cultures ($\mu_{0,\ control}$), using formula $[\mu_{1,\ tox}/\mu_{0,\ control}] \times 100\%$. Concentration that caused 50% growth inhibition was expressed as EC50. Microbial growth was monitored by measuring OD at 620 nm (Shimadzu, 1601PC, Japan). Cultures with and without acetone lacking ILs were serving as controls. No significant influence of acetone supplementation on microbial growth was found. Toxicity test were performed for 6 h, with measurements taken every hour.

Growth conditions for diesel fuel biodegradation tests

Stock cultures contained cell suspension of the consortium from the enrichment flask (1 ml) and diesel fuel (2.0% v/v) and were cultivated at pH 7.0 in a mineral medium as described by Hartmans et al. (1989). After 48 h, appropriate amounts of cell suspension were used for the inoculation of the final culture, with starting optical density of the final culture set to fit 0.1 (measured at 620 nm). Final biodegradation experiments were performed in 300-ml Erlenmeyer flasks containing 50 ml medium supplemented with diesel fuel (2.0% v/v). Samples were incubated at 25°C and shaken at 120 rpm for 7 days. For experiments with 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides for ILs up to C_7 their actual EC50 concentrations was used, whereas 20 μM concentrations were used for the C_8–C_{18} homologues. Simultaneously with biodegradation experiments blanks were investigated for systems: diesel fuel with and without ILs without microorganisms, and mineral medium with microorganisms. The final results have been corrected for the blanks.

Determination of diesel fuel

After 7 days of diesel fuel biodegradation the whole cultivation broth was subjected to residual biomass and hydrocarbons determination. At first the whole broth was centrifuged to separate biomass (15 min at 10,000×*g*). Biomass was washed twice with 10 ml of acetone to remove traces of diesel fuel. The residual

aqueous phase was subjected to double extraction with ethyl acetate. After extraction, organic phase was dried with anhydrous $MgSO_4$, and dripped through a drain (Filtrak 388). After evaporation of ethyl acetate the residual yellowish oil was subject to double extraction with 70% ethanol water solution to remove cationic matter originating from ILs. Diesel residuals were combined with acetone solution of diesel fuel, later resolved in hexane and evaporated. The efficiency of diesel biodegradation was calculated as $[(X_o - X_1)/X_o] \times 100\%$, where X_o is the initial amount of diesel, X_1 is the amount of diesel after biodegradation. This is Polish standard method for gravimetric determination of hydrocarbons (PN-86C-04573/01, 1986), which allows recovering 95% of diesel oil (2.0% v/v) from the control with biomass, with detection error of $\sim$5%. Biodegradation experiments were performed in triplicates; values for biodegradation were calculated as a mean value out of three flasks.

Division of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides between water and diesel fuel phase

1-Alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides content in water and diesel fuel phase was assessed after 7-day biodegradation process. Following systems were investigated: (i) whole cultivation broth, (ii) aqueous phase after centrifugation of the whole cultivation broth, (iii) 70% ethanol water solution resulting from double extraction of diesel fuel phase carefully separated after centrifugation of the whole cultivation broth.

The first system (i) was used for direct 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides content determination without any further preparation. System (ii) was obtained by centrifugation of the whole cultivation broth at $10{,}000 \times g$ for 15 min (Eppendorf R 4501), vials were kept for 24 h at −15°C and solid diesel fuel hydrocarbons were collected from vials. The vials were then kept at room temperature and after melting were subject to the same procedure once again. Later aqueous phase was used for 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides determination. System (iii) was designed to determine the amount of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides which accumulated in diesel fuel after centrifugation of the whole cultivation broth. A separate set of experiments for estimation of the distribution of ILs between diesel fuel and water in the absence of biomass was conducted. Partitioning index was calculated from formula $[C_{oil}/C_{aq}] \times 100\%$, where C_{oil} is concentration in oil phase, and C_{aq} is concentration in aqueous phase.

Determination of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

ILs were analyzed by the disulphine blue active substances (DBAS) method according to the modified procedure for the determination of cationic surfactants (Waters and Kupfer 1976). Samples (i, ii, or iii) were added to separatory funnel and pH was adjusted to 10 with NaOH using phenolphthalein as indicator. The pink colour was discharged by addition of 0.5 M H_2SO_4. Next, 15 ml $CHCl_3$ and 5 ml disulphine blue reagent were added and funnel was shaken vigorously and later let the phases separate. The $CHCl_3$ phase was drawn off into a second separatory funnel, and the funnel was rinsed with 2 ml $CHCl_3$. Two-step extraction was performed using 10 ml $CHCl_3$ each time. Combined extracts were supplemented with wash solution and later shaken vigorously for 1 min. $CHCl_3$ layer was filtered through a funnel containing a plug of glass wool, to make the filtrate clear. Glass wool and funnel was rinsed with $CHCl_3$. Absorbance of disulphine blue base form with cationic surfactant in $CHCl_3$ was determined against a blank of $CHCl_3$ at 628 nm (Spectrophotometer UV–Vis V—530 Jasco, Japan). Corresponding amounts of surfactants were calculated from a calibration curve.

Cell surface hydrophobicity

Cell surface hydrophobicity was assessed by the microbial adhesion to hydrocarbons method (MATH). The experiments were carried out using modified procedure described by Rosenberg et al. (1980). Cells were grown on diesel fuel, diesel supplemented with 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides, or ILs alone. These experiments were carried out at 7th day of biodegradation process. After centrifugation, for each optical density determination measurement, cells were washed twice with a PUM buffer (g l^{-1}; K_2HPO_4, 19.7; KH_2PO_4, 7.26; H_2NCONH_2, 1.8; $MgSO_4 \times 7H_2O$, 0.2) and suspended in the PUM

buffer to fit an optical density of 1.0 (A_o), measured at 620 nm. Next, 500 µl of hexadecane was added to 5 ml of microbial suspension which was vortexed for 2 min. After 15 min the optical density of the aqueous-phase was measured (A_1). Cell surface hydrophobicity was calculated from formula $[(A_0-A_1)/A_0] \times 100\%$.

Results

Synthesis and properties of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

1-Alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides with the desired side alkyl chain were synthesized with average reaction yield of 80% (data not shown). Satisfactory elemental analyses of CHN for all synthesized ILs were acquired, with 0.43% tolerance between the calculated and experimental values (data not shown). The melting point temperature varied from 110 for the compound with C_3 side alkyl chain to 140°C for C_{18}. Results from the determination of cationic active matter content indicated that only compounds with side-chain C_3–C_6 do not present surface active properties (data not shown). For other homologues cationic active matter content was close to 100%.

Octanol-water partition coefficients (log K_{OW}) of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

Preliminary calculations showed significant variations among log K_{OW} values for each salt, with respect to the computing method used (data not shown). However, as in each case log K_{OW} almost linearly increased with side chain length, we decided to use results from KOWWIN software and use them as valid descriptor of ILs' properties (Table 2), although these computed values probably do not correspond precisely to real values.

Utilization of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

No microorganisms were found that were able to grow on any of the investigated ILs as sole carbon sources, regardless the carbon source used to prepare the inoculum for plating. For agar plates covered with diesel fuel, significant growth of microorganisms was observed, although it was not possible to perform enumeration. This indicates that the microorganisms applied in this experiment are not able to utilize any of the homologues as a source of carbon and energy.

Table 2 Properties of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

Substitute R	log K_{OW}[a]	EC50[b] (µM)
C_3H_7	0.42	2.03
C_4H_9	0.91	2.50
C_5H_{11}	1.40	8.93
C_6H_{13}	1.89	10.11
C_7H_{15}	2.38	18.02
C_8H_{17}	2.87	ND
C_9H_{19}	3.36	ND
$C_{10}H_{21}$	3.86	ND
$C_{11}H_{23}$	4.35	ND
$C_{12}H_{25}$	4.84	ND
$C_{14}H_{29}$	5.82	ND
$C_{16}H_{33}$	6.80	ND
$C_{18}H_{37}$	7.78	ND

ND not determined

[a] Calculated using KOWWIN program (available at www.vcclab.org)

[b] Determined experimentally

Toxicity of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

Results from determination of toxic effect exhibited by 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides to the consortium grown on sodium succinate showed that the most toxic were ILs with short chain attached to the pyridinium ring moiety (Table 2). The lowest EC50 value of 2 µM (corresponding to 0.44 mg l^{-1}) was determined for the C_3-homologue. With increase in side-chain length a decrease in toxicity was observed.

Diesel fuel biodegradation and cell surface hydrophobicity in the presence of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides

Previous tests conducted for water soluble compound such as disodium succinate showed that the most

toxic were the homologues with short alkyl chain attached to pyridinium ring (Table 2). Another experiment was performed to examine how supplementation of ILs affects biodegradation of diesel fuel. Here we used ILs at their EC50 concentrations, as determined for cells grown on disodium succinate. For the long-chain homologues, for which determination of EC50 was not possible, we used the concentration which was established as EC50 for the C7-homologue (20 μM). Results showed that surprisingly, the most toxic homologues did not affect significantly biodegradation of diesel fuel (Fig. 1), which for sole diesel fuel reached 70% of applied amount within 7 days. For the higher homologues (C_7–C_{18}), constant level of ~50% biodegradation was achieved.

Simultaneously with biodegradation experiments, cell surface hydrophobicity changes were measured in the presence of investigated ILs. Results showed that those ILs with surface active properties significantly altered cell surface properties of microorganism (Fig. 1), which corresponded to diesel fuel biodegradation. A decrease in cell surface hydrophobicity was observed for C_7–C_8 homologues, whereas for lowest homologues hydrophobicity remained at 68%. For the higher homologues average hydrophobicity was ~51%.

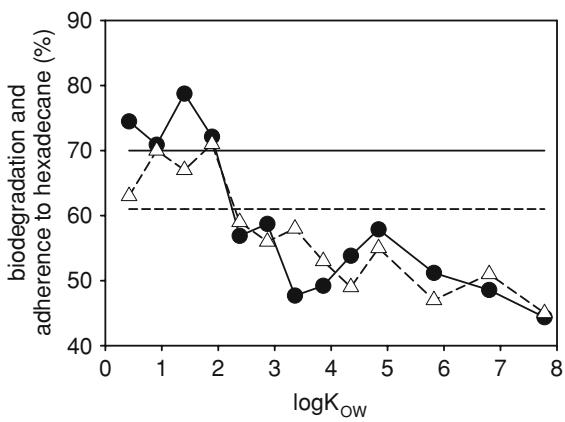


Fig. 1 Biodegradation (●) and adherence to hexadecane (Δ) after 7 days for bacterial consortium cultivated on diesel fuel in relation to calculated log K_{OW}; *solid line* refers to biodegradation without ILs, *dashed line* refers to hydrophobicity without ILs. Average standard error for biodegradation was ±6.0%; average standard error for adherence to hexadecane was ±5.3%

Division of 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides between water and diesel fuel phase

Investigating further the effect exhibited by 1-alkoxymethyl-2-methyl-5-hydroxypyridinium chlorides on hydrocarbons biodegradation, partitioning index of ILs between diesel fuel and aqueous phase was determined (Fig. 2). Results confirmed that division of ILs molecules correlated with their log K_{OW} values. First three homologues (C_3–C_5) had a strong affinity to water phase, whereas increase in log K_{OW} of ILs reflected higher affinity to the diesel fuel phase. However, homologues with long side chain (C_{12}–C_{18}) tend to divide between both phases rather then entirely dissolve in diesel fuel phase. Medium homologues (C_8–C_{11}) exhibited slightly lower affinity to diesel fuel in the presence of biomass, than for pure diesel–water system. This might have been an artifact from adsorption of those compounds on cell surface, however, partitioning index for higher homologues and results from cell surface hydrophobicity tests did not give straight indication that adsorption on cell walls indeed occurred.

Discussion

Biological activity of chemical compounds is related to their chemical structure (Hansch and Leo 1995). In

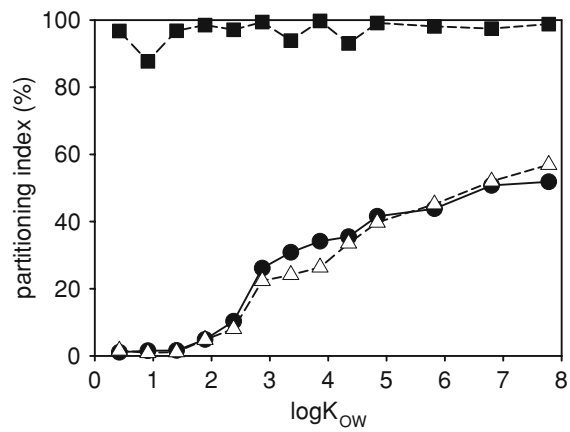


Fig. 2 Partitioning index of ionic liquids between diesel fuel and aqueous phase, determined after 7 days of biodegradation experiment; systems with biomass (Δ), control without biomass (●), total recovery of ILs (■). Average standard error was ±3.4%

case of ILs, parameters influencing their toxicity are the type of head group, the type of side chain attached to the cationic head and in some cased the type of anion moiety combined with cationic head (Ranke et al. 2004; Stock et al. 2004; Docherty and Kulpa 2005; Jastorff et al. 2005; Couling et al. 2006; Stolte et al. 2006, 2007a, b; Matzke et al. 2007). The most sensitive parameter influencing toxicity of ILs is the length of side chain, which is related to the overall lipophilicity of the compound (Ropel et al. 2005). Therefore in the present study, we characterized the IL homologues using computed log K_{OW} values, bearing in mind restrictions it has. As discussed by Belvèze (2004) the difference between log K_{OW} predicted by KOWWIN program (for structures where anion was attached to the cation) may be ten times lower than the measured value. However, based on linear relationship between chain length and log K_{OW}, we decided to use values predicted by KOWWIN program (calculated for cation only).

Toxicity dependency on lipophilicity can be explained by facilitated affinity of lipophilic molecules to the lipophilic membrane (Sikkema et al. 1995). Additionally, surface active properties of ILs may result in the increase in membrane permeability, causing narcosis effect and in extreme cases leading to membrane disruption and cell death (Bernot et al. 2005). It is important to mention here that membrane permeability is an important parameter influencing uptake of lipophilic compounds by microorganism, and in some cases explains mechanism of toxic action for cationic surfactants (Cross 1994; Roberts and Costello 2003). Generally increase in toxicity for ILs was observed with increasing chain length, as determined in standard toxicological assays to water organisms such as algae, crustaceans and bacteria (García et al. 2005; Stolte et al. 2007b). On the other hand, García et al. (2001) observed decrease in toxicity with increase in chain length for homologues of quaternary ammonium-based surfactants to marine bacterium *Photobacterium phosphoreum*. Similar pattern of decreased toxicity for longer side-chain homologues for cells grown on a water soluble carbon source was also observed in our studies. García et al. (2001) attributed this phenomenon to limited bioavailability of higher homologues due to solubility restrictions. Considering presented in this study results this explanation would find confirmation. In the presence of higher homologues of ILs, a remarkable decrease in diesel fuel biodegradation was observed, which was correlated with the chain length. As determined in the partitioning experiment, higher hydrophobicity of longer chain ILs makes them more favorable for solubilization in diesel fuel. Relatively high cell surface hydrophobicity ($\sim$60%, expressed as adherence to hexadecane) for cells grown on diesel fuel gave evidence that at least a part of the cells from the consortium may exhibit direct interfacial uptake mechanism of diesel fuel hydrocarbons (Bouchez-Naïtali et al. 1999). Therefore, we conclude that the decrease in biodegradation of diesel fuel was caused by enhanced bioavailability of toxic compounds, which could partition to the membrane together with hydrocarbons from the oil droplets. Significant amounts of diesel fuel in the culture flask (16.5 g/l) in relation to the amounts of ILs (maximum 8.56 mg/l for the C_{18}-homologue) ensure that mass transfer limitations caused by the presence of ILs (such as entrapment into micelles) were not likely to occur. Also for that reason, we do not attribute the cell-ILs interactions as a main reason for biodegradation decrease. Although modification of cell surface hydrophobicity may be altered in the presence of amphiphilic, surface active compounds, considering the cell densities used in biodegradation experiments and amounts of diesel fuel in the cultures, usually much higher amounts of surfactant are necessary to cause significant changes in cell surface hydrophobicity and/or degradation of hydrocarbons (Chrzanowski et al. 2009). It is important to note that change in cell hydrophobicity is amid few parameters which may be modified by cells to minimize toxic effect exhibited by chemical compounds (Sikkema et al. 1995; Isken and de Bont 1998). Modification of cell surface hydrophobicity in the presence of toxic compounds has been previously reported, and usually a pattern with decreasing hydrophobicity in the presence of toxic compounds was observed. Explanation for that is that is that less hydrophobic cells have lower affinity to toxic lipophilic compounds, thus alteration in cell hydrophobicity allows them to grow better under stress conditions caused by toxic compounds (Sikkema et al. 1995). In this context, we attribute the decreases in cell surface hydrophobicity and diesel fuel biodegradation to the toxic effect exhibited by ILs as a result of solubilization in diesel fuel. Decrease in cell surface hydrophobicity as a result of

the action of toxic compounds would be an additional, but not the main factor responsible for the decrease in diesel fuel biodegradation.

Another issue which should be raised here is possible biodegradation of ILs by the consortium of interest. As determined in preliminary experiments, neither of the homologues, nor pyridine, serving as sole sources of carbon and energy were utilized by microorganisms grown on agar plates. Therefore, it is very unlikely that investigated ILs were degraded in toxicity test, where cells were grown on readily available disodium succinate. On the other hand, it was previously observed that for pyridinium ILs, biodegradation might occur, especially for ILs with long alkyl chains supplemented as sole sources of carbon and energy (Docherty et al. 2007). Although high recovery of ILs after 7 day biodegradation process might not necessarily reflect their complete resistance to biodegradation by hydrocarbon-degrading microorganism, it is unlikely that in the presence of more readily utilized hydrocarbons from diesel fuel, the investigated ILs were degraded.

Presented in this study results indicate, that in the presence of hydrophobic medium constituents the influence of ILs on diesel oil biodegradation is negative. We attribute this effect to the enhanced bioavailability of the low-water-soluble ILs in a hydrophobic phase of the medium. The development of ILs as potentially ground-breaking chemicals will undoubtedly lead to the development of new formulations and will result in new applications. Although at current state of development and their presence in such mixtures might be scare, future applications and may lead to such formulations where ILs are combined with petroleum mixtures, or other hydrophobic solvents. As this study indicates, in case of environmental pollution such formulations might be less susceptible to biodegradation processes, and are likely to pose higher risk than predicted for single compounds. This should be considered in risk characterization of ILs. Future studies are aimed at investigating microbial community dynamics as a result of exposure to toxic ILs.

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