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Biodegradability of imidazolium and pyridinium ionic liquids by an activated sludge microbial community

Kathryn M. Docherty · JaNeille K. Dixon · Charles F. Kulpa Jr

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Abstract Ionic liquids (ILs) are novel organic salts that have enormous potential for industrial use as green replacements for harmful volatile organic solvents. Varying the cationic components can alter the chemical and physical properties of ILs, including solubility, to suit a variety of industrial processes. However, to complement designer engineering, it is crucial to proactively characterize the biological impacts of new chemicals, in order to fully define them as environmentally friendly. Before introduction of ILs into the environment, we performed an analysis of the biodegradability of six ILs by activated sludge microorganisms collected from the South Bend, Indiana wastewater treatment plant. We examined biodegradability of 1-butyl, 1-hexyl and 1-octyl derivatives of 3-methyl-imidazolium and 3-methyl-pyridinium bromide compounds using the standard Organisation for Economic Cooperation and Development dissolved organic carbon Die-Away Test, changes in total dissolved nitrogen concentrations, and ^{1}H-nuclear magnetic resonance analysis of initial and final chemical structures. Further, we examined microbial community profiles throughout the incubation period using denaturing gradient gel electrophoresis (DNA-PCR-DGGE). Our results suggest that hexyl and octyl substituted pyridinium-based ILs can be fully mineralized, but that imidazolium-based ILs are only partially mineralized. Butyl substituted ILs with either cation, were not biodegradable. Biodegradation rates also increase with longer alkyl chain length, which may be related to enhanced selection of a microbial community. Finally, DGGE analysis suggests that certain microorganisms are enriched by ILs used as a carbon source. Based on these results, we suggest that further IL design and synthesis include pyridinium cations and longer alkyl substitutions for rapid biodegradability.

Keywords Ionic liquids · Imidazolium · Pyridinium · Microbial biodegradability · Green chemistry

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Introduction

Over the past several years, there has been a dramatic increase in both academic and industrial interest in synthesis of novel ''green'' imidazolium-based and pyridinium-based ionic liquids (ILs). In an effort to address the Pollution

Prevention Act of 1990 and enhance the field of green chemistry, engineers have been developing ILs to replace conventional volatile organic solvents that contribute to air pollution which can result in photochemical smog, ozone depletion and global climate change. ILs are one class of chemicals that have potential as benign industrial alternatives. These organic salts have vanishingly low vapor pressures, are liquid at ambient conditions, and do not evaporate or cause air pollution (Brennecke and Maginn 2001). Substitution of ILs for traditional solvents could potentially improve environmental health while saving industry billions of dollars in future environmental mitigation and clean-up (Allen and Shonnard 2002).

Although dramatic reductions in air pollution would result from adopting these new solvents, water pollution may increase because many ILs are water soluble and will inevitably be released into wastewater, groundwater and aquatic environments. For example, the IL 1-butyl-3-methyl imidazolium chloride (bmimCl), is predicted to be minimally retained by geologic adsorption in non-interlayer clay systems, which would result in unimpeded transport of the chemical through subsurface groundwater (Gorman-Lewis and Fein 2004). A similar problem occurred with the use of methyl tertiary butyl ether (MTBE) in the late 1990s which was manufactured as a green chemical. MTBE, which ranked second (at 24 billion pounds produced) among all organic chemicals manufactured in the United States in 1993, was a green additive to gasoline to reduce engine knocking and auto emissions and improve air quality (USGS-NAWQA 1995). However, it was later found to have potential carcinogenic effects and to leach into and pollute groundwater. Because of its water solubility, MTBE plumes have been shown to extend well beyond the area of less soluble organic pollutant plumes in California (e.g., Scow 2002). As a result, MTBE use has been banned and expensive in situ bioremediation studies have begun using microorganisms found to degrade MTBE (Smith et al. 2005).

Ionic liquids have not been released into the environment on a large scale, but have been studied extensively and are currently being used in innovative commercial and industrial applications. They provide a perfect opportunity to take a proactive research approach and determine their biodegradability properties before water pollution becomes a problem. ILs are designed with large organic cations, such as imidazolium or pyridinium, with alkyl chain substituents that alter the hydrophobicity of the molecule (Fig. 1). In this study, we have focused on ILs with only the bromide (Br^-) anion, but other common anions include hexafluorophosphate (PF_6^-), tetrafluoroborate (BF_4^-), chloride (Cl^-) and nitrate (NO_3^-). ILs have been engineered to suit a variety of applications, including electrolytes, solvents in liquid–liquid extractions, acid-scavengers, and many reactions including hydrogenations, oxidations, Diels-Alder cycloadditions, Friedel-Crafts acylations and alkylations and Heck reactions (Allen and Shonnard 2002; Dupont et al. 2002; Liao and Hussey 1996; Marsh et al. 2002; Sheldon 2005; Sigma-Aldrich-Fluka 2001).

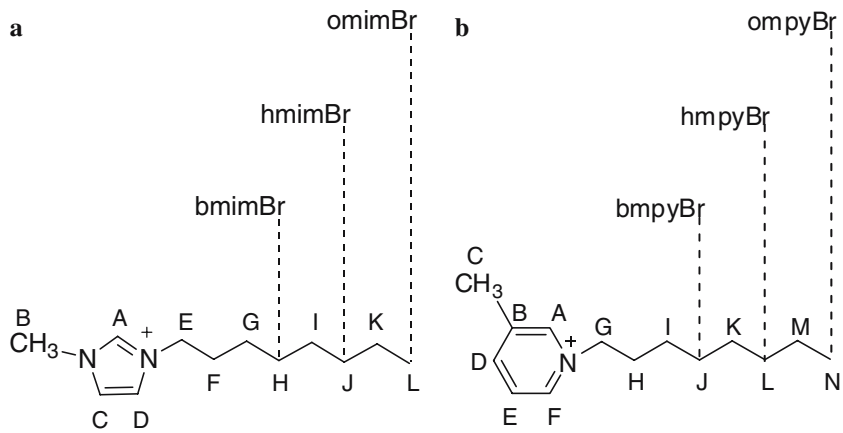

Fig. 1 Chemical structures of the six (**a**) imidazolium and (**b**) pyridinium ILs examined in this study. *Letters* correspond to carbon atoms indicated in the ^{1}H-NMR figures (Figs. 3, 6)

To address the issue of green chemical design and environmental safety, many studies in the past few years have examined the toxicity of a variety of ILs prior to widespread industrial use. Pyridinium, imidazolium and pyrrolidinium-based ILs have recently been nominated to the United States National Toxicology Program (NTP) for toxicological testing, based upon their widespread interest as possible alternatives to organic solvents (NTP-NIEHS 2004). The antibacterial potential of ILs has been the source of several recent publications. The most commonly observed trend is increasing toxicity to various bacteria with an increase in the C-1 alkyl chain length substituent in pyridinium, imidazolium and quaternary ammonium salts (Pernak and Chwala 2003; Pernak et al. 2001, 2004). This trend was also observed in ILs containing propyl-mim to decyl-mim cations and BF_4^-, PF_6^-, Cl^- and Br^- anions to the fluorescent marine bacterium, *Vibrio fischeri*, IPC-81 cells and C_6 glioma cells (Docherty and Kulpa 2005; Ranke et al. 2004). Toxicity tests examining the acute and chronic effects of ILs upon higher organisms are extensive and include: algae (*Scenedesmus* spp.), plants (*Lemna minor*), cladocerans (*Daphnia magna*), mollusks (*Physa acuta*, *Dreissena polymorpha*), nematodes (*Caenorhabditis elegans*) and fish (*Danio rerio*, *Pimephales promelas*) (Bernot et al. 2005a, b; Pretti et al. 2006; Swatloski et al. 2004). ILs have not been found to be significantly mutagenic in the Ames Test, however, no carcinogenicity studies exist to date (Docherty et al. 2006).

While rapid toxicity tests have provided a wealth of information leading to the design of less toxic ILs, very few studies exist examining the biodegradability of ILs. Full biodegradation by active microorganisms, which results in full mineralization of a compound to CO_2 and biomass, can yield completely non-toxic products from potentially persistent and toxic solvents. Combining knowledge about trends in IL toxicity as well as biodegradability and biodegradation pathway information is crucial to the green chemical design and synthesis process.

Recent studies have proposed theoretical biodegradation pathways for imidazolium cation ILs, which are based upon lipophilicity measurements and possibilities of the molecule reaching the cytochrome P_{450} of a cell and suggest that they are not readily biodegradable and will persist in the environment (Jastorff et al. 2003; Stepnowski and Storoniak 2005). In a recent experimental study, this prediction was verified. Gathergood et al. (2004) examined the biodegradability of ethyl ester and amide imidazolium ILs using standard Organisation for Economic Cooperation and Development (OECD) Sturm and Closed-Bottle test protocols, and found that ester imidazoliums were more biodegradable than amide imidazoliums (Gathergood et al. 2004). However, no imidazolium compounds tested could be classified as readily biodegradable according to OECD standards, which requires 60–70% or greater biodegradation by activated sludge microbial inoculate, within a 10-day window in a 28-day test period. Additionally, bmimBr, bmimPF_6 and bmimBF_4 were resistant to biodegradation, but the anion did not affect biodegradability (Gathergood et al. 2004). A later study examining imidazolium-based compounds containing an octylsulfate anion are the only ILs that have been examined thus far that exhibited any significant level of biodegradation and could be classified as readily biodegradable by OECD standards (Gathergood et al. 2006). Introduction of ester groups in the substituted side chain have also enhanced biodegradability of imidazolium-based ILs (Gathergood et al. 2006). Fewer studies have examined pyridinium-based IL biodegradation, but aerobic and anaerobic degradation pathways for pyridine are well known, suggesting that pyridinium-based ILs will be more readily biodegradable than imidazolium-based ILs (Kaiser et al. 1996). One study investigating 1-dodecylthiomethyl pyridinium Cl^- metabolism by activated sludge communities has shown complete ring mineralization (Grabinska-Sota and Kalka 2004).

In this study, we have examined the biodegradability of six ILs: 1-butyl-3-methyl imidazolium bromide (bmimBr), 1-hexyl-3-methyl imidazolium bromide (hmimBr), 1-octyl-3-methyl imidazolium bromide (omimBr), 1-butyl-3-methyl pyridinium bromide (bmpyrBr), 1-hexyl-3-methyl pyridinium bromide (hmpyrBr) and 1-octyl-3-methyl pyridinium bromide (ompyrBr). Our results suggest

that pyridinium ILs are generally more readily biodegradable than imidazolium ILs. Further, longer alkyl chain length compounds are more readily degradable than compounds with shorter alkyl chains.

Experimental

Synthesis

The materials used in IL synthesis, including source, grade and purification method (if any), are as follows: 1-bromobutane (Aldrich 99.5%, redistilled), 1-bromohexane (Aldrich ≥98.0%, redistilled), 1-bromooctane (Aldrich ≥98.0%, redistilled), 1-methylimidazole (Aldrich 99%, redistilled over KOH) and 3-methylpyridine (Aldrich ≥99.0%, redistilled over KOH).

All the ILs were synthesized in our laboratory using standard procedures (Bonhôte et al. 1996; Cammarata et al. 2001; Fredlake et al. 2004). Synthesis involved mixing equal molar amounts of a nitrogen base and alkyl halide in a flask then stirring the mixture overnight. Ethyl acetate was frequently used as a solvent for reactions for the synthesis of pyridinium-based ILs, but the imidazolium-based ILs were done neat. If solvent was used, it was removed from the IL under vacuum before purification. If the IL was a solid at room temperature, it was recrystallized from acetonitrile/ethyl acetate. We dissolved ILs that were liquid at room temperature in methylene chloride and stirred them over activated charcoal to remove any colored impurities. Nitrogen containing impurities were removed by passing the ILs through a column of acidic alumina. The solutions were then filtered and the solvent removed in vacuo. We confirmed the identity of all ILs by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR). NMR results indicate that amine impurities were below the detection limit.

Biodegradability

We used a modified OECD Guideline for Testing of Chemicals standard dissolved organic carbon (DOC) Die-Away Test to assess the biodegradability of six ILs: bmimBr, hmimBr, omimBr, bmpyrBr, hmpyrBr and ompyrBr (OECD 1992). An activated sludge microbial community collected from the South Bend Wastewater Treatment Facility was pre-conditioned by aerating at room temperature for 1 week. At the end of the aeration period, the total suspended solids of the sludge inoculate was determined to be 2.89 g L^{-1}. Three replicate 1-L bottles were prepared with sterile mineral medium containing: 0.085 g L^{-1} KH_2PO_4, 0.2175 g L^{-1} K_2HPO_4, 0.334 g L^{-1} $Na_2HPO_4{\cdot}2H_2O$, 5 mg L^{-1} NH_4Cl, 36.4 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 22.5 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 0.25 mg $FeCl_3{\cdot}6H_2O$, as per the OECD guidelines (1992). ILs were added in concentrations of 40 mg C L^{-1} of each of the six chemicals tested. We inoculated these with 10.38 mL of activated sludge sample, to yield a final concentration of 30 mg suspended solids per 1-L treatment flask. We inoculated three replicate 1-L flasks containing mineral medium with no-IL as biotic controls. Additionally, we created one replicate flask for each IL treatment with mineral medium and no bacterial inoculate, to control for abiotic degradation of the ILs. Finally, three replicate controls were prepared with sodium acetate as a carbon source, to test inoculate viability.

All 30 treatment and control flasks were shaken aerobically, at room temperature. We removed 10 mL of sample four times per week from all bottles for DOC concentration analysis. The samples were syringe filtered (0.22-μm) and acidified using 100 μL of 2 N HCl. We measured non-purgeable DOC and total dissolved nitrogen (TDN) concentrations within 48-h of collecting the samples by combustion on a Shimadzu TOC5000 with an autosampler. When a ≥ 20% decrease in DOC concentration was reached, we removed 10-mL samples every day for 14 days or until DOC concentrations became constant or did not decrease for ten measurement-days. After each test, DOC data were analyzed using repeated measures ANOVA, and then one-way ANOVA with a Tukey's pairwise comparison test at significant time points. For the TDN data, we calculated experimental-to-control ratios, used an arcsin square root transformation for normality and then used repeated measures ANOVA to determine differences through time.

At the time of inoculation with activated sludge, we prepared one set of 1-L bottles of inoculated IL-medium for each of the six ILs and one blank background inoculate, and filtered them immediately (0.22-μm) for initial NMR spectrum analysis. After the final incubation day, we removed the remaining IL treatments (~400 mL), which were also filtered. All 12 initial and final samples were rotary evaporated to remove water and then dissolved in 2 mL of D_2O. Resuspended samples were filtered through a 0.22-μm syringe filter, and then placed in NMR sample tubes. We analyzed ^{1}H- and ^{13}C-NMR spectra of the 12 samples using a Varian Unity*Plus* 300 spectrometer. ^{1}H-NMR spectra were analyzed at ambient temperatures. The spectra of the initial samples required no more than eight scans to produce a clean spectrum in the ^{1}H-NMR and typically 512 scans for the ^{13}C-NMR. The final samples were much more dilute than the initial samples and required between 16 and 32 scans for the ^{1}H-NMR. These samples were too dilute to achieve resolvable peaks from the baseline in the ^{13}C-NMR even allowing for nearly 24-h accumulation times.

Microbial communities

Once each week during the incubation period, we removed 50-mL sub-samples from each of the treatments for molecular microbial community characterization. Each sample was filtered (0.22-μm Nucleopore) and seston trapped by these filters was used for DNA extraction with an UltraClean Soil DNA Kit (MoBio Labs, Solana Beach, CA, USA). Extracted DNA (5-μL per sample) was PCR-amplified for 40 cycles of 95°C denaturing/55°C annealing/72°C extension using universal Eubacterial primers (Eub GC-341f; Eub 534r), which target a 193 bp portion of the 16S rDNA (Muyzer et al. 1993). The forward primer contains a GC-clamp to improve band separation (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGGG–3′) (Muyzer et al. 1993). We used denaturing gradient gel electrophoresis (DGGE) to characterize microbial community structure once per week during the incubation period. Triplicate gels were run for each test replicate for 14 h at 60 V, with a 20 min/20 V start-up period, in a DCode (Biorad, Hercules, CA, USA) apparatus using 8% acrylamide with a 40–60% urea-formamide denaturing gradient. A 100% denaturing solution contains 6.99 M urea and a 0.4:1 ratio of formamide-to-water (Biorad 1996). Gradient gels were poured using a mini-pump variable flow + gradient maker (CBS Scientific Co. Inc., Del Mar, CA, USA). We stained the gels in 10 μL of ethidium bromide and 100 mL 1 × Tris–Acetate–EDTA buffer (pH 8.5) for 1 h and viewed and digitally photographed them using a Kodak EDAS 290 imaging system (Eastman Kodak Co., Rochester, NY, USA). Gels for each replicate within an IL-test exhibited similar microbial community banding patterns.

Results

We measured biodegradability of six imidazolium and pyridinium-based ILs using the OECD DOC Die-Away Test. In this test, 70% removal of DOC must be reached in a 10-day window within the standard 28-day period of the test, in order for the chemical to be designated as readily biodegradable. No significant decreases in DOC concentrations were observed in uninoculated controls, indicating that abiotic degradation of ILs does not occur under our test conditions. The biotic controls contained less than 3 mg C L^{-1}, and we corrected all experimental data for the biotic carbon inputs.

Imidazolium ionic liquids

None of the imidazolium-based ILs fit the criteria for the DOC Die-Away Test, so none can be classified as readily biodegradable (Fig. 2a; Table 1). However, we extended the incubation period of the test past the standard 28-days and found that hmimBr and omimBr can be partially mineralized by the activated sludge microbial community. DOC concentrations with these two ILs differed significantly from the abiotic controls through time (repeated measures ANOVA, $p = 0.012$, $df = 21$ for hmimBr and $p = 0.013$, $df = 21$ for omimBr). In the hmimBr test, DOC concentrations decreased by 54% after day 25 (Tukey's HSD, $p = 0.024$), and then remained constant for the remainder of the test (day 38)

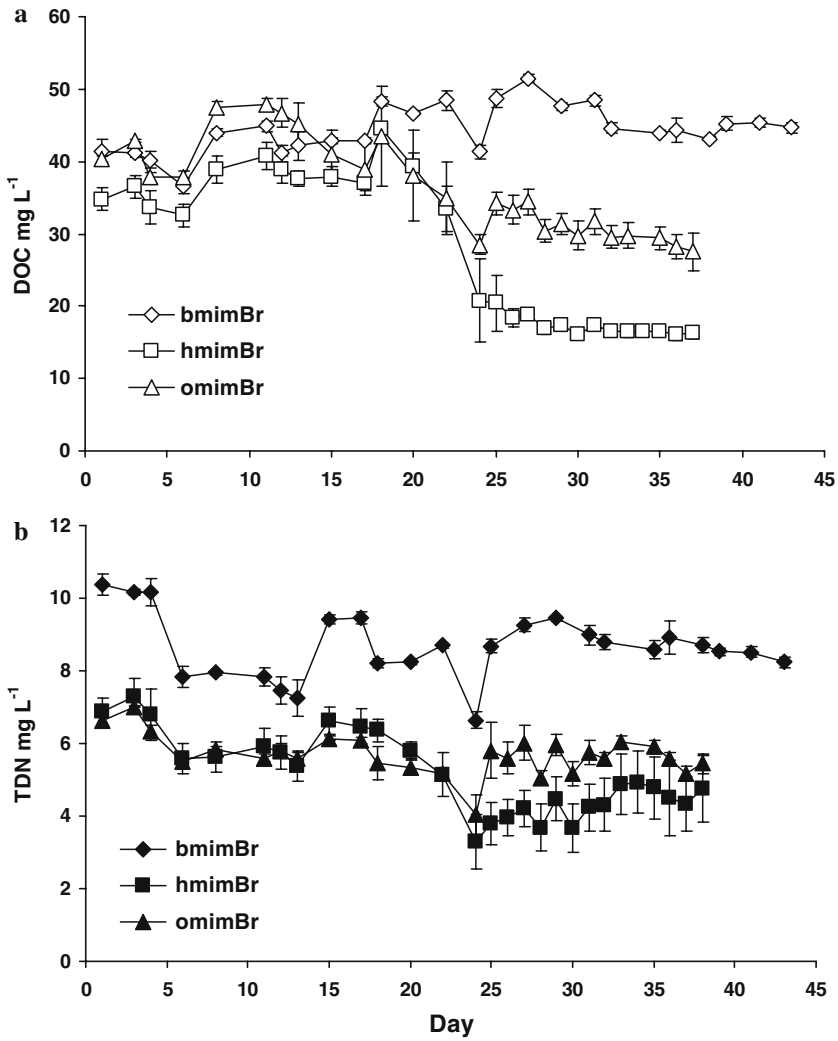

Fig. 2 **(a)** Dissolved organic carbon (DOC mg C L^{-1} ± standard deviation) and **(b)** total dissolved nitrogen (TDN mg N L^{-1} ± standard deviation) for bmimBr, hmimBr and omimBr biodegradability tests through time. For all treatments, $n = 3$

(Fig. 2a; Table 1). In the omimBr test, DOC concentrations decreased by 41% after day 22 (Tukey's HSD, $p = 0.007$), and similarly remained constant for the rest of the incubation period (day 38). We did not observe any decreases in DOC concentration in the bmimBr

Table 1 Summary of results from six IL biodegradation tests using the OECD DOC Die-Away Test

IL	Days of incubation	Final [DOC] mg L^{-1}	Maximum % of degradation	Classification
bmimBr	43	44.68 ± 0.75	0%	Not readily biodegradable
hmimBr	37	16.27 ± 0.70	54%	Not readily biodegradable, partially mineralized
omimBr	38	23.87 ± 5.79	41%	Not readily biodegradable, partially mineralized
bmpyrBr	43	41.18 ± 5.12	0%	Not readily biodegradable
hmpyrBr	49	1.16 ± 0.43	97%	Not readily biodegradable, fully mineralized
ompyrBr	25	1.82 ± 0.14	96%	Readily biodegradable, fully mineralized

A 70% removal of carbon within a 10-day window during a 28-day incubation period with an activated sludge microbial community is required for a test compound to be classified as readily biodegradable

test (repeated measures ANOVA, $p = 0.095$, $df = 22$) throughout the 43-day incubation period, indicating that bmimBr is not biodegradable. We did not observe any changes in TDN concentrations throughout the incubation periods in hmimBr and omimBr tests (repeated measures ANOVA, $p = 0.111$, $df = 1$ for hmimBr and $p = 0.725$, $df = 1$ for omimBr) indicating that any mineralization that occurred did not affect the nitrogen atoms present in the imidazolium ring structure (Fig. 2b). Dissolved nitrogen concentrations in the bmimBr test did vary significantly (repeated measures ANOVA, $p = 0.011$, $df = 1$) but this result was due to changes in the bmimBr control during days 12–20, and not due to microbial activity in the bmimBr tests.

We examined initial and final ^{1}H- and ^{13}C-NMR spectra of the imidazolium-based test chemicals. Our analyses confirmed that bmimBr did not undergo any changes in chemical structure during the incubation period, and was not mineralized. However, we did observe changes in chemical structure in both the hmimBr and omimBr tests. ^{1}H-NMR analysis indicated that hmimBr structure was altered from containing 13 hydrogen atoms on the hexyl side chain to containing less than 5 hydrogen atoms (Fig. 3). This corresponds to a loss of between four and five carbon atoms (labeled G, H, I and J in Figs. 1, 3) from the side chain (^{13}C-NMR, data not shown). The imidazolium ring, however, remained intact and was not utilized as a carbon source by the microbial community. ^{1}H-NMR analysis of omimBr indicated that the chemical structure was altered from containing 15 hydrogen atoms on the octyl side chain to containing 6 hydrogen atoms (Fig. 3). This corresponds to a loss of five carbon atoms (labeled G, H, I, J, K and L in Figs. 1, 3) from the side chain (^{13}C-NMR, data not shown). Similarly, the imidazolium ring remained intact and was not metabolized.

Throughout the test periods we collected samples for microbial community analysis using DNA-PCR-DGGE. Each replicate DGGE profile was similar within the IL tests (data not shown). In the bmimBr test, we did not observe any changes in the DGGE banding pattern of the microbial community through time indicating that no microorganisms were enriched by the IL carbon source (Fig. 4). However, in the hmimBr test, the microbial community was altered after day 22, corresponding to the time period when DOC concentrations were decreasing, and resulting in enrichment of organisms presumed to be capable of metabolizing hmimBr (Fig. 4). In the omimBr test, the microbial community was also altered after day 22, corresponding with the decrease in DOC concentration in that treatment (Fig. 4).

Pyridinium ionic liquids

For the three pyridinium-based ILs, only ompyrBr fit the criteria for ready and full biodegradability established by the OECD test guidelines (Table 1). However, with an extended incubation period, hmpyrBr was also fully mineralized though could not be classified as readily biodegradable. DOC concentrations in the hmpyrBr and ompyrBr tests differed significantly through time from the abiotic controls (repeated measures ANOVA, $p = 0.078$, $df = 27$ and $p = 0.002$, $df = 13$). In the hmpyrBr test, DOC concentrations decreased by 97% after day 38 (Tukey's HSD, $p = 0.048$) and remained at a minimum until day 49 (Fig. 5a; Table 1). In the ompyrBr test, DOC concentrations decreased by 96% by day 25. Degradation of ompyrBr began on day 12 (Tukey's HSD, $p = 0.001$) and continued to decrease to a minimum concentration until day 25 (Fig. 5). In the bmpyrBr test, DOC and TDN concentrations did not differ from the abiotic control (repeated measures ANOVA, $p_{DOC} = 0.503$, $df = 21$; $p_{TDN} = 0.399$, $df = 1$) throughout the 43-day incubation period, indicating that bmpyrBr is not biodegradable. We also observed changes in TDN concentrations in the hmpyrBr and ompyrBr tests (repeated measures ANOVA, $p = 0.027$, $df = 1$ for hmpyrBr and $p = 0.004$, $df = 1$ for ompyrBr) corresponding with the days DOC concentrations decreased, indicating that the nitrogen atom in the pyridinium ring was being metabolized (Fig. 5b).

Our analyses of ^{1}H- and ^{13}C-NMR spectra confirmed that bmpyrBr chemical structure did not change from initial to final samples, and therefore was not metabolized (Fig. 6). For both hmpyrBr and ompyrBr tests, final ^{1}H-NMR

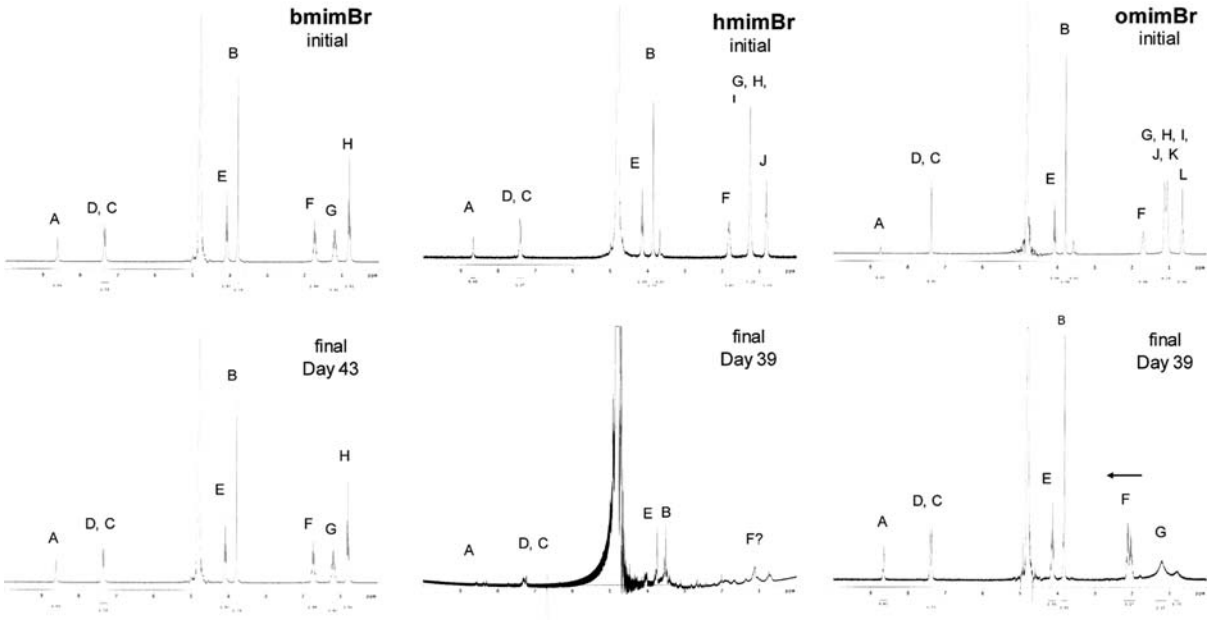

Fig. 3 ^{1}H-NMR spectra at the initial (*top*) and final (*bottom*) time points of the incubation periods for bmimBr, hmimBr and omimBr biodegradability tests. *Letter labels* represent the carbon atoms indicated in Fig. 1

(Fig. 6) and 13C-NMR (data not shown) spectra indicated that none of the original compound remained in solution and that both ILs were fully mineralized by the activated sludge microbial community during the incubation period.

Analysis of DGGE profiles revealed that there were no changes in the microbial community exposed to bmpyrBr (Fig. 7). However, the hmpyrBr test DGGE profile indicated changes and selection of certain bands on days 29–36 (Fig. 7). In the ompyrBr test, we observed community profile changes on days 8–15 (Fig. 7). Each DGGE profile replicate exhibited a similar banding pattern (data not shown). These community changes corresponded with the time of most rapid DOC concentration decrease, and suggest that certain organisms are enriched through biodegradation and IL-carbon source utilization.

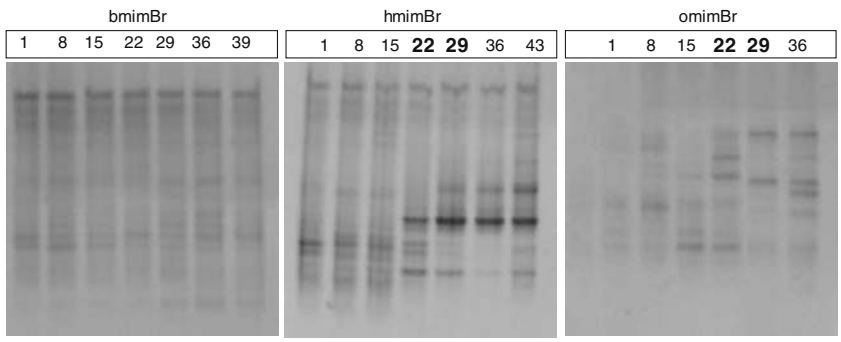

Fig. 4 Denaturing gradient gel electrophoresis profiles of activated sludge Eubacterial microbial communities growing in bmimBr, hmimBr and omimBr media through time. *Numbers* above each *lane* represent the incubation day the sample was removed; *numbers* indicated in *bold* correspond to the days during which the most rapid decreases in DOC concentration occurred

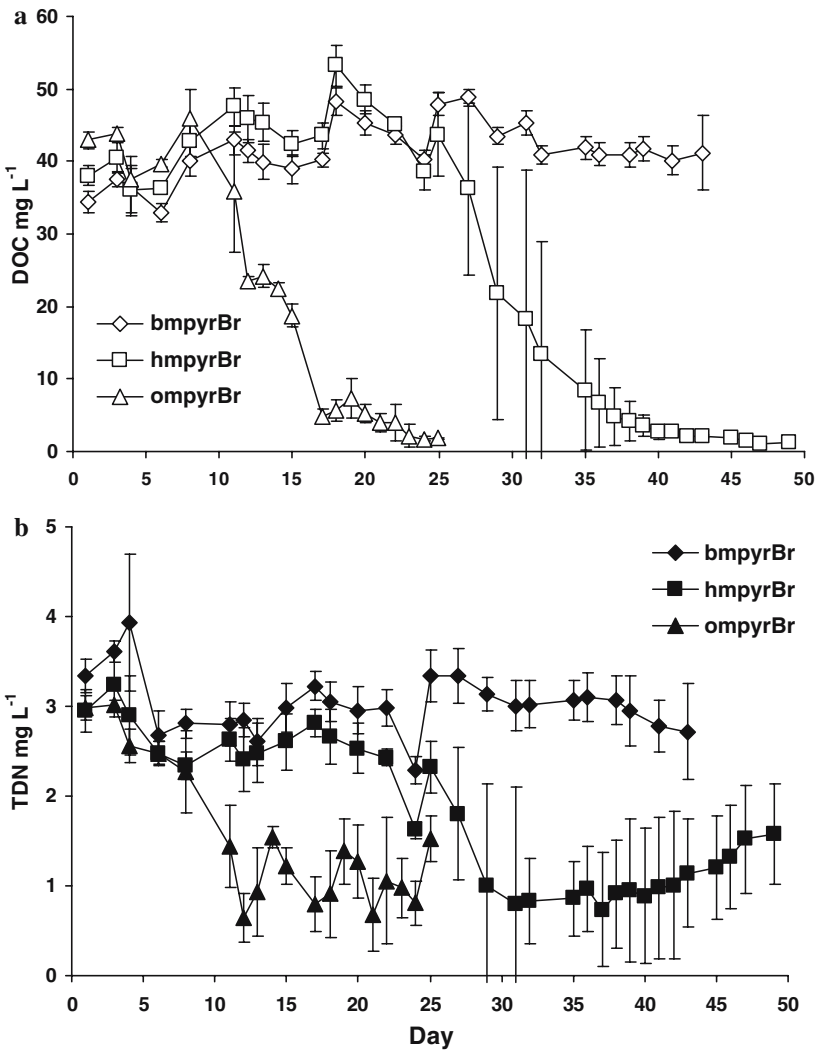

Fig. 5 **(a)** Dissolved organic carbon (DOC mg C L^{-1} ± standard deviation) and **(b)** total dissolved nitrogen (TDN mg N L^{-1} ± standard deviation) for bmpyrBr, hmpyrBr and ompyrBr biodegradability tests through time. For all treatments, $n = 3$

Discussion

We examined the biodegradability of three imidazolium-based and three pyridinium-based ILs by an activated sludge microbial community. The OECD criterion for ready biodegradability states that a compound must be at least 70% biodegraded within a 10-day window and within 28 days of incubation. Only the ompyrBr IL fit these criteria for ready biodegradability. We indirectly determined that this compound was fully degraded (96%) within 15–25 days of incubation by measuring decrease in DOC concentration (Fig. 5a). Further, we observed that none of the compound remained in solution by ^{1}H-NMR analysis after the final sample day (day 25).

While none of the other compounds fit the OECD criteria for ready biodegradability, hmpyrBr was also fully mineralized (97%) after 35–49 days of incubation (Fig. 5a). This suggests that, while hmpyrBr is not as readily biodegradable as ompyrBr, it should not persist in the environment and could be treated and fully removed using the appropriate treatment system.

None of the imidazolium-based ILs could be characterized as readily biodegradable. However, the hmimBr treatment lost 54% carbon by day 37 and the omimBr treatment lost 41% carbon by day 38 (Fig. 2). ^{1}H-NMR analysis indicated that these losses in carbon were the result of metabolism of some of the hexyl and octyl side chains, and that the imidazolium ring was not metabolized.

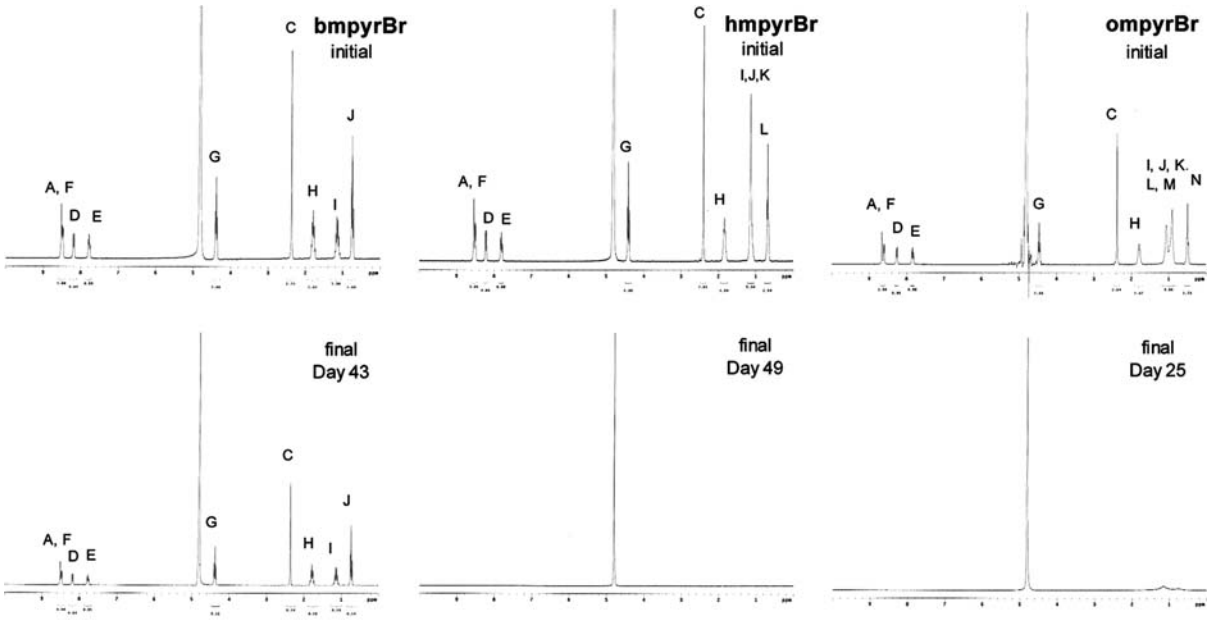

Fig. 6 ^{1}H-NMR spectra at the initial (*top*) and final (*bottom*) time points of the incubation periods for bmpyrBr, hmpyrBr and ompyrBr biodegradability tests. *Letter labels* represent the carbon atoms indicated in Fig. 1

Secondarily, TDN concentrations also did not vary, indicating that the nitrogen atoms in the imidazolium ring were not affected. However, since the test media does contain nitrogen sources, this evidence is less straightforward than DOC concentration and ^{1}H-NMR data. We are uncertain of the identity of the terminal functional group at the end of the metabolized alkyl chain. ^{1}H-NMR shows a new chain length consistent with a propyl chain and integration and chemical shifts suggests the new structure to be $-CH_2CH_2CH_2X$, where X is an electron withdrawing group. We believe that the most likely identity of X is an alcohol, but it could be a halogen, or another functional group that exchanges quickly with D_2O. Because of the extremely dilute nature of the sample, ^{13}C-NMR analysis did not yield any additional evidence to

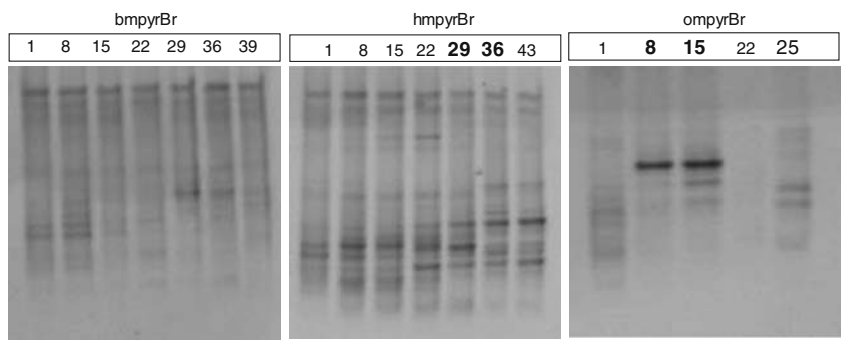

Fig. 7 Denaturing gradient gel electrophoresis profiles of activated sludge Eubacterial microbial communities growing in bmpyrBr, hmpyrBr and ompyrBr media through time. *Numbers* above each *lane* represent the incubation day the sample was removed; *numbers* indicated in *bold* correspond to the days during which the most rapid decreases in DOC concentration occurred

help in the complete identification of the biodegradation compound formed.

Neither bmimBr or bmpyrBr were metabolized by the activated sludge microbial community within the 43–49-day incubation periods. DOC concentrations did not decrease within this time period and the chemical structures remained intact (Figs. 2a, 3, 5a, 6). Theoretical and experimental studies have also examined biodegradability of the bmim cation and found it to be resistant to biodegradation (Gathergood et al. 2004, 2006; Jastorff et al. 2003; Stepnowski and Storoniak 2005).

Our results suggest that pyridinium-based ILs are readily biodegradable, and that imidazolium-based ILs can only be partially metabolized. Additionally, the length of the alkyl chain has an effect upon how quickly the pyridinium compounds are metabolized. There are two possible explanations for this observation. First, the longer alkyl chain could be more easily accessible to bacteria and may eliminate structural hindrance between the pyridinium ring and any potential binding sites. The presence of linear alkyl chains, particularly containing four or more carbons, is an important factor in designing biodegradable compounds because they can act as potential sites for attack by oxygenases (Boethling 1996; Gathergood et al. 2004). Second, ILs with longer chain lengths are more toxic, and may act as selecting agents for microorganisms capable of utilizing them as a carbon source. For example, ompyrBr has effective concentration at 50% (EC-50) of 1.8 mg L^{-1} to *Vibrio fisheri*, and would more likely kill off any non-metabolizing bacteria more quickly than hmpyrBr (EC-50 = 30.0 mg L^{-1}) and allow more rapid growth of biodegrading microorganisms which can out-compete other microorganisms for carbon in the medium (Docherty and Kulpa 2005).

This second explanation is supported by our microbial community analyses using DNA-PCR-DGGE. These preliminary profiles suggest that only a few bacteria in the community are enriched and capable of utilizing the ILs as a carbon source. For example, in the ompyrBr test, one band becomes more predominant during the period of most rapid decrease in DOC (days 8–15), suggesting that potentially a single microorganism population is growing as a result of using the IL as a carbon source (Fig. 7). Similarly, we observed enrichment of multiple bands in the hmpyrBr treatment on days 29–36, suggesting that more than one microorganism may be working in a consortium to metabolize hmpyrBr. This suggests that different microbial communities may be responsible for the metabolism of these two different alkyl chain length pyridinium-based ILs. Multiple bands were also enriched in the hmimBr and omimBr treatments during the time period when the alkyl side chains were being metabolized. Finally, no changes were observed in the bmimBr and bmpyrBr DOC concentrations or ^{1}H-NMR, so no microorganisms actively metabolized these carbon sources.

The results presented here represent novel biodegradation data and may be used as guidelines for further synthesis and testing. Based upon these results, we suggest that pyridinium ILs are, in general, more environmentally friendly than imidazolium ILs, because it is possible to fully biodegrade hexyl and octyl substituted pyridiniums. We found that, although alkyl chain length is directly related to an increase in toxicity, increased alkyl chain length increases the rate of degradation, so a tradeoff exists between controlling for level of toxicity and speed of biodegradability. Our DGGE results suggest that one or more microorganisms are working to metabolize the pyridinium ILs. Future studies will enrich and examine the metabolic capabilities of these active communities. Finally, we obtained direct chemical analyses via NMR for initial and final time points only. We have not examined the toxicity of metabolic products of partial degradation of IL solvents. While metabolism generally ends in less-toxic products, there are cases where metabolism can result in a more-toxic byproduct. For example, polybrominated diphenyl ether (PBDE)-degrading *Dehalococcoides* cultures metabolized octa-BDE to more toxic congeners hexa-154, penta-99, tetra-49 and tetra-47 (He et al. 2006). While it was beyond the scope of this study to determine specific biodegradation pathway products, preliminary results suggest that omimBr and ompyrBr are non-toxic to *V. fisheri* after microbial degradation. Future studies will further investigate intermediate chemical products and

their toxicity and investigate potential differences in biodegradation pathways between differing alkyl chain length pyridinium compounds.

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References

Allen DT, Shonnard DR (2002) Green engineering. Prentice-Hall, Upper Saddle River

Bernot RJ, Brueseke MA, Evans-White MA, Lamberti GA (2005a) Acute and chronic toxicity of imidazolium-based ionic liquids on *Daphnia magna*. Environ Toxicol Chem 24(1):87–92

Bernot RJ, Kennedy EE, Lamberti GA (2005b) Effects of ionic liquids on the survival, movement, and feeding behavior of the freshwater snail, *Physa acuta*. Environ Toxicol Chem 24(7):1759–1765

Biorad Life Science Group (1996) The Dcode Universal Mutation Detection System. Biorad Life Science Group, Hercules

Boethling RS (1996) Designing biodegradable chemicals. Designing safer chemicals: ACS symposium series 640:156–171

Bonhôte P, Dias AP, Papgeorgiou N, Kalyanasundaram K, Gratzel M (1996) Hydrophobic, highly conductive ambient-temperature molten salts. Inorg Chem 35(5):1168–1178

Brennecke JF, Maginn EJ (2001) Ionic liquids: innovative fluids for chemical processing. AIChE J 47:2384–2389

Cammarata L, Kazarian SG, Salter PA, Welton T (2001) Molecular states of water in room temperature ionic liquids. Phys Chem Chem Phys 3(23):5192–5200

Docherty KM, Kulpa CF (2005) Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. Green Chem 7:185–189

Docherty KM, Hebbeler SZ, Kulpa CF (2006) An assessment of ionic liquid mutagenicity using the Ames Test. Green Chem 8(6):560–567

Dupont J, deSouza RF, Suarez PAZ (2002) Ionic liquid (molten salt) phase organometallic catalysis. Chem Rev 102:3667–3692

Fredlake CP, Crosthwaite JM, Hert DG, Aki SNVK, Brennecke JF (2004) Thermophysical properties of imidazolium ionic liquids. J Chem Eng Data 49(4): 954–964

Gathergood N, Garcia MT, Scammells PJ (2004) Biodegradable ionic liquids: part I. Concept, preliminary targets and evaluation. Green Chem 6:166–175

Gathergood N, Scammells PJ, Garcia MT (2006) Biodegradable ionic liquids: part III. The first readily biodegradable ionic liquids. Green Chem 8:56–160

Gorman-Lewis DJ, Fein JB (2004) Experimental study of the adsorption of an ionic liquid onto bacterial and mineral surfaces. Environ Sci Technol 38:2491–2495

Grabinska-Sota E, Kalka J (2004) An assessment of the toxicity of pyridinium chlorides and their biodegradation intermediates. Environ Int 28:687–690

He J, Robrock KR, Alvarez-Cohen L (2006) Microbial reductive debromination of polybrominated diphenyl ethers (PBDEs). Environ Sci Technol 40:4429–4434

Jastorff B, Störmann R, Ranke J, Mölter K, Stock F, Oberheitmann B, Hoffmann W, Hoffmann J, Nüchter M, Ondruschka B, Filser J (2003) How hazardous are ionic liquids? Structure–activity relationships and biological testing as important elements for sustainability evaluation. Green Chem 5:136–142

Kaiser JP, Feng YC, Bollag JM (1996) Microbial metabolism of pyridine, quinoline, acridine, and their derivatives under aerobic and anaerobic conditions. Microbiol Rev 60(3):483–498

Liao Q, Hussey L (1996) Densities, viscosities and conductivities of mixtures of benzene with the Lewis acidic aluminum chloride: 1-methyl-3-ethylimidazolium chloride molten salt. J Chem Eng Data 41:1126–1130

Marsh KN, Deev A, Wu ACT, Tran E, Klamt A (2002) Room temperature ionic liquids as replacements for conventional solvents—a review. Korean J Chem Eng 19(3):357–362

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

National Toxicology Program (NTP) and National Institute of Environmental Health Sciences (NIEHS) (2004) Review of toxicological literature for ionic liquids. Prepared by Integrated Laboratory Systems Inc., Research Triangle Park

Organisation for Economic Co-operation and Development (OECD) Guideline for Testing of Chemicals 301 A-F. Adopted July 17, 1992

Pernak J, Chwala P (2003) Synthesis and anti-microbial activities of choline-like quaternary ammonium chlorides. Eur J Med Chem 38:1035–1042

Pernak J, Rogoza J, Mirska I (2001) Synthesis and antimicrobial activities of new pyridinium and benzimidazolium chlorides. Eur J Med Chem 36:313–320

Pernak J, Goc I, Mirska I (2004) Anti-microbial activities of protic ionic liquids with lactate anion. Green Chem 6:323–329

Pretti C, Chiappe C, Pieraccini D, Gregori M, Abramo F, Monni G, Intorre L (2006) Acute toxicity of ionic liquids to the zebrafish (*Danio rerio*). Green Chem 8:238–240

Ranke J, Molter K, Stock F, Bottin-Weber U, Poczobutt J, Hoffmann J, Ondruschka B, Filser J, Jastorff B (2004) Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays. Ecotoxicol Environ Saf 58(3):396–404

Scow KM (2002) Microbial ecology and in situ bioremediation of MTBE-contaminated groundwater. Bioremediation and biodegradation conference paper, Pacific Grove

Sheldon RA (2005) Green solvents for sustainable organic synthesis: state of the art. Green Chem 7:267–278

Sigma-Aldrich-Fluka (2001) Green chemistry ChemFiles: ionic liquids, fluorous phase organic synthesis (FPOS). Phase Transfer Catalysis (PTC) 1(7):1–18. (http://www.sigmaaldrich.com/img/assets/15469/chemfiles_v1n7_greenchemistry_small.pdf)

Smith AE, Hristova K, Wood I, Mackay DM, Lory E, Lorenzana D, Scow KM (2005) Comparison of biostimulation versus bioaugmentation with bacterial strain PM1 for treatment of groundwater contaminated with methyl tertiary butyl ether (MTBE). Environ Health Perspect 113(3):317–322

Stepnowski P, Storoniak P (2005) Lipophilicity and metabolic route prediction of imidazolium ionic liquids. Environ Sci Pollut Res Int 12(4):199–205

Swatloski RP, Holbrey JD, Memon SB, Caldwell GA, Caldwell KA, Rogers RD (2004) Using *Caenorhabditis elegans* to probe toxicity of 1-alkyl-3-methylimidazolium chloride based ionic liquids. Chem Commun 6:668–669

United States Geological Survey, National Water Quality Assessment Program (USGS NAWQA) (1995) Occurrence of the gasoline additive MTBE in shallow ground water in urban and agricultural areas. USGS Fact Sheet FS-114–95