Toxicity of imidazolium and pyridinium based ionic liquids towards algae. *Bacillaria paxillifer* (a microphytobenthic diatom) and *Geitlerinema amphibium* (a microphytobenthic blue green alga)

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Many studies done so far on the aquatic ecotoxicity of ionic liquids have dealt with phytoplankton organisms. Nonetheless, benthic organisms are just as important if we are to fully understand the prospective fate and effects of ionic liquids in the aquatic environment. An investigation was therefore undertaken into the toxicity of 1-alkyl-3-methylimidazolium ionic liquids towards the diatom *Bacillaria paxillifer* and the cyanobacterium *Geitlerinema amphibium*, species characteristic of the benthic environment. As in the first part of this study (A. Latała, M. Nędzi and P. Stepnowski, *Green Chem.*, 2009, **11**, 580–588), five 1-alkyl-3-methylimidazolium chlorides (from ethyl to decyl) were chosen as the test kit compounds for examining the expected alkyl chain length effect, together with 1-butyl-3-methylimidazolium tetrafluoroborate, dicyanamide, trifluoromethanesulfonate and methyl sulfate and α-methyl[poly(oxy-1,2-ethanediyl)]sulfate for investigating the influence of anions on the toxicity of ionic liquids towards the algal species under investigation. A pronounced alkyl chain effect was found with both organisms. The results indicate that *P. amphibium* is more sensitive than *B. paxillifer.* It was also found that the use of trifluoromethanesulfonate and dicyanamide as counteranions in the IL structure gave rise to the most pronounced toxic effects in comparison with the other anions tested. It is assumed that the greater toxicities of long alkyl chain ILs will most probably not pose a greater threat to benthic organisms, since these compounds will undergo rapid and very strong adsorption to sedimentary matter. Short-chain compounds, which are known to be reversibly bound to sediments, may still pose a significant risk to benthic ecosystems, however.

Introduction

The first part of this series of papers reported on the toxicity of ionic liquids towards two green algae (*Chlorella vulgaris* and *Oocystis submarina*), characteristic of freshwater and brackish environments, as well as two marine diatoms (*Cyclotella meneghiniana* and *Skeletonema marinoi*).[1] A pronounced alkyl chain effect was found with all four organisms. The results also showed that diatoms were far more sensitive than green algae, the observed difference being due to the cell wall structure, *i.e.*, the silica-based cell wall of the diatoms and the cellulose wall of the green algae. Cell size also plays an important part in the intoxication process; a tenfold difference in cell size results in 100% more sensitive reaction to ionic liquids on the part of both the green algae and the diatoms.

Many studies done so far on the aquatic ecotoxicity of ionic liquids have dealt with phytoplankton organisms. Nonetheless, benthic organisms are just as important if we are to fully understand the prospective fate and effects of ionic liquids in the aquatic environment. Benthic algae are important because they are the primary source of fixed carbon in shallow waters or littoral zone. In many bodies of water, marine coastal ecosystems and inland freshwaters, benthic algae contribute more productivity than do the phytoplankton.[2] In the case of benthic organisms, it is not only water, but also sedimentary matter that is a significant source of chemicals previously sorbed in this environmental compartment.

The rate at which organic compounds become bioavailable from sediments depends mainly on the strength and reversibility of sorption. Current studies in risk assessment of ionic liquids have also covered sorption issues. Gorman-Lewis and Fein[3] investigated the adsorption of 1-butyl-3-methylimidazolium chloride onto a range of surfaces representing those usually found in the near-surface environment: ILs were found to interact with the mineral montmorillonite, a process attributed solely to electrostatic interaction between the cation and the outer and interlayer surface of the clay. Moreover, the sorption isotherms and equilibrium sorption coefficients of selected imidazolium IL cations with different alkyl side-chain lengths have been determined and compared for several types of soils and sediments with different physico-chemical properties.[4] All the compounds were found to be strongly sorbed onto these substrates, and the longer the alkyl chain, the greater the sorption capacity. In most cases, maximum achievable surface concentrations were well above the cation exchange capacity (CEC). This observation may indicate that initially sorbed solutes modify the sorbent, a process favouring further sorption through dispersive

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interactions. Therefore, the more hydrophobic compounds, the stronger sorption was observed. The desorption of sorbed ILs, which decreases with increasing alkyl chain length (thus hydrophobicity), has also been determined. In practice, therefore, compounds such as 1-hexyl- or 1-octyl-3-methylimidazolium salts become very strongly bound to the sedimentary matter. Extremely strong sorption onto fine-textured marine sediments has also been reported, which highlights the importance of the mineral component of the soil (sediment) in the sorption mechanism. Sorption strength (expressed as sorption coefficient) of imidazolium–based ionic liquids was also found to be positively correlated with the content of organic matter in aquatic sediments.[5] The mechanism of IL sorption onto kaolinite clay has been investigated in detail.[6] The results indicated a multilayer type of adsorption: long-alkyl chain molecules had the highest distribution coefficients at final saturations. Mean free energies were below the values from a typical ion-exchange process, which suggests that the adsorption mechanism is a combination of electrostatic and hydrophobic interactions.

It can be assumed that, after the sorption and preconcentration of ionic liquids in the sedimentary matter, sorbates (especially those with shorter alkyl chain lengths) may still undergo desorption, and will thus be available to benthic organisms in relatively higher concentrations, as compared to water as a source. In this study, therefore, it was decided to study the toxicity of 1-alkyl-3-methylimidazolium ionic liquids towards the diatom *Bacillaria paxillifer* (O.F. Müller) Hendey and the cyanobacterium *Geitlerinema amphibium* (C. Agardh) Anagnostidis, species characteristic of the benthic environment. The test kit of IL compounds consisted of five 1-alkyl-3-methylimidazolium chlorides (from -ethyl to -decyl) investigating the expected alkyl chain length effect, together with 1-butyl-3-methylimidazolium tetrafluoroborate, dicyanamide, trifluoromethanesulfonate, methyl sulfate and α -methyl[poly(oxy-1,2-ethanediyl)]sulfate for assessing the influence of the anion on IL toxicity towards algal species.

Results

Influence of alkyl chain length on ionic liquid toxicity

In order to examine the well-known effect of the chain length (lipophilicity) of ionic liquids on toxicity, the test kit of 1-alkyl-3-methylimidazolium chlorides varied from ethyl to decyl.[7,8,13] The influence of ionic liquids on algal growth in relation to the toxicity was deduced from the ratio between the treated cells and the control sample. Fig. 1 presents examples of dose response relationships obtained during this study, and Tables 1, and 2 show the results of algal toxicity expressed as EC_{50} values. The EC_{50} values for the exposure of the two organisms to 1-ethyl-3-methylimidazolium chloride were quite similar (30.90–34.40 μM). In the case of *B. paxillifer*, elongation of the alkyl chain in the cationic moiety of the ionic liquids by a further two, four, six or eight carbon atoms did not cause any sharp decrease in EC_{50} values; growth inhibition of this algae exposed to 1-decyl-3-methylimidazolium chloride occurred at 0.99 μM.

Table 1 Influence of ionic liquids on the growth of *Bacillaria paxillifer*, expressed as EC_{50} values ± standard deviation (μM)

Chemical name	Abbreviation	EC_{50} ± SD/μM	log EC_{50}
1-R-3-methylimidazolium chloride			
R – ethyl	EMIM Cl	34.40 ± 1.72	1.54
R – butyl	BMIM Cl	6.48 ± 0.32	0.81
R – hexyl	HMIM Cl	2.01 ± 0.10	0.30
R – octyl	OMIM Cl	1.52 ± 0.07	0.18
R–decyl	DMIM Cl	0.99 ± 0.05	−0.004
1-Butyl-3-methyl imidazolium X			
X – tetrafluoroborate	BMIM BF_4	8.04 ± 0.04	0.91
X – dicyanamide	BMIM DCNA	5.16 ± 0.26	0.71
X – trifuoromethanesulfonate	BMIM TFMS	6.99 ± 0.34	0.84
X – methylsulfate	BMIM $MeSO_4$	16.05 ± 0.80	1.21
X – α-methyl[poly(oxy-1,2-ethanediyl)]sulfate	BMIM $MPEGSO_4$	17.03 ± 0.85	1.23

Table 2 Influence of ionic liquids on the growth of *Geitlerinema amphibium*, expressed as EC_{50} values ± standard deviation (μM)

Chemical name	Abbreviation	EC_{50} ± SD/μM	log EC_{50}
1-R-3-methylimidazolium chloride			
R – ethyl	EMIM Cl	30.90 ± 1.54	1.49
R – butyl	BMIM Cl	3.65 ± 0.18	0.56
R – hexyl	HMIM Cl	0.97 ± 0.04	−0.01
R – octyl	OMIM Cl	0.10 ± 0.005	−1.00
R – decyl	DMIM Cl	0.02 ± 0.001	−1.70
1-Butyl-3-methyl imidazolium X			
X – tetrafluoroborate	BMIM BF_4	5.38 ± 0.26	0.73
X – dicyanamide	BMIM DCNA	6.91 ± 0.34	0.84
X – trifuoromethanesulfonate	BMIM TFMS	2.48 ± 0.12	0.39
X – methylsulfate	BMIM $MeSO_4$	5.08 ± 0.25	0.71
X – α-methyl[poly(oxy-1,2-ethanediyl)]sulfate	BMIM $MPEGSO_4$	4.90 ± 0.24	0.69

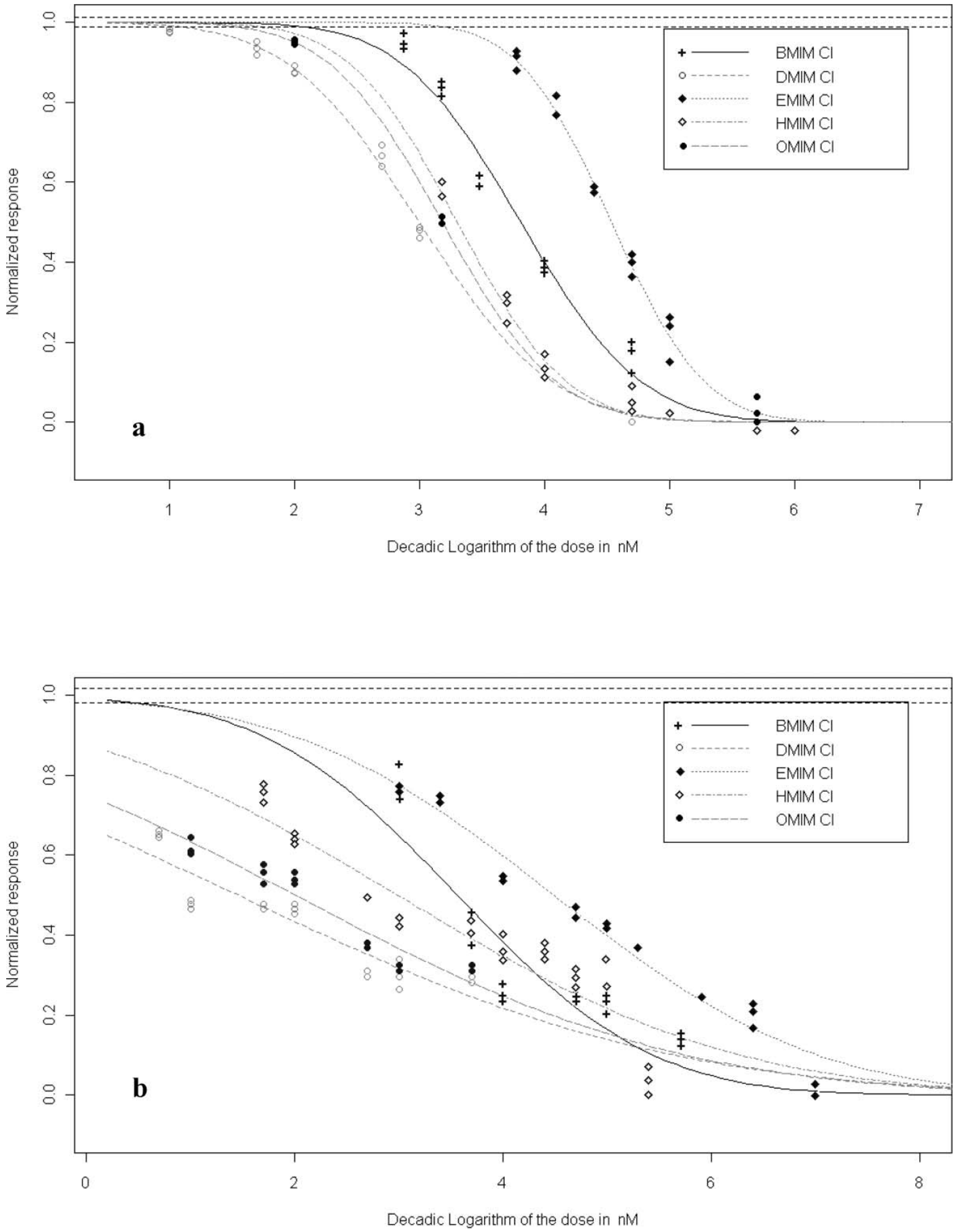

Fig. 1 Dose response relationships plotted for (a) diatom *Bacillaria paxillifer* and (b) cyanobacterium *Geitlerinema amphibium* exposed to 1-alkyl-3methylimidazolium chlorides with varying alkyl chain lengths (EMIM Cl 1-ethyl-3-methylimidazolium chloride, BMIM Cl 1-butyl-3-methylimidazolium chloride, HMIM Cl 1-hexyl-3-methylimidazolium chloride, OMIM Cl 1-octyl-3-methylimidazolium chloride and DMIM Cl 1-decyl-3-methylimidazolium chloride).

In contrast, in the case of *G. amphibium* for every two carbon atoms elongating the chain, there was a fall in the EC_{50} value by nearly one order of magnitude. The longest ionic liquid inhibited growth of this cyanobacterium at concentrations as low as 0.02 μM.

Influence of anions on the toxicity of ionic liquids

The effect of various anions in the IL structure on toxicity towards algae was also studied: six 1-butyl-3-methylimidazolium salts containing tetrafluoroborate, dicyanamide, trifluoromethanesulfonate, methyl sulfate, chloride and α-methyl[poly(oxy-1,2-ethanediyl)]sulfate were compared. Tables 1 and 2 lists the results of algal toxicity expressed as EC_{50} values, and Fig. 2 presents dose-response relationships recorded for the series of ILs varying with the type of anion. Generally, both algae are far less sensitive to anion changes in the IL structure than to changes in alkyl chain length. 1-butyl-3-methylimidazolium dicyanamide exerted the greatest toxic effect on *B. paxillifer* (EC_{50} – 5.16 μM); the effects of ILs containing tetrafluoroborate and trifluoromethanesulfonate (EC_{50} 8.04 and 6.99 μM respectively) on this species were also relatively strong. The least toxic ILs in this test kit

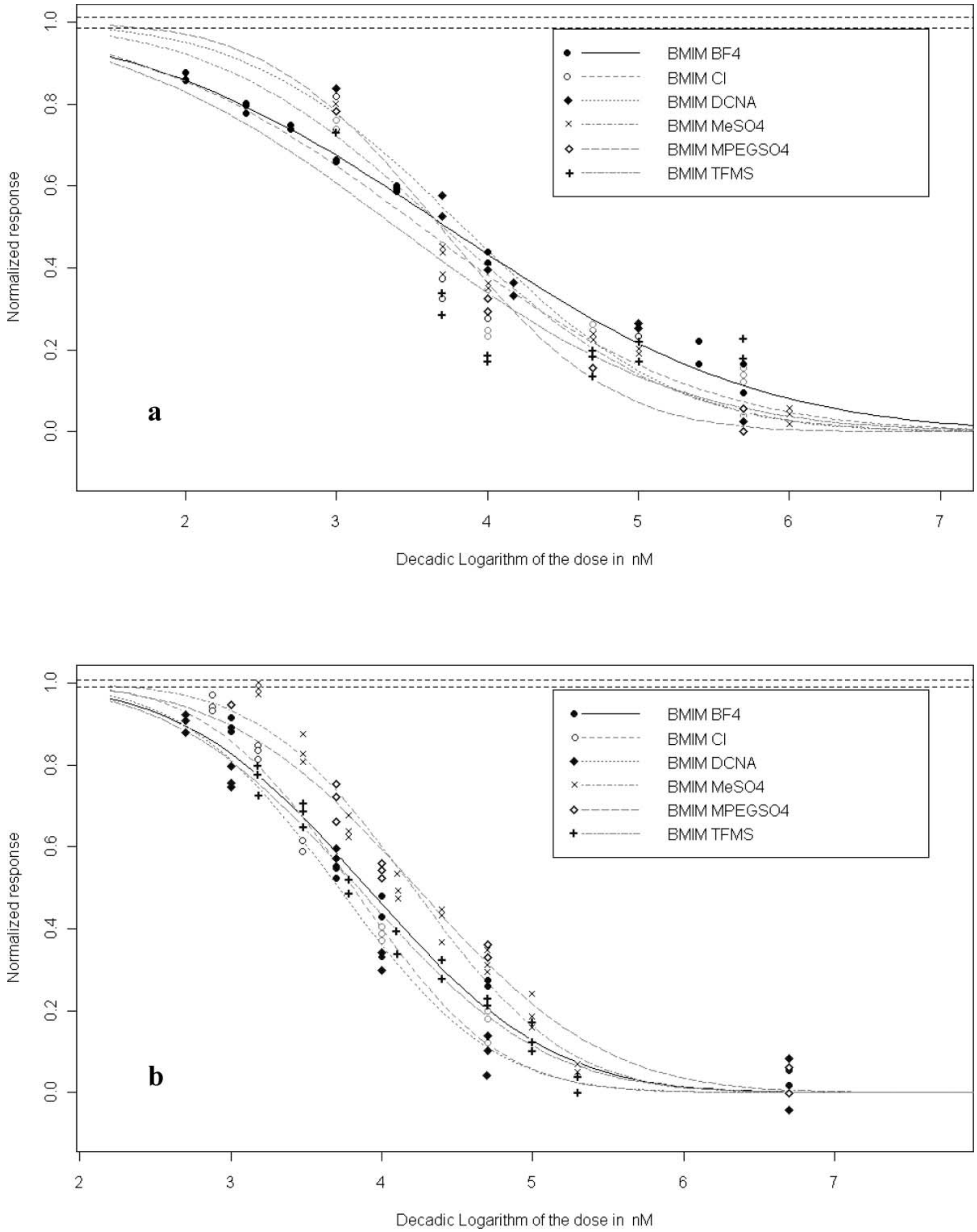

Fig. 2 Dose response relationships plotted for (a) diatom *Bacillaria paxillifer* and (b) cyanobacterium *Geitlerinema amphibium* exposed to 1-butyl-3methylimidazolium chloride (BMIM Cl), -tetrafluoroborate (BMIM BF_4), -dicyanamide (BMIM DCNA), -trifuoromethanesulfonate (BMIM TFMS), -methylsulfate (BMIM $MeSO_4$) and -α -methyl[poly(oxy-1,2-ethanediyl)]sulfate (BMIM $MPEGSO_4$).

were 1-butyl-3-methylimidazolium methyl sulfate and a-methyl[poly(oxy-1,2-ethanediyl)]sulfate.

The most effective growth inhibitor of *G. amphibium* in this series was 1-butyl-3-methylimidazolium trifluoromethanesulfonate (EC_{50} = 2.48 μM), the other anions also effectively inhibited the growth of this algae, with EC_{50} values ranging from 4.90 to 6.91 μM.

Discussion

The results show that both species are very sensitive to alkylimidazolium ionic liquids: the EC_{50} values obtained in the presence of ILs with alkyl chains containing from 4 to 10 carbon atoms ranged from 0.02 to 6.5 μM for both organisms. The same set of compounds was effective in a similar EC_{50} range (from 0.08 to 7.21 μM) for the diatoms *Cyclotella meneghiniana* and *Skeletonema marinoi* investigated in part one of this study series.[1] Not only are diatoms highly sensitive to these compounds; the blue-green algae inhabiting benthic environments are even more susceptible to the toxic action of ILs. Differences in sensitivity, however, were recorded only with the most effective ionic liquids – OMIM Cl and DMIM Cl. The differences between the effects due to compounds with shorter alkyl moieties or containing various anions were much smaller. The more lipophilic ionic liquids (1-hexyl- or 1-octyl-3-methylimidazolium salts) are known to undergo rapid and strong adsorption to sedimentary matter,[4–6] so the risk to their exposure will be quite limited in benthic environments. However, in the case of compounds with shorter alkyl chains, there is still the possibility of previously sorbed ILs being desorbed: *e.g.* 1-propyl- or 1-butyl-3-methylimidazolium salts are desorbable at the level of 20 to 30%, depending on the type of solid matrix.[2] This in turn may even elevate the environmental concentration of ionic liquids within the sedimentary environment and pose a greater risk to the organisms inhabiting the benthic environment. For example, since 1-butyl-3-methylimidazolium chloride, weakly bound to sediments, effectively inhibits the growth of *B. paxillifer* and *G. amphibium* already at respective EC_{50} values of 6.48 and 3.65 μM, we may speculate that the hazard posed by short-chain imidazolium ionic liquids reaching aquatic sediments and their biota may be significant. Additionally, comparison of these effective concentrations with EC_{50} values obtained for planktonic species (Table 3) clearly indicate that benthic organisms are very sensitive to short-chain ILs. This is paradoxical, since so far, short-chain ILs have been assumed to be far safer in the environmental context than highly lipophilic compounds.[7] These assumptions, however, are always made on the basis of very simple dose–response experiments, which take no account of the complexity of aquatic environmental compartments (water, particulate matter, sediments), themselves players of crucial roles in the intoxication pathways of chemicals. A very recent study on bioavailability of ionic liquids from soils showed that not only chemical structure itself, but also clay type and concentration have a great influence on growth of wheat exposed to these compounds.[14]

Table 3 Comparison of EC_{50} values of BMIM Cl and DMIM Cl obtained for planktonic[a] and benthic algae

	$EC_{50} \pm SD/\mu M$	
Algae	BMIM Cl	DMIM Cl
Chlorella vulgaris	1 026.24 ± 51.31	3.68 ± 0.18
Oocystis submarina	2 224.48 ± 111.20	8.02 ± 0.39
Skeletonema marinoi	3.32 ± 0.17	0.08 ± 0.003
Cyclotella meneghiniana	7.21 ± 0.36	0.27 ± 0.01
Bacillaria paxillifer	6.48 ± 0.32	0.99 ± 0.05
Geitlerinema amphibium	3.65 ± 0.18	0.02 ± 0.001

[a] Data for *C. vulgaris*, *O. submarina*, *S. marinoi*, and *C. meneghiniana* from ref. 1.

The potential persistence and toxicity of ionic liquids is also a function of their biodegradability. According to the recent work in this field, commonly used alkylimidazolium entities are characterised by poor to negligible biodegradability.[15–17] The biodegradation rates increase with longer alkyl chain length,[18] thus short chain entities are not only more bioavailable but also potentially more persistent.

Table 3 compares EC_{50} values of BMIM Cl and DMIM Cl obtained for planktonic species[1] and benthic organisms investigated in this paper. It is clear that effective concentrations of the short chain ILs.

The influence of IL anions on algal toxicity was also tested. 1-butyl-3-methylimidazolium trifluoromethanesulfonate exerted the greatest toxic effect on *G. amphibium.* We have previously postulated that in the case of this entity elevated toxicity is likely to be caused by relatively high lipophilicity of this anion, additionally known to be strongly associated with alkylimidazolium cations, that in turn may enhance cell wall penetration.[1]

In the case of *B. paxillifer* 1-butyl-3-methylimidazoilum dicyanamide the most effectively inhibited growth of this species; the differences between this anion and chloride, tetrafluoroborate and trifluoromethanesulfonate, however, were practically negligible.

Conclusion

The diatom *Bacillaria paxillifer* and the cyanobacterium *Geitlerinema amphibium*, species characteristic of the benthic environment, were used to investigate the toxic effects of five 1-alkyl-3-methylimidazolium chlorides with different alkyl chain lengths and five 1-butyl-3-methylimidazolium salts with different counteranions. The test kit of IL compounds consisted of five 1-alkyl-3-methylimidazolium chlorides (from ethyl to decyl) for examining the expected alkyl chain length effect, along with 1-butyl-3-methylimidazolium tetrafluoroborate, dicyanamide trifluoromethanesulfonate, methyl sulfate and α -methyl[poly(oxy-1,2-ethanediyl)]sulfate for investigating the effect of the anion on the toxicity of ionic liquids towards the algal species under investigation. A pronounced alkyl chain effect was found for both organisms, and the cyanobacterium *G. amphibium* was more sensitive than the benthic diatom *B. paxillifer.* The greater toxicities of long alkyl chain ionic liquids will most probably not pose a greater threat to benthic organisms, since these compounds will undergo rapid and strong adsorption to the sedimentary matter. However, the still high sensitivity of benthic algae to BMIM or even EMIM entities, compounds known to bind reversibly to soils and sediments, may pose an extreme risk to benthic ecosystems, where the bioavailability of short-chain ILs may be much higher than in the water column.

Further studies on the ecotoxicity of ionic liquids are still needed, especially in the context of the variety of biotic and abiotic components that may have a considerable influence on the intoxication of aquatic organisms by ionic liquids.

Material and methods

Test organisms

Two taxonomically different benthic algal species were used in this study: diatom *Bacillaria paxillifer* (BA-0014) and blue-green algae *Geitlerinema amphibium* (BA-0013). They were isolated from coastal waters of the Baltic Sea and maintained as unialgal cultures in the Culture Collection of Baltic Algae (CCBA) http://ocean.univ.gda.pl/~ccba/at the Institute of Oceanography of the University of Gdańsk.[9,10]

Ionic liquids

All ionic liquids were obtained from Merck (Darmstadt, Germany). They were: 1-ethyl-3-methylimidazolium chloride (EMIM Cl), 1-butyl-3-methylimidazolium chloride (BMIM Cl), 1-hexyl-3-methylimidazolium chloride (HMIM Cl), 1-octyl-3-methylimidazolium chloride (OMIM Cl) and 1-decyl-3-methylimidazolium chloride (DMIM Cl). Also used were 1-butyl-3-methylimidazolium tetrafluoroborate (BMIM BF_4), -dicyanamide (BMIM DCNA), -trifluoromethanesulfonate (BMIM TFMS), -methyl sulfate (BMIM $MeSO_4$) and -α-methyl[poly(oxy-1,2-ethanediyl)]sulfate (BMIM $MPEGSO_4$, mean molecular weight 514,63 g/mol). All chemicals were graded *for synthesis* and characterized by >98% purity.

Batch cultures and toxicity tests

The test algae were batch-cultured in f/2 medium[11] prepared in distilled water. The culture salinity of 8 PSU was similar to that in the southern Baltic Sea, the original environment of the test organisms. The salinity was made up using TropicMarin® sea salt (Tropic Marin, Wartenberg, Germany).

The stock cultures of test organisms were acclimatised for 10 days at 20 °C and illuminated with 25 μmol photons m^{-2} s^{-1} from daylight type fluorescent lamps (L:D photoperiod 16:8). The irradiance was measured with a LiCor (LI-189) quantum meter (*LiCor*, Lincoln, Neb., USA).

The acute toxicity tests of ILs towards algae were carried out using modified versions of the methods recommended in the European Committee for Standardization's guidelines.[11,12] The main modifications were the use of f/2 medium, the photoperiod and the choice of the test strains. The final batch cultures used in the experiments were obtained by mixing a known amount of cells in the log growth phase with sterile medium. The initial cell number was constant and was measured as Optical Density (OD) at two wavelengths (665 and 750 nm).

The initial cell number of *B. paxillifer* was approximately 6.5×10^4 cells/ml and *G. amphibium* was 1.5×10^5 cells/ml. The relationship between OD and cells number was calculated.

9.5 cm^3 aliquots of algal suspension were transferred to glass conical flasks (25 ml), and to each of these 0.5 cm^3 of different concentrations of an aqueous IL solution or distilled water (control cultures) was added. Final concentrations ranged from 50 mM to 0.000005 mM. All experiments were run in triplicate.

After 72 h incubation the number of cells in the cultures was determined by OD measurement. The variability of the results did not exceed 5% on the inhibition scale. The ILs were tested on a wide range of concentrations, which enabled the EC_{50} values to be calculated.

Statistical analysis

To calculate the EC_{50} values, dose response curves were fitted with the non-linear least squares method using a linear logistic model.[13] Calculations were carried out with the R-language in the statistical environment, version 2.7.2 (see: http://www.r-project.org). The data are the means of independent experiments conducted in triplicate for each compound.

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