Toxicity of imidazolium and pyridinium based ionic liquids towards algae. *Chlorella vulgaris*, *Oocystis submarina* (green algae) and *Cyclotella meneghiniana*, *Skeletonema marinoi* (diatoms)

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This paper reports on an investigation into the toxicity of 1-alkyl-3-methylimidazolium ionic liquids (ILs) towards two green algae (*Chlorella vulgaris* and *Oocystis submarina*) characteristic of freshwater and brackish environments, as well as two brackish and marine diatoms (*Cyclotella meneghiniana* and *Skeletonema marinoi*). The test kit of IL compounds consisted of five 1-alkyl-3-methylimidazolium chlorides (from -ethyl to -decyl) for evaluating the expected alkyl chain length effect, together with 1-butyl-3-methylimidazolium tetrafluoroborate, dicyanamide, trifluoromethanesulfonate, methyl sulfate an α-methyl[poly(oxy-1,2-ethanediyl)]sulfate for investigating the influence of the anion on IL toxicity towards various algal species. A pronounced alkyl chain effect was confirmed with all the organisms studied. EC_{50} values were linearly very well correlated with the number of carbon atoms in the IL alkyl chains. The results indicate that diatoms are far more sensitive than green algae to ILs. Cell size also plays an important part in the intoxication process: a tenfold difference in cell size results in a 100% more sensitive reaction to ionic liquids in both the green algae and diatoms. No significant differences were observed between alkylimidazolium salts and an alkylpyridinium compound of similar lipophilicity. It was also found that the use of tetrafluoroborate and trifluoromethanesulfonate as counteranions in the IL structure gave rise to the most pronounced toxic effects in comparison with the other anions tested. In the case of tetrafluoroborate this is probably caused by the potential hydrolysis of this entity, which leads to the formation of fluoride and a further increase in toxicity. Whereas in the case of trifluoromethanesulfonate it is likely 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.

Introduction

Ionic liquids (ILs) are a new class of organic salts with melting points below 100 °C. They typically consist of nitrogen-containing organic cations such as 1-alkyl-3-methylimidazolium or *N*-alkylpyridinium together with an inorganic or organic anion commonly containing fluorine, for example, BF_4^-, PF_6^-, CF_3COO^-, SbF_6^-, or $(CF_3SO_2)_2N^-$. Current research indicates that replacing conventional molecular solvents with these media can bring about remarkable improvements in well-known processes: ILs have already been applied in organic synthesis and catalysis, in the separation sciences, as electrolytes in batteries and solar cells, and as alternative lubricants.[1–2]

ILs are often uncritically regarded as intrinsically environmentally friendly, since their vapour pressure is negligible, and hence are a good alternative to the emissions of toxic vapours from conventional molecular organic solvents. Merely reducing such gaseous discharges, however, does not automatically make a process greener, and many other facts have to be taken into account before such a statement can be made.[3]

There are numerous reports covering the toxicity of ionic liquids. They evaluate this toxicity on the basis of test systems at different levels of biological complexity, starting with the Ames mutagenicity test[4] and the inhibition of acetylcholinesterase[5] or AMP deaminase[6] enzymes, through growth inhibition tests performed on bacterial, algal and mammalian cell cultures[7–11] as well as developmental toxicity assessment in mice,[12] to ecotoxicological tests done on soil roundworms,[13] freshwater crustaceans[14] and freshwater snails.[15] Most of these test systems indicate that the longer the *n*-alkyl chain in the ionic liquid cation, the greater the toxicity. The importance of side chain length (and hence lipophilicity) as a factor contributing to the overall observed biological effect has also been examined in detail.[16–17] The potential persistence and toxicity of ionic liquids is also a function of their biodegradability: several investigations in this area have shown that the biodegradability of the most commonly used alkylimidazolium ionic liquids is poor to negligible.[18–21] The most recent reports, however, indicate that the incorporation of oxygen functional groups in the cation and anion structures significantly enhances biodegradation and lowers toxicity.[22]

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In view of the chemical industry's great interest in these compounds, one has to anticipate a number of risk scenarios, including those in which certain amounts of ionic liquids are present in industrial effluents where, because of their great stability, they could become persistent pollutants and break through classical treatment systems into natural waters. Recent studies have demonstrated that only the most advanced techniques are effective in totally removing ionic liquids from such systems.[23–25] Since these methods are not usually included in conventional wastewater treatment processes, there is a considerable risk of aquatic organisms becoming exposed to ILs. This is a problem that should be addressed as a matter of urgency.

There are many bioassay techniques using fish, invertebrates, phytoplankton and bacteria for detecting the toxic effects of chemicals in fresh and seawaters. Among these, algal assays have become widely used as biological tools in environmental impact studies. Current assessment procedures are based on the premise that algal tests can be used to predict the effects of chemical compounds in aquatic environments.[26] Growth-inhibition tests with freshwater algae[27] and seawater algae[28] are included in the base set of ecotoxicological tests recommended by the European Committee for Standardization (CEN), the International Organization for Standardization (ISO), and the Organization for Economic Cooperation and Development (OECD).

So far, the toxicity of ILs towards algae has only been tested to a preliminary extent in a few research projects. The first report published by our group indicated that the growth of two Baltic algae was effectively inhibited by even very low concentrations of alkylimidazolium ionic liquids.[9] That study also demonstrated the evident influence of water salinity on toxicity towards algae; in general the higher the salinity, the lower the toxicological effects. Wells and Coombe[29] tested several imidazolium, phosphonium and ammonium ILs for their toxicity towards the green algae *Pseudokirchneriella subcapitata* and the invertebrate *Daphnia magna.* In general, the toxicity levels of a particular substance to algae and *Daphnia* were surprisingly similar, and toxicities of the most and least toxic ionic liquids were separated by more than four orders of magnitude for both trophic levels. In contrast, Kulacki and Lamberti,[30] in their study of the toxicity of imidazolium ionic liquids towards the freshwater algae *Scenedesmus quadricauda* (EC_{50} = 0.005–13.23 mg L^{-1}) and *Chlamydomonas reinhardtii* (EC_{50} = 4.07–2138 mg L^{-1}), demonstrated with clarity that ionic liquids can elicit a range of algal responses, suggesting that it is difficult to extrapolate the results of these bioassays across taxa. Stolte *et al.*[31] examined the influence of different head groups, functionalised side chains and anions of ionic liquids on the freshwater green alga *Scenedesmus vacuolatus*. The set of 40 ILs these authors analysed confirmed the relation between the lipophilicity and toxicity of these compounds: the toxicity was clearly lower when short, functionalised compounds were used instead of long non-polar alkyl chains. The same group also investigated the toxic effects of mixtures of ILs on the same algae as well as on wheat:[32] green algae were approximately two orders of magnitude more sensitive to the mixture scenarios than wheat, thus proving to be a good reference test system for evaluating the measured effects. Recently, Pham *et al.*[33] found that the length of the alkyl chain in the cation of imidazolium and pyridinium ionic liquids has a direct influence on the photosynthetic response of *P. subcapitata*. Apart from the alkyl chain effect, which was very distinct in all these studies, the type of anion was also found to influence the toxicity towards the alga *Selenastrum capricornutum*:[34] the most toxic anions were SbF_6^- and PF_6^-, which is probably due to them being hydrolysed to fluoride.

The cited results clearly demonstrate the wide diversity of responses of algae exposed to various ionic liquids. This is due not only to the different sensitivities of algal organisms, but also to the complexities of the chemical structures of ILs, where alkyl chain length, the various functional groups, cationic head groups and types of anions are active variables as far as the mode of toxic action is concerned. Moreover, since most of the studies undertaken so far have been done with freshwater algae, we possess only limited knowledge of the fate of ILs in the marine environment. It is important to know this fate if we wish to predict the full consequences of the release of these compounds into the environment.

The present study therefore addressed the problem of the toxicity of 1-alkyl-3-methylimidazolium ionic liquids towards two Baltic green algae—*Chlorella vulgaris* and *Oocystis submarina*—as well as two diatoms—*Cyclotella meneghiniana* and *Skeletonema marinoi*. Two of these species (*O. submarina* and *C. meneghiniana*) had previously been studied by our group in a very preliminary manner, so no EC_{50} values were obtained and only four ILs were tested on these species.[9] In addition, *S. marinoi* was tested to see whether the previously observed higher sensitivity of diatoms to IL exposure was taxon-specific. The test kit of IL compounds consisted of five 1-alkyl-3-methylimidazolium chlorides (from ethyl to decyl) for evaluating the expected alkyl chain length effect, as well as of 1-butyl-3-methylimidazolium tetrafluoroborate, dicyanamide, trifluoromethanesulfonate, methyl sulfate and α-methyl[poly(oxy-1,2-ethanediyl)]sulfate in order to investigate the influence of the anion on IL toxicity towards various algal species (Fig. 1).

Results

Influence of alkyl chain length on toxicity of ionic liquids

In order to examine the well-known effect of IL alkyl chain length (lipophilicity) on toxicity towards the algal organisms under investigation, a test kit consisting of 1-alkyl-3-methylimidazolium chlorides, with alkyl varying from ethyl to decyl, was used. The influence of ILs on algal growth in relation to their toxicity was deduced from the ratio of the treated cells to the control sample. Fig. 2 exemplifies the dose response relationships obtained during this study, and Tables 1–4 set out the toxicities towards algae expressed as EC_{50} values. These data show that there is a distinct relationship between alkyl chain effect and toxicity: in nearly all cases, a two-carbon increase in chain length results in EC_{50} falling by nearly one order of magnitude. In the case of the green algae exposed to 1-ethyl-3-methylimidazolium chloride, the effective concentration of this compound remains in the millimolar range (6330.51–13 573.01 μM) but effectively inhibits the growth of both diatoms at levels from 59.24 to 112.36 μM. This trend is maintained

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

Chemical name	Abbreviation	EC_{50} ± SD/μM	log EC_{50}
1-R-3-methylimidazolium chloride			
R – ethyl	EMIM Cl	6330.51 ± 178.56	3.80
R – butyl	BMIM Cl	1026.24 ± 51.31	3.01
R – hexyl	HMIM Cl	64.52 ± 3.23	1.81
R – octyl	OMIM Cl	15.14 ± 0.76	1.18
R – decyl	DMIM Cl	3.68 ± 0.18	0.57
1-Butyl-3-methyl imidazolium X			
X – tetrafluoroborate	BMIM BF_4	425.33 ± 21.27	2.63
X – dicyanamide	BMIM DCNA	2650.98 ± 132.55	3.42
X – trifuoromethanesulfonate	BMIM TFMS	1417.75 ± 70.89	3.15
X – methylsulfate	BMIM $MeSO_4$	1011.70 ± 50.59	3.01
X – α-methyl[poly(oxy-1,2-ethanediyl)]sulfate	BMIM $MPEGSO_4$	930.81 ± 46.54	2.97
1-Butyl-4-methyl pyridinium chloride	MBPy Cl	2109.74 ± 105.49	3.32

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

Chemical name	Abbreviation	EC_{50} ± SD/μM	log EC_{50}
1-R-3-methylimidazolium chloride			
R – ethyl	EMIM Cl	13 573.01 ± 678.63	4.13
R – butyl	BMIM Cl	2224.48 ± 111.20	3.35
R – hexyl	HMIM Cl	753.60 ± 37.64	2.88
R – octyl	OMIM Cl	79.13 ± 3.93	1.90
R – decyl	DMIM Cl	8.02 ± 0.39	0.89
1-Butyl-3-methyl imidazolium X			
X – tetrafluoroborate	BMIM BF_4	707.81 ± 35.35	2.85
X – dicyanamide	BMIM DCNA	2984.40 ± 149.18	3.47
X – trifuoromethanesulfonate	BMIM TFMS	1689.88 ± 84.44	3.23
X – methylsulfate	BMIM $MeSO_4$	3292.98 ± 164.59	3.52
X – α-methyl[poly(oxy-1,2-ethanediyl)]sulfate	BMIM $MPEGSO_4$	878.04 ± 43.92	2.94
1-Butyl-4-methyl pyridinium chloride	MBPy Cl	1924.31 ± 96.21	3.28

Table 3 Influence of ionic liquids on the growth of *Skeletonema marinoi*, expressed as EC_{50} ± standard deviation (μM)

Chemical name	Abbreviation	EC_{50} ± SD/μM	log EC_{50}
1-R-3-methylimidazolium chloride			
R – ethyl	EMIM Cl	112.36 ± 5.61	2.05
R – butyl	BMIM Cl	3.32 ± 0.17	0.52
R – hexyl	HMIM Cl	1.01 ± 0.050	0.00
R – octyl	OMIM Cl	0.41 ± 0.02	–0.39
R – decyl	DMIM Cl	0.08 ± 0.003	–1.10
1-Butyl-3-methyl imidazolium X			
X – tetrafluoroborate	BMIM BF_4	2.63 ± 0.13	0.42
X – dicyanamide	BMIM DCNA	2.66 ± 0.13	0.42
X – trifuoromethanesulfonate	BMIM TFMS	1.77 ± 0.08	0.25
X – methylsulfate	BMIM $MeSO_4$	3.01 ± 0.15	0.48
X – α-methyl[poly(oxy-1,2-ethanediyl)]sulfate	BMIM $MPEGSO_4$	3.76 ± 0.18	0.58

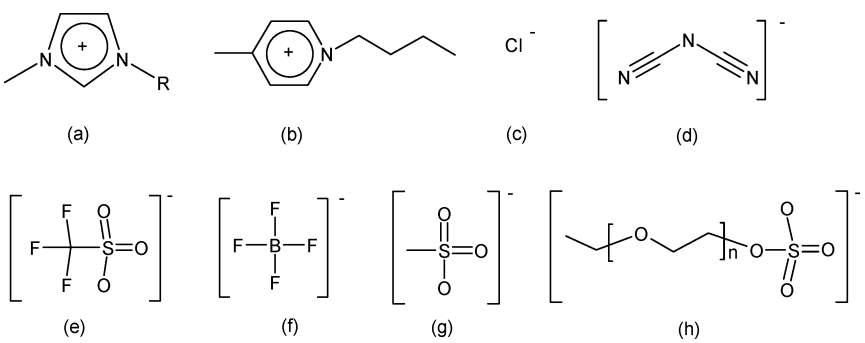

Fig. 1 Chemical structures of the ionic liquids: (a) 1-alkyl-3-methylimidazolium, (b) 1-butyl-4-methylpyridinium, (c) chloride, (d) dicyanamide, (e) trifluoromethanesulfonate, (f) tetrafluoroborate, (g) methyl sulfate, (h) methyl[poly(oxy-1,2-ethanediyl)]sulfate.

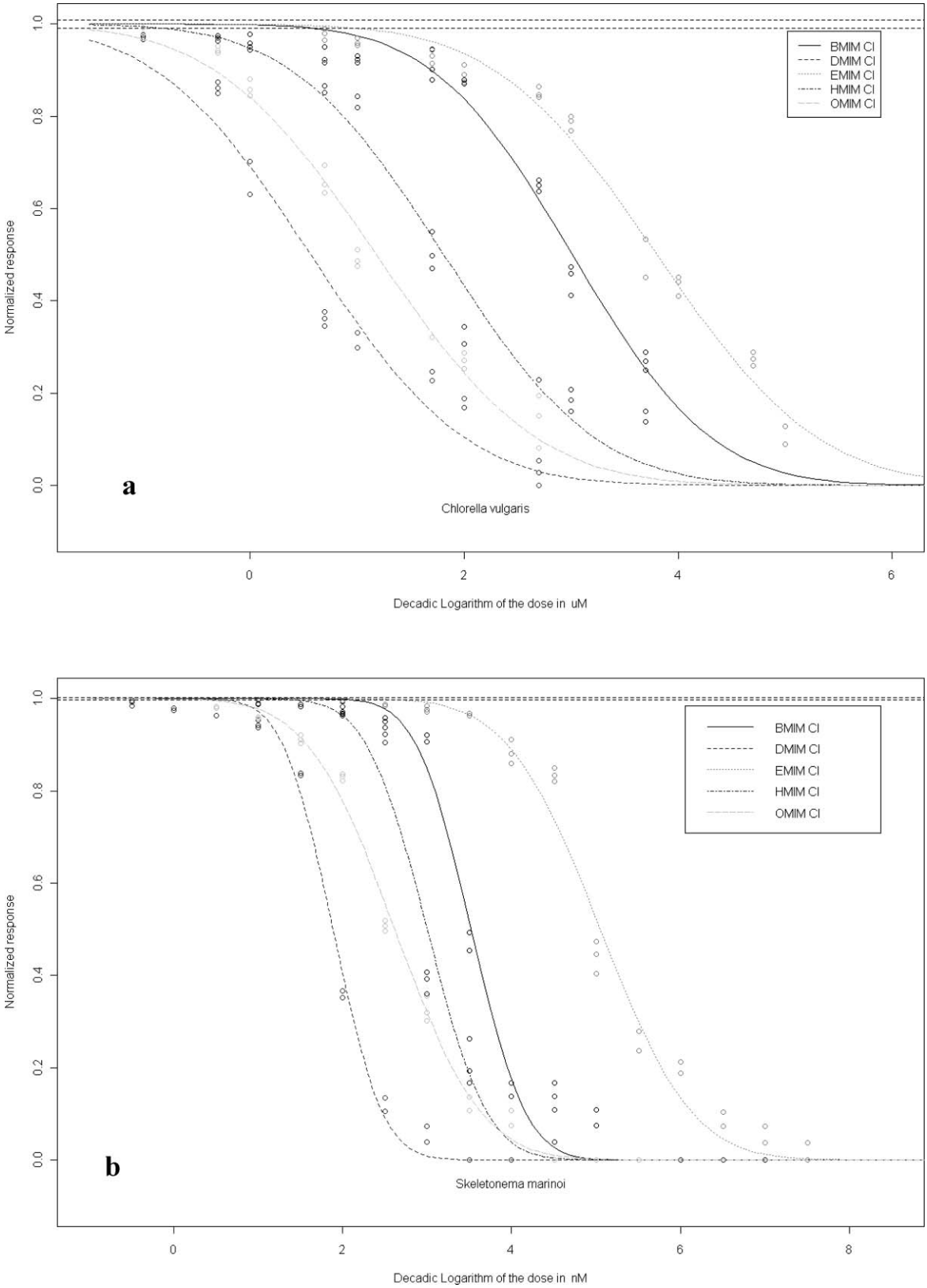

Fig. 2 Dose–response relationships plotted for (a) the green alga *Chlorella vulgaris* and (b) the diatom *Skeletonema marinoi* exposed to 1-alkyl-3-methylimidazolium chlorides with different alkyl chain lengths (EMIM Cl-1-ethyl-3-methylimidazolium chloride, BMIM Cl-1-butyl-3-methylimidazolium chloride, HMIM Cl-1-hexyl-3-methylimidazolium chloride, OMIM Cl-1-octyl-3-methylimidazolium chloride and DMIM Cl-1-decyl-3-methylimidazolium chloride).

Table 4 Influence of ionic liquids on the growth of *Cyclotella meneghiniana*, expressed as EC_{50} ± standard deviation (μM)

Chemical name	Abbreviation	EC_{50} ± SD/μM	log EC_{50}
1-R-3-methylimidazolium chloride			
R – Ethyl	EMIM Cl	59.24 ± 2.96	1.77
R – Butyl	BMIM Cl	7.21 ± 0.36	0.86
R – Hexyl	HMIM Cl	2.39 ± 0.12	0.38
R – Octyl	OMIM Cl	0.73 ± 0.04	–0.14
R – Decyl	DMIM Cl	0.27 ± 0.01	–0.57
1-Butyl-3-methyl imidazolium X			
X – tetrafluoroborate	BMIM BF_4	2.75 ± 0.13	0.44
X – dicyanamide	BMIM DCNA	10.95 ± 0.54	1.04
X – trifuoromethanesulfonate	BMIM TFMS	5.72 ± 0.28	0.76
X – methylsulfate	BMIM $MeSO_4$	11.64 ± 0.58	1.07
X – α-methyl[poly(oxy-1,2-ethanediyl)]sulfate	BMIM $MPEGSO_4$	9.68 ± 0.48	0.99

Table 5 Linear correlation between the alkyl chain length of the imidazolium cation in the ionic liquid (expressed as the number of carbon atoms C_n) and log EC_{50}

	$C_n = a \log EC_{50} + b$		
Species	Slope (a)	Intercept (b)	Linear correlation R^2
Chlorella vulgaris	–0.414	4.561	0.9837
Oocystis submarina	–0.396	5.009	0.9839
Skeletonema marinoi	–0.362	2.379	0.9295
Cyclotella meneghiniana	–0.284	2.163	0.9766

with the other ILs, with the diatoms exhibiting a far higher sensitivity than the green algae. The toxicity of the next shortest alkyl chain compound (BMIM Cl) is still in the millimolar range as regards green algae and, as in the case of EMIM Cl, the effect can be treated as insignificant. In the case of the *C. meneghiniana* and *S. marinoi*, EC_{50} dropped to 3.32 and 7.21 μM of EMIM Cl respectively; with 1-hexyl-3-methylimidazolium chloride, effective concentrations were still of the same order of magnitude (1.01–2.39 μM). This was not the case with *Chlorella vulgaris*, where the fall in effective concentration with HMIM Cl was more than one order of magnitude (64.52 μM). The other green alga (*O. submarina*) was relatively more resistant to the toxic effect of this IL, cultures of which were inhibited by 753.60 μM. The toxic effect of the longest alkyl chain compounds (OMIM Cl and DMIM Cl) in green algae was apparent already at very low concentrations (3.68–79.13 μM). In the diatoms, however, EC_{50} values as low as 0.08–0.73 μM were recorded with these latter two compounds.

One alkylpyridinium salt—1-butyl-4-methylpyridinium chloride—was also tested for its toxicity towards *C. vulgaris* and *O. submarina*: in both cases the EC_{50} values lay between those of EMIM Cl and BMIM Cl.

EC_{50} values (expressed as log EC_{50}) were correlated linearly with the number of carbon atoms (C_n) in the IL alkyl chain. Table 5 presents the resultant intercepts (b), slopes (a) and linear correlation coefficients (R^2) of the functions. The EC_{50} values for both green algae were very well correlated with the length of the alkyl chain ($R^2 \sim 0.984$), and, in the case of *C. meneghiniana*, the resultant correlation coefficient was reasonably good ($R^2 = 0.9766$). The weakest relation was definitely that for *S. marinoi* ($R^2 = 0.9295$); this was probably due to the relatively high resistance of this species to the shorter-chain compounds and its greater sensitivity to the longest-chain IL being investigated. The slopes obtained appear to be characteristic of particular taxa, at least as far as the green algae are concerned. The similar intercepts within the taxonomic groups (~ 0.5 for green algae, ~ 0.2 for diatoms) reflect the sensitivities of these organisms.

Influence of anions on ionic liquid toxicity

The effects of different anions in the IL structure on toxicity towards algae were also studied. To this end six 1-butyl-3-methylimidazolium salts containing tetrafluoroborate, dicyanamide, trifluoromethanesulfonate, methyl sulfate, chloride and α-methyl[poly(oxy-1,2-ethanediyl)]sulfate anions were compared. Tables 1–4 list the toxicities towards algae expressed as EC_{50} values, and Fig. 3 shows examples of the dose-response relationships recorded for the series of ILs with different anions. Generally, both green algae were far less sensitive than the diatoms to the changes in anion. In the case of *C. vulgaris* and *O. submarina*, the toxicity levels were highest with 1-butyl-3-methylimidazolium tetrafluoroborate (EC_{50} = 425.33 and 707.81 μM, respectively); fairly strong effects were also observed with ILs containing the α-methyl[poly(oxy-1,2-ethanediyl)]sulfate anion, a polymeric entity (EC_{50} = 930.81 and 871.04 μM, respectively). The least toxic ILs in this test kit were 1-butyl-3-methylimidazolium dicyanamide (*C. vulgaris*) and 1-butyl-3-methylimidazolium methyl sulfate (*O. submarina*).

This study of the toxic effects of the 1-butyl-3-methylimidazolium cation with various anions on the two diatoms showed that the most effective growth inhibitor of *S. marinoi* was 1-butyl-3-methylimidazolium trifluoromethanesulfonate (EC_{50} = 1.77 μM), while that of *C. meneghiniana* was 1-butyl-3-methylimidazolium tetrafluoroborate (EC_{50} = 2.75 μM). Varying the anions when testing IL toxicity on diatoms also showed that *S. marinoi* was generally more sensitive than *C. meneghiniana*.

Discussion

The test kit of IL compounds was used to investigate the toxicity of 1-alkyl-3-methylimidazolium ionic liquids with different alkyl chain lengths. Moreover, the choice of algal species enabled the various effects of ILs on green algae (*Chlorella vulgaris* and *Oocystis submarina*) and diatoms (*Cyclotella meneghiniana* and *Skeletonema marinoi*) to be distinguished. The alkyl chain effect was pronounced in all four organisms. The length of the alkyl chain is the main structural feature of ILs determining

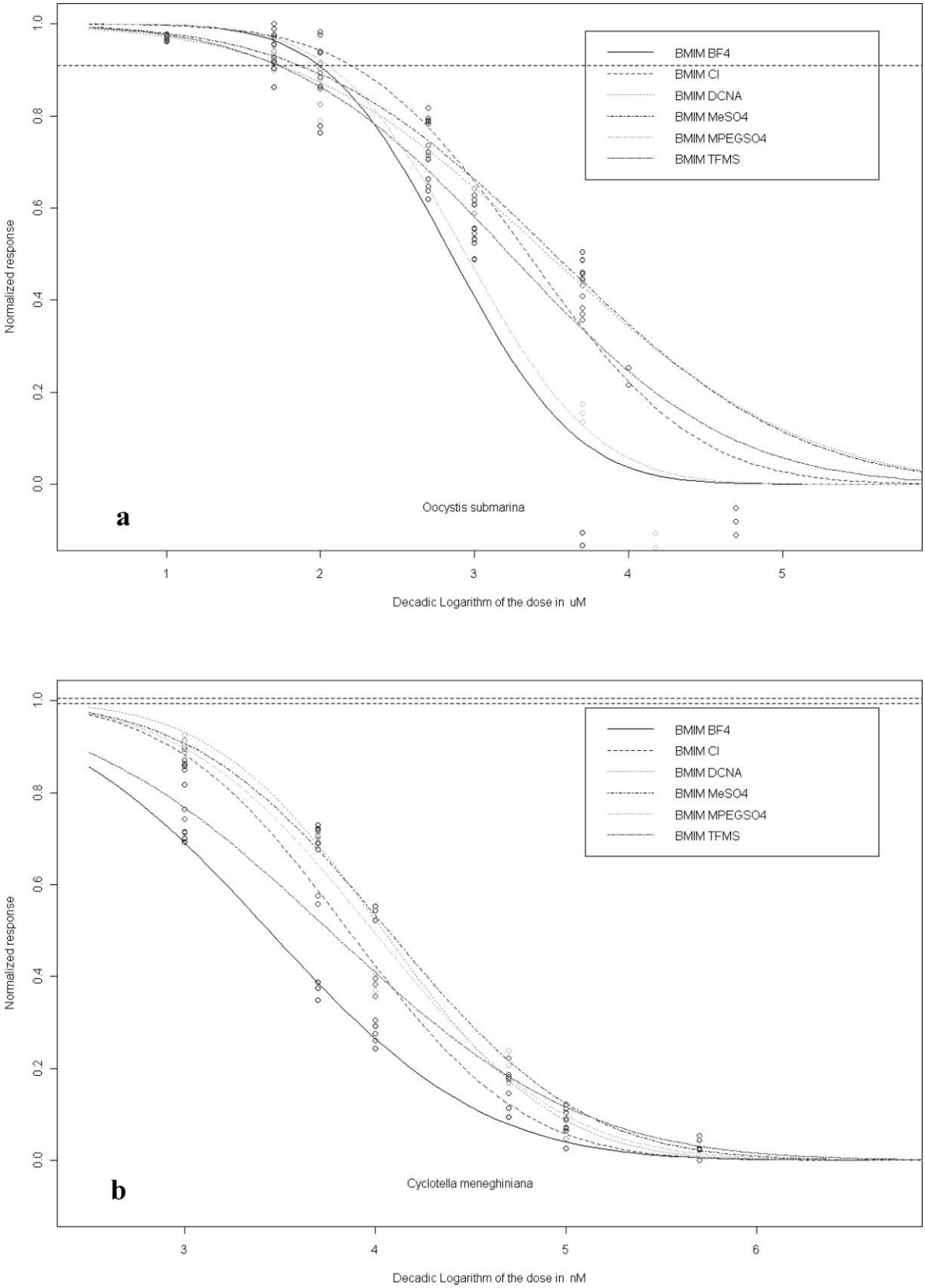

Fig. 3 Dose–response relationships plotted for (a) the green alga *Oocystis submarina* and (b) the diatom *Cyclotella meneghiniana* exposed to 1-butyl-3-methylimidazolium chloride (BMIM Cl), -tetrafluoroborate (BMIM BF_4), -dicyanamide (BMIM DCNA), -trifluoromethanesulfonate (BMIM TFMS), -methyl sulfate (BMIM $MeSO_4$) and -α-methyl[poly(oxy-1,2-ethanediyl)]sulfate (BMIM $MPEGSO_4$).

their lipophilicity.[35] The relation between a compound's toxicity towards aquatic organisms and its lipophilicity is known as the baseline toxicity: this causes a non-specific disturbance of the structure and functioning of the biological membranes.[36] Since ILs containing longer alkyl chains exhibit an adsorption potential through simultaneous hydrophobic and ionic interactions (in the same way as surfactants), they have a greater ability to accumulate at the algal membrane–water interface, which causes the disruption of the integral membrane.[37] The relation between the alkyl chain length and the toxicity of ILs towards freshwater algae has also been confirmed in other studies.[29–32] In this report, however, we deliver, for the first time, systematic data on IL toxicity towards two marine diatom species. From this data it is clear that diatoms are far more sensitive to ILs than green algae. This concurs with the results of our preliminary study, where the greater sensitivity of diatoms exposed to ILs was postulated:[9] on exposure to ILs, the *Cyclotella meneghiniana* culture, unlike the green algae population, did not acclimatise even to the low ionic liquid concentrations tested, and growth was inhibited throughout the experiment. Obtained results in this study may therefore suggest that the reason for the observed difference in sensitivity is to be found in the cell wall structure. Likely diatoms are much more sensitive to the toxic action of ILs because their cell walls are silica-based; in contrast, green algae, with their cellulose-based cell walls, are possibly more resistant to such action. Diatom cells have a range of features that make them highly divergent from the classical cellular structure of green algae and higher plants. As a consequence, one could almost consider diatoms as photosynthetic animals rather than unicellular plants.[38] The cell wall of diatoms is a composite of amorphous hydrated silica, the product of a partial condensation of orthosilicic acid, and various organic components mainly proteins. In the last decade, with the discovery of further classes of organic components involved in silicification and maintaining the integrity of the cell wall, three families of cell wall proteins were investigated: frustulins,[39] silaffins[40] and pleuralins.[41] Analysis of these cell-surface protein sequences reveals stretches of amino acids highly enriched in acidic residues.[38,42] These all make diatoms highly capable biosorbents for charged chemical entities including heavy metals and ionized organic substances. In comparison, the cell wall of green algae is a composite of indigestible cellulose, far stronger, insensitive and far less negatively charged than diatoms. It is known, for example, that the sorption capacity of copper is five times higher in the case of diatom *C. meneghiniana* than green algae *C. reinhardtii*.[43] If ionic liquid cations are of concern, it is highly possible that much more effective electrostatic biosorption on the diatom surface in comparison to green algae will be decisive in the further effectiveness of the toxic action in this taxonomic group.

Differences in the sensitivity of algae exposed to ILs were also observed by Kulacki and Lamberti:[30] they found that algae with cellulose-based cell walls (*S. quadricauda*) were more sensitive than *C. reinhardtii*, the cell wall of which is composed primarily of glycoproteins. In conjunction with our results, this provides ample demonstration of the baseline toxicity mode action of ionic liquids. Hence, the sensitivity of particular organisms will depend entirely on IL availability within the biological membrane structure; this availability, in turn, is governed by the membrane's surface chemistry.

Interestingly, the IL toxicity ranged widely not only between taxonomic groups but also between species from the same group. Among the green algae, *C. vulgaris* was twice as sensitive to ILs as *O. submarina*; likewise, among the diatoms, *S. marinoi* was twice as sensitive as *C. meneghiniana*. These differences in sensitivity within taxonomic groups correlate well with the size of the algal cells: those of *C. vulgaris* range between 15 and 20 μm^3, whereas those of *O. submarina* are around ten times larger (170 μm^3). Among the diatoms, the more sensitive *S. marinoi* cells are from 100 to 150 μm^3 in size, and those of *C. meneghiniana* between 1350 and 2000 μm^3.[44–45] The relation between cell size and sensitivity again underscores the significance of the cell wall component in the toxic action of ionic liquids. Algal cultures consisting of smaller cells have a relatively larger cell wall surface area, which in turn enables toxins to be transported more efficiently into the cells. It is well known that intertropical oceanic waters, poor in nutrients and ions, are inhabited mainly by picoplanktonic species, which are able to absorb these deficient substances more efficiently as a result of the privileged ratio of cell wall surface to cell volume. In the present study, the tenfold difference in cell size resulted in a 100% more sensitive reaction to ionic liquids by both the green algae and the diatoms. This observation should be taken into consideration when further ecotoxicological risk assessment experiments with ionic liquids are designed.

Apart from the alkyl chain length, two other structural factors of ionic liquids may contribute to the toxic effects on algae: the type of cationic head group and the type of anion. In the case of the head group one alkylpyridinium salt (1-butyl-4-methylpyridinium chloride), the lipophilicity of which lies between that of BMIM Cl and HMIM Cl, was additionally tested. Its EC_{50} values towards both green algae species were in the same range as that of the four-six carbon alkyl chains of the imidazolium ILs. Stolte *et al.*[31] likewise found that pyridinium and imidazolium ILs inhibited the growth of green algae in the same concentration range.

The effect of IL anions on their toxicity towards algae was also tested: in general, both diatoms turned out to be far more sensitive than the green algae to changes of IL anion. 1-butyl-3-methylimidazolium tetrafluoroborate and trifluoromethanesulfonate had the greatest toxic effect on *S. marinoi* and *C. meneghiniana.* In one of our first studies of IL toxicity, it was postulated that tetrafluoroborate could undergo hydrolysis, which would lead to the formation of fluoride and the consequent potentiation of the toxic effect.[11] This was confirmed by other authors, who reached the unequivocal conclusion that the elevated toxicity towards various species of algae was due to fluoride formation.[30–33] In the case of trifluoromethanesulfonate 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-butyl-3-methylimidazolium tetrafluoroborate most effectively inhibited the growth of both green algae, probably for the same reasons as in the case of diatoms. Relatively stronger effects with ILs containing α-methyl[poly(oxy-1,2-ethanediyl)]sulfate were also recorded. The main difference between this anion and the others tested lies in its polymeric nature. Therefore, the generally observed baseline toxicity of lipophilic cations may

well be potentiated by a polymeric anion, but this will have to be confirmed in further studies.

In the case of the green algae, the EC_{50} value of none of the compounds used fell below the millimolar range, which further confirms that these organisms are less sensitive to the toxic effects of ionic liquids.

Conclusion

Two green algae (*Chlorella vulgaris* and *Oocystis submarina*) and two diatoms (*Cyclotella meneghiniana* and *Skeletonema marinoi*) 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. A pronounced alkyl chain effect was found with all the organisms. EC_{50} values were very well correlated linearly with the number of carbon atoms in the IL alkyl chain, and diatoms used in this study were found to be far more sensitive to ILs than green algae. Cell size also played an important role in the intoxication of green algae and diatoms; the smaller the cell, the greater the sensitivity to ILs. There were no significant differences between the alkylimidazolium salts and an alkylpyridinium compound of a similar lipophilicity. The use of tetrafluoroborate and trifluoromethanesulfonate as counteranions in the IL structure gave rise to the most pronounced toxic effects in comparison with the other anions tested. In the case of tetrafluoroborate this was probably due to the hydrolysis of these entities, which leads to the formation of fluoride and consequently a rise in toxicity. Whereas in the case of trifluoromethanesulfonate it is likely 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.

Material and methods

Test organisms

Two taxonomically different planktonic algal species were used in this study: the green algae *Oocystis submarina* (BA-0001) and *Chlorella vulgaris* (BA-0002), and the diatoms *Cyclotella meneghiniana* (BA-0010) and *Skeletonema marinoi* (BA-0098). 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.[46–47]

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 $MPEGSO_4$), as well as 1-butyl-4-methylpyridinium chloride (MBPy Cl).

Batch cultures and toxicity tests

The test algae were batch-cultured in f/2 medium[48] 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, NE, 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.[25–26] 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 respective densities of green algae *C. vulgaris* and *O. submarina* were c. 0.022/665 nm and 0.019/750 nm; those of the diatoms *C. meneghiniana* and *S. marinoi* were 0.062/665 nm and 0.043/750 nm.

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.[10] 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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