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Aquatic Toxicology 73 (2005) 91–98

AQUATIC TOXICOLOGY

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Marine toxicity assessment of imidazolium ionic liquids: Acute effects on the Baltic algae *Oocystis submarina* and *Cyclotella meneghiniana*

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Received 10 February 2005; received in revised form 15 March 2005; accepted 22 March 2005

Abstract

Interest in ionic liquids for their potential in different chemical processes is increasing, as they are claimed to be environmentally benign and are very good non-volatile solvents for a wide range of applications. With regard to their physical and chemical characteristics, the properties of ionic liquids can be modified over a wide range because the cation's fine structure and the anion's identity can be altered. Since millions of ion combinations are possible it is of the highest importance to outline rational guidelines to develop technologically suitable but also environmentally harmless ionic liquids. This paper presents the results of a preliminary assessment of the toxicity of selected imidazolium ionic liquids towards marine algae. The selection of chemical entities was based on the t-SAR approach (thinking in terms of structure–activity relationships) focusing on the length ($C_2 < R1 < C_6$) or type (aliphatic–aromatic) of the side chain whereas head group (imidazolium) remained the same. The acute effect of ionic liquids was measured using the green alga *Oocystis submarina* and the diatom *Cyclotella meneghiniana* inhabiting the southern Baltic Sea. Standard algal testing procedures revealed significant differences in the responses of the two species. *O. submarina* appeared to acclimatize to the lower concentrations used: after ca. 5 days their ability to grow recovered, and initial densities were eventually restored. In the case of *C. meneghiniana*, growth in batch cultures was effectively inhibited throughout the experiment regardless of the ionic liquid concentration applied. Additionally, it was found that at higher salinities, the toxicity of 1-butyl- and 1-hexyl-3-methylimidazolium entities towards *O. submarina* was significantly lower than at low salinities.

Keywords: Ionic liquids; Toxicity; Algal tests; Salinity; Baltic sea

1. Introduction

Typically consisting of nitrogen-containing organic cations and inorganic or organic anions, imidazolium ionic liquids have been widely investigated as possible "green" replacements for organic solvents (Suarez et

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doi:10.1016/j.aquatox.2005.03.008

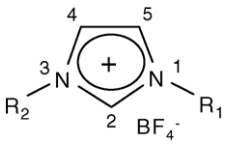


Fig. 1. Generic structure and atomic numbering of 1-alkyl-3-methylimidazolium ionic liquid cations. R1: methyl–decyl or aromatic; R2: methyl–ethyl.

al., 1998; Welton, 1999; Wasserscheid and Keim, 2000; Dupont et al., 2002). With regard to polarity, hydrophobicity and solvent miscibility behavior, the properties of ionic liquids are tunable over a wide range because the cation's fine structure and the anion's identity can be altered; millions of ion combinations are possible. So far, the most commonly applied ionic liquids have been 1-alkyl-3-methylimidazolium salts, which remain in the fluid state over a broad range of temperatures. The generic structures of these compounds are presented in Fig. 1. Nonvolatile, nonflammable, and thermally very stable, they are excellent solvents for a wide range of inorganic and organic materials (Gordon, 2001; Sheldon, 2001; Rantwijk et al., 2003). The "green" aspect of ionic liquids derives mainly from their practically undetectable vapor pressure, but this is obviously insufficient justification for calling a technology "cleaner". Since millions of ion combinations are possible it is of the highest importance to outline rational guidelines to develop technologically suitable but also environmentally harmless ionic liquid.

Even though ionic liquids have a considerable technical and commercial potential, there are, as yet, only a few preliminary reports on their toxicological properties, a knowledge which is essential to any comprehensive risk assessment of these compounds. It has recently been discovered that some of imidazolium ionic liquids are relatively resistant to photodegradation (Stepnowski and Zaleska, 2005), and are biodegradable only to a very small degree (Gathergood and Scammells, 2002). Initial predictions and data on the toxicological and ecotoxicological characteristics of imidazolium ionic liquids have been based on theoretical considerations (structure–activity relationships) and the experimental evaluation of their biological activity (Jastorff et al., 2003; Ranke et al., 2003; Stepnowski et al., 2004; Stock et al., 2004; Składanowski et al., 2005). In all these test systems it was found that the longer is the *n*-alkyl chain length of the ionic liquids, the greater is their toxicity. In addition, structural similarities were found between imidazolium-type ionic liquids and biologically active plant growth regulators or cationic surfactants of known negative environmental impact. In a recent study, the antimicrobial activity of selected 1-alkyl-3-metylimidazolium entities was evaluated against several bacterial strains (Pernak et al., 2003). It was found that ionic liquids possess good antimicrobial activity against strains of Gram-positive and -negative bacteria and fungi.

Once their large-scale implementation has begun, it will not be long before ionic liquids become a permanent component of industrial effluents. In view of their great stability, they could move through classical treatment systems to become persistent pollutants of natural waters. The long-term consequences of their presence in the environment are still unknown. As the effects of pollutants on biological processes in the aquatic environment cannot be adequately assessed solely with the aid of chemical or physical parameters, several biological indicators and methodologies have been developed. A variety of bioassay techniques employing fish, invertebrates, phytoplankton and bacteria for detecting the toxic effect of chemicals in fresh- and seawater are currently being studied (Maciorowski et al., 1981). Among these, algal assays have become widely used as biological tools in environmental impact studies. The justification for using algae is related to their ecological role as primary producers in transferring energy to higher trophic levels (Wong and Couture, 1986). In addition, algal assays are relatively simple, quick and inexpensive in comparison with bioassays with other organisms. Algal assays are intended to provide information about the availability of chemical compounds to the organism and their inhibitory effects. Current assessment procedures are based on the premise that algal tests can be used to predict the effects of chemical compounds in aquatic environments (Skulberg, 1995). Growth-inhibition tests with freshwater algae (EN ISO, 1995) and seawater algae (EN, 1993) 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, 1993). In this study, the first to report on the toxicity of imidazolium ionic liquids in the marine environment, we used a growth-inhibition algal test. The

selection of ionic liquids entities was based on the t-SAR approach (thinking in terms of structure–activity relationships) (Jastorff et al., 2003) focusing on the length ($C_2 < R1 < C_6$) or type (aliphatic–aromatic) of the side chain whereas head group (imidazolium) remained the same. The acute effect of ionic liquids was measured in two algal species typically inhabiting brackish waters like those of the southern Baltic Sea: the green alga *Oocystis submarina* and the diatom *Cyclotella meneghiniana.* Since the salinity in the Baltic Sea is subject to considerable variation—from 2 PSU in the Bothnian Bay, through 7 PSU in the Gulf of Gdańsk up to 18 PSU in the Kattegat—the additional influence of different salinities on the biological impact of some ionic liquids on algae was also examined.

2. Material and methods

2.1. Batch cultures

Two taxonomically different algal species were used in this study: the green alga *O. submarina* (BA-0002) and the diatom *C. meneghiniana* (BA-0010). They were isolated from coastal waters of the Baltic Sea and maintained as unialgal cultures in the Culture Collection of Baltic Algae (CCBA) at the Institute of Oceanography of the University of Gdańsk (Latała, 2003).

The test algae were batch-cultured in F/2 medium (Guillard, 1975) prepared from Baltic water (PSU = 7). When required, higher salinities were obtained by evaporation, lower ones by dilution with distilled water. Stock cultures of the test organisms were acclimatized for 10 days at 20 °C in the presence of photosynthetically active radiation (PAR) at 25 μmol photons m^{-2} s^{-1} using an L:D photoperiod of 16:8 h. Irradiance was measured using a quantummeter LiCor (LI-189) with a cosine collector. The final batch cultures used in the experiments were obtained by mixing a known amount of the stock culture in the log growth phase with sterile medium. The initial density of *O. submarina* was approximately 50,000 cells ml^{-1} than that of *C. meneghiniana* 26,000 cells ml^{-1}. 9.9 ml of algal suspension were transferred into glass Erlenmeyer flasks (25 ml). To each of these, 0.1 ml of different concentrations of an aqueous ionic liquid solution was added to obtain final concentrations of 5, 50 or 500 μM. All the flasks were incubated in culture chambers for 3–11 days at 20 °C. Stock cultures of the control organisms were acclimatized similarly to the test organisms, i.e., for 10 days at 20 °C in the presence of (PAR) at 25 μmol photons m^{-2} s^{-1} using a L:D photoperiod of 16:8 h. The final batch cultures used as controls were obtained by mixing a known amount of the stock culture in the log growth phase with sterile medium. The algal suspension were transferred into glass Erlenmeyer flasks (25 ml). To each of these, 0.1 ml of deionized water (instead ionic liquid) was added. All experiments were run minimally in triplicate. The variability of the results did not exceed 5% on the inhibition scale. The number of cells were determined microscopically every day in a Bürker counting chamber.

2.2. Ionic liquids

Except for 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM][BF_4]), which was purchased from Fluka (Buchs, Switzerland), all the remaining compounds selected for these studies: 1-butyl-, 1-benzyl- and 1-hexyl-3-methylimidazolium tetrafluoroborates ([BMIM][BF_4], [BzMIM][BF_4] and [HMIM][BF_4])—were obtained from Merck (Darmstadt, Germany). The ionic liquids were used as supplied, without any additional pre-treatment. All compounds were checked for their purities by HPLC analysis following a method of Stepnowski et al. (2003). No nonvolatile impurities were found in this assay. Volatile contaminants (up to 300 °C bp) of ionic liquids were analyzed by gas chromatography headspace technique during routine checks of synthesized products in the Prof. Bernd Jastorff s laboratory at the Centre for Environmental Research and Technology, UFT, University of Bremen. They were at the level of 0.1%.

2.3. Toxicity tests

The ionic liquid toxicity tests were carried out using modified versions of the methods recommended in the European Committee for Standardization's guidelines (EN ISO, 1995; EN, 1993). The percentage inhibition of the cell growth I_n was determined for the each point in time from the equation:

$$I_n\,(\%) = \frac{N_c - N_t}{N_c} \times 100 \qquad (1)$$

where n is the each point of the measurement in time, N_c the cell number in the control and N_t the cell number in the culture incubated with toxicant (an ionic liquid). Ionic liquids were tested in three different concentrations (5, 50 and 500 μM) added to the algal media of both species. A further two compounds ([HMIM][BF_4] and [BMIM][BF_4]) were additionally tested for their activity with respect to *O. submarina* in varying salinities of 0, 8, 16, 24 and 32 PSU.

Potential adhesion of the ionic liquid cations to the walls of flasks used was measured using analytical methods for determination of ionic liquids (Stepnowski et al., 2003; Stepnowski and Mrozik, 2005; Stepnowski, 2005). Within the time period of the experiment solute concentration in the aqueous phase did not change.

3. Results

Fig. 2A–D illustrates the growth inhibition of *O. submarina* during the 11-day exposure to three different concentrations—from 5 to 500 μM—of ionic liquids added to the media. The pattern of algal cell inhibition was similar for all the ionic liquids, regardless of the compound used. The effects of the 5 and 50 μM concentrations were immediate but also reversible: in these cases, the growth abilities of these cell cultures were restored, so that their densities eventually reached the same level as that of the control sample. In contrast, the highest concentration of ionic liquids (500 μM) was sufficient to inhibit cell growth throughout the experiment. The first 3 days exposure to all concentrations of 1-butyl-3-methylimidazolium ([BMIM][BF_4]) decreased growth by 20–30%. With the 5 μM concentration, cell numbers gradually increased during the next 4 days of the experiment, finally reaching the level of the control sample (Fig. 2B). With 50 μM of [BMIM][BF_4] 7 days had to elapse before the initial cell density was restored. When 500 μM of [BMIM][BF_4] were employed, algal cell numbers fell to 70% of the ini-

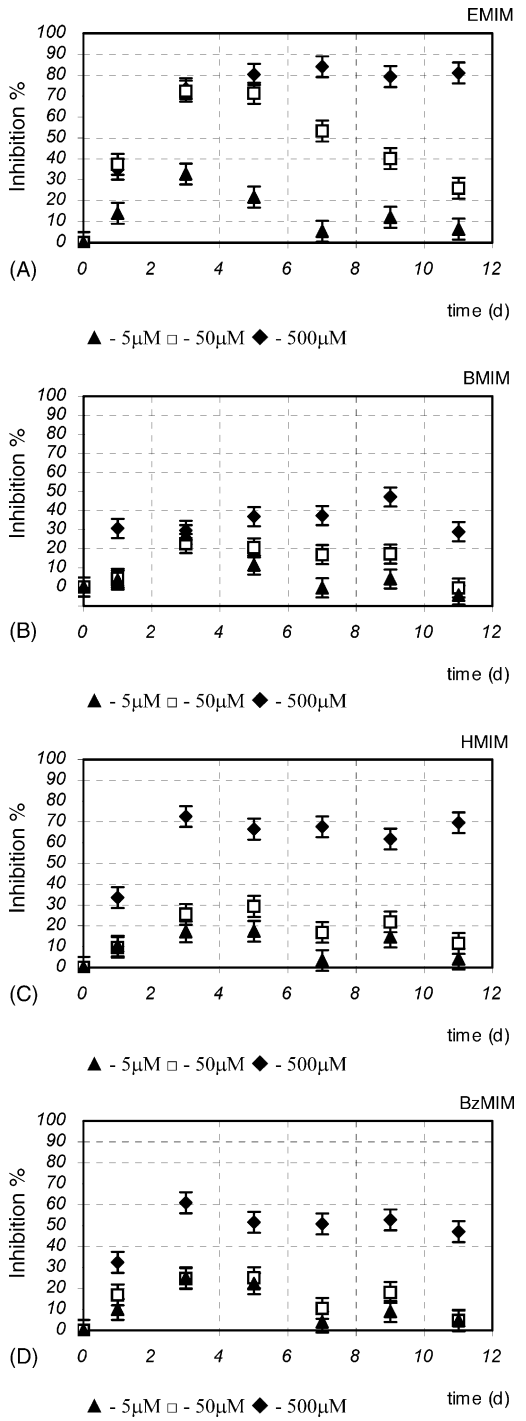
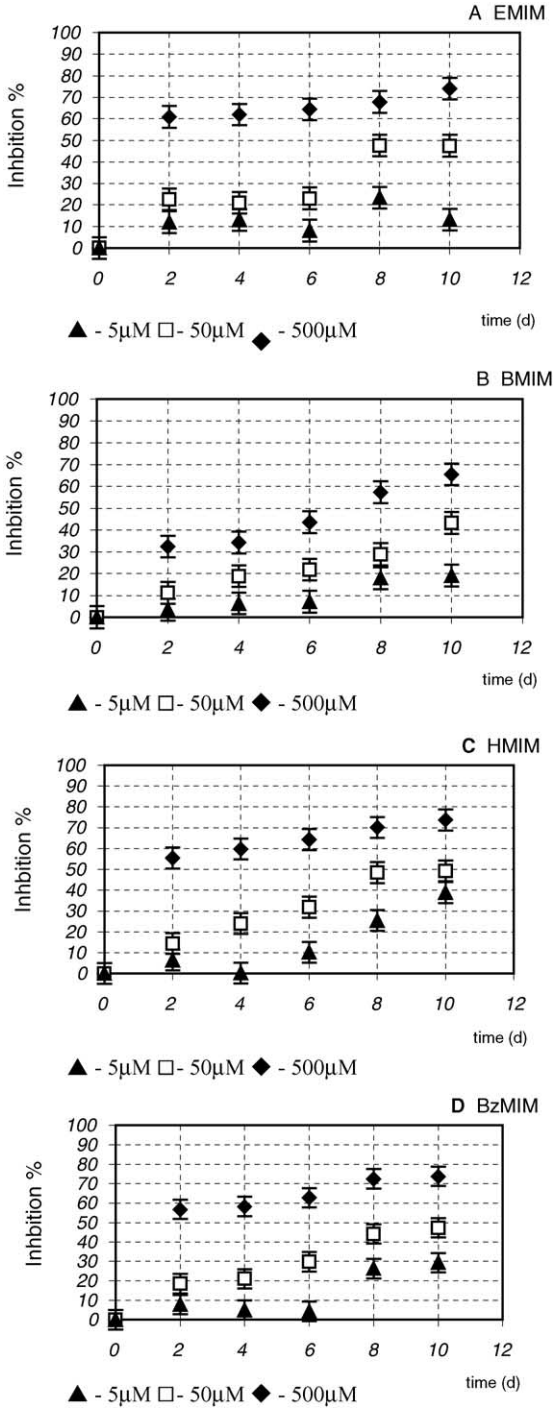


Fig. 2. Mean values of the percentage inhibition of *O. submarina* cell number by imidazolium ionic liquids during the experiment. (A) 1-Butyl-3-methylimidazolium, (B) 1-ethyl-3-methylimidazolium, (C) 1-hexyl-3-methylimidazolium, (D) 1-benzyl-3-methylimidazolium salts. Concentrations used: (▲) 5 μM; (□) 50 μM; (♦) 500 μM.

tial value already after the first day, and remained at the level of 50% for the duration of the experiment. Growth inhibition of *O. submarina* cultures with 1-ethyl-3-methylimidazolium ([EMIM][BF_4]) was much stronger than in cultures with [BMIM][BF_4]. After 3 days, 5 μM of [EMIM][BF_4] had reduced algal numbers by one-third, although during the next few days this effect appeared to be reversed, as was the case with [BMIM][BF_4]. As Fig. 2A shows, concentrations of 50 and 500 μM inhibited growth of the *Oocystis* culture much more significantly, with cell numbers dropping to 25% of the control value after 72 h of exposure. In the case of the 50 μM concentrations, algal growth recommenced during the following few days. The cell density of cultures with the ionic liquid reached 75% of the control cell density by the end of the experiment. The highest [EMIM][BF_4] concentration (500 μM) prevented any growth of *O. submarinal*, causing at least 80% inhibition throughout the experiment. With regard to the 1-hexyl-3-methylimidazolium entity ([HMIM][BF_4]), growth inhibition was very much in evidence after just 3 days exposure: 18, 25 and 71% by 5, 50 and 500 μM of [HMIM][BF_4], respectively (Fig. 2C). As was the case with [BMIM][BF_4] and [EMIM][BF_4], the 5 μM concentration lost its inhibitory potency during the experiment, so that the *O. submarina* culture recovered to attain a final density close to the initial value. While the pattern of inhibition variation was similar for the 50 μM concentration, exposure to 500 μM of ionic liquid ([HMIM][BF_4]) brought cell growth to a standstill. The aromatically substituted imidazolium ionic liquid ([BzMIM][BF_4]) inhibited *Oocystis* cells in the same way as [HMIM][BF_4], although the highest concentration used was comparably less effective in allowing the number of algal cells to remain at 50% of the control density (Fig. 2D).

On the basis of the data obtained, it is not possible to estimate any effective concentration values. However it can be observed that after 72 h of the exposure, the lowest concentration inhibiting the cell growth was obtained for [EMIM][BF_4], the entity with the shortest alkyl chain length. After the same exposure period, [BzMIM][BF_4] and [HMIM][BF_4] were about one order of magnitude less effective. The data obtained for [BMIM][BF_4] were insufficient to make even a very rough estimate of this value.

Fig. 3A–D illustrates the growth inhibition of *C. meneghiniana* during 10 days of culture as a result of exposure to 3 different concentrations of ionic liquids (concentrations 5–500 μM). The response of the algae to the presence of spiked ionic liquids at all concentrations is clearly visible. Unlike the population of *Oocystis*, the *Cyclotella* culture did not acclimatize to even the lowest ionic liquid concentration tested and growth was inhibited throughout the experiment. With 5 and 50 μM of [BMIM][BF_4] (Fig. 3B), cell densities decreased gradually during the first 6 days of the experiment, but during the next 4 days growth inhibition was much stronger. With 500 μM of [BMIM][BF_4], cell numbers dropped to 70% of the control value after 2 days, after which they continued to decrease gradually to a level of 35%. The pattern of *C. meneghiniana* cell growth inhibition by 5 and 50 μM of [EMIM][BF_4] (Fig. 3A) or [BzMIM][BF_4] (Fig. 3D) salts was similar to that of inhibition by [BMIM][BF_4], but was much stronger with the 500 μM concentration. After 2 days, 500 μM ionic liquids reduced the density of these cultures to ca. 40% of the control cell density and prevented all growth during the remaining days of exposure. Inhibition of the algae by [HMIM][BF_4] was already very strong after only 2-day exposure to 500 μm, whereas the 5 and 50 μm concentrations caused a retardation in the growth of the culture so that the cell density was ca 50% of that in control cultures by the end of the experiment (Fig. 3C). Except for the [BMIM][BF_4] entity, the effective concentrations within a 48 h time period were at the same level for [EMIM][BF_4], [HMIM][BF_4] and [BzMIM][BF_4] ionic liquids. Cultures of green alga *O. submarina* were additionally exposed to 500 μM of [BMIM][BF_4] and [HMIM][BF_4] at salinities ranging from 0 to 32 PSU. The results demonstrate the moderating influence of salinity on the biological activity of [HMIM][BF_4] in the *O. submarina* viability assay (Fig. 4). HMIM's acute toxicity weakened with increasing ambient salinity. In freshwater cultures the control cell density was reduced to 50% after 3 days exposure to the ionic liquid solution. However, at a salinity of 8 PSU, cell growth inhibition was only 30%, and in the most saline waters (16, 24 and 32 PSU) it was a mere 10%. In the case of [BMIM][BF_4], cell growth inhibition was also reduced with increasing water salinity: the cell density fell by 30% in response to a 3-day exposure to this ionic liquid in fresh water, but dropped by only 10% at 16 PSU, and was unaffected at the highest salinity applied (32 PSU).



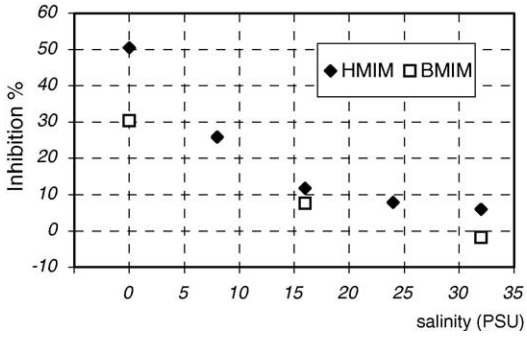


Fig. 4. Influence of 500 μM of 1-hexyl and 1-butyl-3-methylimidazolium tetrafluoroborates on the density of the *O. submarina* population in different salinities, presented as the percentage of growth inhibition on day 3 of the culture: (□) 1-butyl-3-methylimidazolium tetrafluoroborate, (♦) 1-hexyl-3-methylimidazolium tetrafluoroborate.

4. Discussion

Inspection of the temporal relationships between algal population density and ionic liquid concentrations in batch cultures of the Baltic species *O. submarina* and *C. meneghiniana* shows that ionic liquids effectively inhibit algal growth. The *Oocystis* cultures appeared to acclimatize to the imidazolium ionic liquids in concentrations of up to 50 μM, because after ca. 10 days they recovered their growth ability, finally reaching the same density as control cultures. In contrast, the response of *C. meneghiniana* was different: the growth of these cultures was effectively inhibited throughout the 10-day test period regardless of the ionic liquid concentration applied.

The 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM][BF_4]), the shortest alkyl-substituted derivative of the imidazolium ionic liquids used in these experiments, was effective in inhibiting growth of an *O. submarina* batch culture at the smallest concentrations. These values for the other compounds and batch cultures of the diatom *C. meneghiniana* were one order of magnitude higher. Though preliminary, this informa-

Fig. 3. Mean values of the percentage inhibition of *C. meneghiniana* cell number by imidazolium ionic liquids during the experiment. (A) 1-Ethyl-3-methylimidazolium, (B) 1-butyl-3-methylimidazolium, (C) 1-hexyl-3-methylimidazolium, (D) 1-benzyl-3-methylimidazolium salts. Concentrations used: (▲) 5 μM; (□) 50 μM; (♦) 500 μM.

tion is very interesting, since all the prokaryotic and eukaryotic cell systems tested so far appear to have exhibited the opposite relationship, that is, the longer is the *n*-alkyl chain, the greater is the toxicity of the compound in question. This property has usually been attributed to enhanced membrane permeability (Ranke et al., 2003; Stepnowski et al., 2004).

The observed differences between algal species in their response to exposure to ionic liquids can be initially attributed to structural differences in the cell walls. In the case of green algae, cellulose is the main structural element of the wall, whereas diatoms are surrounded by a frustule composed of silica.

The organisms most frequently used in freshwater ecotoxicological tests are green algae (EN, 1993), especially *Selenastrum capricornutum*, a common eutrophic lake species that is easy to culture and moderately sensitive (Stauber, 1995; Walsh and Merrill, 1984), or *Scenedesmus subspicatus*, which is similarly sensitive to toxic substances (Nyholm and Källqvist, 1989). Where marine algae are under investigation, diatoms are the usual test organisms (EN ISO, 1995), since they are the taxonomic group that is the most sensitive to the metals and organic compounds being examined (Hornstrom, 1990). Widespread and of ecological significance in the ocean, *Skeletonema costatum* is recommended as the standard test organism, as it is generally sensitive to pollutants. The results obtained in our experiments also indicate that diatom is more sensitive than green alga as biomarker of ionic liquid contamination.

The discovery that the toxicity of ionic liquids is reduced in more saline waters is of major importance in the further-fate assessment of these compounds in marine environments. The lower toxicity is probably due to the reduced permeability of ionic liquid cations through the algal cell walls. Higher chloride concentrations offer a good ion-pairing environment for imidazolium cations, which therefore compete with hydroxyl (green alga) or silanol (diatom) functional groups in cell-wall structures. The possible specificity of this effect should be investigated further in more detailed studies. In particular, a much broader range of concentrations needs to be covered in order to obtain complete concentration-response curves. Future studies on a larger pool of anions are also needed so that a true assessment of their influence on cationic toxicity can be made.

Acknowledgments

This work was supported by the NATO Collaborative Linkage Grant (EST) “Designing molecular test systems for toxicological and ecotoxicological risk assessment”. Financial support was provided by the Polish Ministry of Scientific Research and Information Technology under grants DS 8000-4-0141-5 and BW 8000-5-0285-5.

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