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A study into the species sensitivity of green algae towards imidazolium-based ionic liquids using flow cytometry

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Abstract: The sensitivity of individual organisms towards toxic agents is an important indicator of environmental pollution. However, organism-specific quantification of sensitivity towards pollutants remains a challenge. In this study, we determined the sensitivity of *Chlorella vulgaris* (*C. vulgaris*) and *Scenedesmus quadricauda* (*S. quadricauda*) towards three ionic liquids (ILs), 1-alkyl-3-methyl-imidazolium chlorides [$C_n$mim][Cl] (n=4,6,8). We kept all external parameters constant to identify the biotic parameters responsible for discrepancies in species sensitivity, and used flow cytometry to determine four conventional endpoints to characterise cell viability and cell vitality. Our results demonstrate that after exposure to the ILs, cell proliferation was inhibited in both species. At the same time, the cell size, complexity and membrane permeability of both algae also increased. However, while Chl *a* synthesis by *S. quadricauda* was inhibited, that of *C. vulgaris* was enhanced. *S. quadricauda* has evolved a metabolic defense that can counteract the decreased esterase activity that has been shown to occur in the presence of ILs.

While it is likely that *S. quadricauda* was less sensitive than *C. vulgaris* to the ILs because of this metabolic defense, this alga may also exhibit better membrane resistance towards ILs.

Keywords: species sensitivity; toxic effect; ionic liquid; *Chlorella vulgaris*; *Scenedesmus quadricauda*

1. Introduction

Large-scale applications of ionic liquids (ILs), especially imidazolium-based ILs, are anticipated to increase in the near future (Chatel et al., 2017) within a variety of domains, including organic synthesis and (bio)catalysis, electrochemistry, analytical chemistry, separation technology, nanotechnology, renewable resource utilization, and functional fluids (e.g. lubricants, heat transfer fluids, corrosion inhibitors) (Bubalo et al., 2014). Nevertheless, the rapid growth in production and application of ILs will inevitably result in their discharge into environment (Torrecilla et al., 2009; Abramenko et al., 2020) through various pathways, such as accidental spills, leaching of landfill sites and wastewater discharge (Liu et al., 2015 a). An ecological risk assessment (ERA) of their effect on aquatic life is therefore essential (Thamke et al., 2017). In an ERA, determination of toxicity, expressed as an EC_{50} (the half-maximal effective concentration) is the approach that is most often employed, but establishing the chronic toxic effects of the pollutant on organisms represents a more sophisticated level of analysis (Wen et al., 2018; Zhang et al., 2017). The sensitivity of a diversity of species towards environmental contaminants is another

important factor when carrying out an ERA (Barron et al., 2012), in order to avoid either “insufficient” or “excessive” assessment (Zhang et al., 2017; Park et al., 2018). Nevertheless, in spite of their importance, the latter two fields are frequently disregarded.

Since algae are an important part of the aquatic ecosystem, and provide a rapid and sensitive response to toxins (Pham et al., 2010; Moro et al., 2012), numerous reports have determined the EC_{50} and/or described the toxicity effects of ILs towards algae. Because they comprise a highly diverse group of photosynthetic eukaryotes, spanning up to 15 phyla, the sensitivities of algae towards toxins can vary widely. The EC_{50} value of a single IL towards diverse species may differ by several orders of magnitude (Kulacki et al., 2008). For example, the 96 h EC_{50} values for $[C_4mim][Br]$ towards *Scenedesmus quadricauda* (*S. quadricauda*) and *Chlamydomonas reinhardtii* are 22 μM and 4898 μM, respectively. The 72 h EC_{50} values for $[C_4mim][Cl]$ towards *Scenedesmus vacuolatus* and *Chlorella vulgaris* (*C. vulgaris*) are 140 μM and 1026 μM, respectively (Petkovic et al., 2011). Furthermore, the toxic effects may present in a variety of ways, including growth inhibition (Hu et al., 2013), membrane damage and membrane disruption (Chen et al., 2014), changes in morphology and structure (Liu et al., 2015 b), or abnormalities in photosynthesis (Deng et al., 2015) and metabolism (Chen et al., 2014).

It is widely recognized that discrepancies in species sensitivity are a natural consequence of interspecies variation in cell cycle, morphology, size, pigment profile and membrane morphology (Stulik et al., 2000). In addition to these innate,

biotic factors, the sensitivity of a species to contaminants is also dependent on external abiotic parameters. It has been demonstrated that changes to the conditions of an assay, such as initial cell density and salinity of the culture medium, can lead to distinct differences in sensitivity (Tsarpali et al., 2016). In addition, for ILs, less predictable abiotic parameters, such as the diversity of IL forms in water, potential interference of ILs with the culture medium tested and IL absorption to vessel walls, might also lead to differing toxic effects (Stulik et al., 2000). In this context, the determination of species sensitivity continues to pose a significant challenge. However, if all external, abiotic parameters were to remain constant, then any possible biotic parameters responsible for discrepancies in species sensitivity could be identified.

In this study, we compared the physiological response of two algal species against 1-alkyl-3-methyl-imidazolium chlorides [$C_n$mim][Cl] (n = 4, 6 or 8), under nearly the same assay conditions, while varying the concentration of IL tested, which resulted in around 10% - 90% inhibition of cell density in a pre-experiment. [$C_n$mim] are representative cations of the imidazolium family that are regularly used in synthetic, catalytic, biomechanical, degradative, and analytical applications (Habibul et al., 2020). The anion, Cl^-, was selected to represent hydrophilic ILs since it shows no intrinsic toxicity effect (Mena, 2020; Matzke et al., 2007). As test organisms, we chose *C. vulgaris* and *S. quadricauda*, which are the two major algae in the fresh water environment of China. The cell walls of both species are composed primarily of cellulose, and chlorophyll *a* is their main photosynthetic pigment. The same

conditions (illumination, photoperiod, initial inoculum and nutrient concentration) were used in all assays for both species, in order to avoid discrepancies arising from the experimental conditions. We used flow cytometry (FCM), a relatively new technique in toxicity studies of ILs, to determine four conventional endpoints that characterise cell viability (capacity for growth) and cell vitality (manifestation of life). A preliminary identification of the biotic factors responsible for varying species sensitivity towards ILs is given that will help to inform future ERA studies into the environmental risk posed by these polar solvents.

2. Materials and methods

2.1. Reagents

The ionic liquids were purchased from Shanghai Chengjie Chemical Co. Ltd (China). IL purity in terms of the cation was > 95%, as determined by ^{1}H NMR (Bruker AV500) while the purity of the anion was also > 95% by ion chromatography (Metrohm, Switzerland). The dyes fluorescein diacetate (FDA) and propidium iodide (PI) were purchased from the Sigma-Aldrich Chemical Co. *C. vulgaris* (FACHB-71) and *S. quadricauda* (FACHB-1297) were purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. The water used in this study was deionized, and came from an ultra-pure water machine (EPED-20TH, China).

2.2. Algal culture and IL exposure

Water and materials to come into contact with the algae were first sterilised under 0.1 MPa pressure at 121 °C for 30 mins, and all operations were carried out in a biosafety cabinet (ESCO AC2-4S1). Algae in the log phase of growth were inoculated in 100 ml portions of freshly sterilized standard BG11 medium (Ma et al., 2010) within 250 mL Erlenmeyer flasks, in order to obtain an initial cell density of 1×10^5 cells mL^{-1}.

All ILs were completely miscible with water and stock solutions of each IL at 2 g/L were prepared. The stock solutions were dispensed into flasks of the medium, to make up the final IL concentrations (C_{IL}) represented in Table 1, resulting in around 10% - 90% inhibition of cell density in a pre-experiment.

Table 1 Exposure concentrations of ILs (C_{IL}/$mg.L^{-1}$) on algae.

	$[C_4mim][Cl]$	$[C_6mim][Cl]$	$[C_8mim][Cl]$
S.quadricauda	20, 50, 100, 150, 200	5, 10, 20, 50, 100	1, 5, 10, 20, 40
C.vulgaris	2, 5, 10, 20, 30, 50	2, 5, 10, 30, 50	0.2, 0.5, 1, 2, 5, 10

Experiments at each concentration were carried out in triplicate. The flasks were each plugged with a wad of cotton wool to protect the medium from contamination, then cultured on a shaker at 120 r/min, at 26 ± 1 °C at an illumination intensity of 56 ± 1 μmol/m^2/s on a 12 h:12 h light: dark cycle.

Aliquots were withdrawn from the cultures in a biosafety cabinet on the 0^{th} and 96^{th} hour of the experiment. The cultures were examined under a microscope over the entire course of the experiment, to ensure that no microbial contamination had taken place.

2.3. Flow cytometric analysis

Flow cytometric analysis was performed using a BD Accuri™ C6 flow cytometer (Bectone Dickinson, USA) equipped with 488 nm and 640 nm argon lasers at a flow rate of 1 mL/s, as described in our previous report (Deng et al., 2015). Aggregated cells, non-algal particles and dead cells were excluded by gating in FSC-height/FSC-area diagrams and SSC/FL4 diagrams. Aliquots taken from the culture were filtered through a 48 μm membrane filter and analysed by FCM to monitor cell density, membrane permeability, chlorophyll *a* autofluorescence and FDA stained fluorescence. Cell density and chlorophyll *a* autofluorescence were measured directly. Aliquots taken from the culture were stained with a solution of PI at 30 μmol/L for *S. quadricauda* and 20 μmol/L for *C. vulgaris*, then allowed to stand for 20 min at 25 °C in the dark. The concentrations of stain with FDA were 30 μmol/L for *S. quadricauda* and 25 μmol/L for *C. vulgaris*. The stained cells were then left to stand for 10 min. Autofluorescence data were collected using the FL4 channel (675 ± 12 nm), and fluorescence data from PI and FDA stained cells were collected using the FL2 (585 ± 20 nm) and FL1 channels, (533 ± 15 nm) respectively. The data were expressed as mean fluorescence intensities (*MFI*s).

2.4. Data analysis

The results collected in the reports represent mean values of triplicate samples. The inhibition rate (*IR*) of each IL towards each alga was calculated using the

equation:

$$IR(\%) = (1 - \mu_i/\mu_c) \times 100\% \quad (1)$$

In which *IR* is the inhibition rate, μ_i and μ_c are the growth rates of the sample and the control after the same incubation time, calculated using Equation (2):

$$\mu = (\ln n_L - \ln n_0)/(t_L - t_0) \quad (2)$$

in which n_0 is the initial cell density, n_L is the cell density at time t_L, t_0 is the time of test start and t_L is the time of sampling.

The ErC_{50} represents the concentration of toxicant that results in a 50% reduction in growth rate compared with the control after 96 h incubation time. The ErC_{50} values were calculated using Analyze-Regression Probit procedure in IBM SPSS (version 19.0; Armonk, NY, USA) (Li et al., 2015). The properties of cells exposed to different C_{IL} were analysed using the One-way Analysis of Variance (ANOVA) procedure and Duncan's new multiple range test in POST HOC in IBM SPSS. $P \leqslant 0.05$ is the criterion for significant difference.

3. Results and discussion

3.1. Cell growth

The cell densities plotted against time are depicted in Fig. 1 and the calculated 96 h ErC_{50} values are reported in Table 2. Previous researchers have concluded that the "side-chain effect" is the main chemical factor determining the toxicity of ILs (Pham et al., 2010; Saraiva et al., 2017). In this study, the ErC_{50} value of ILs towards both species decreased with increasing length of the side chain, supporting this conclusion.

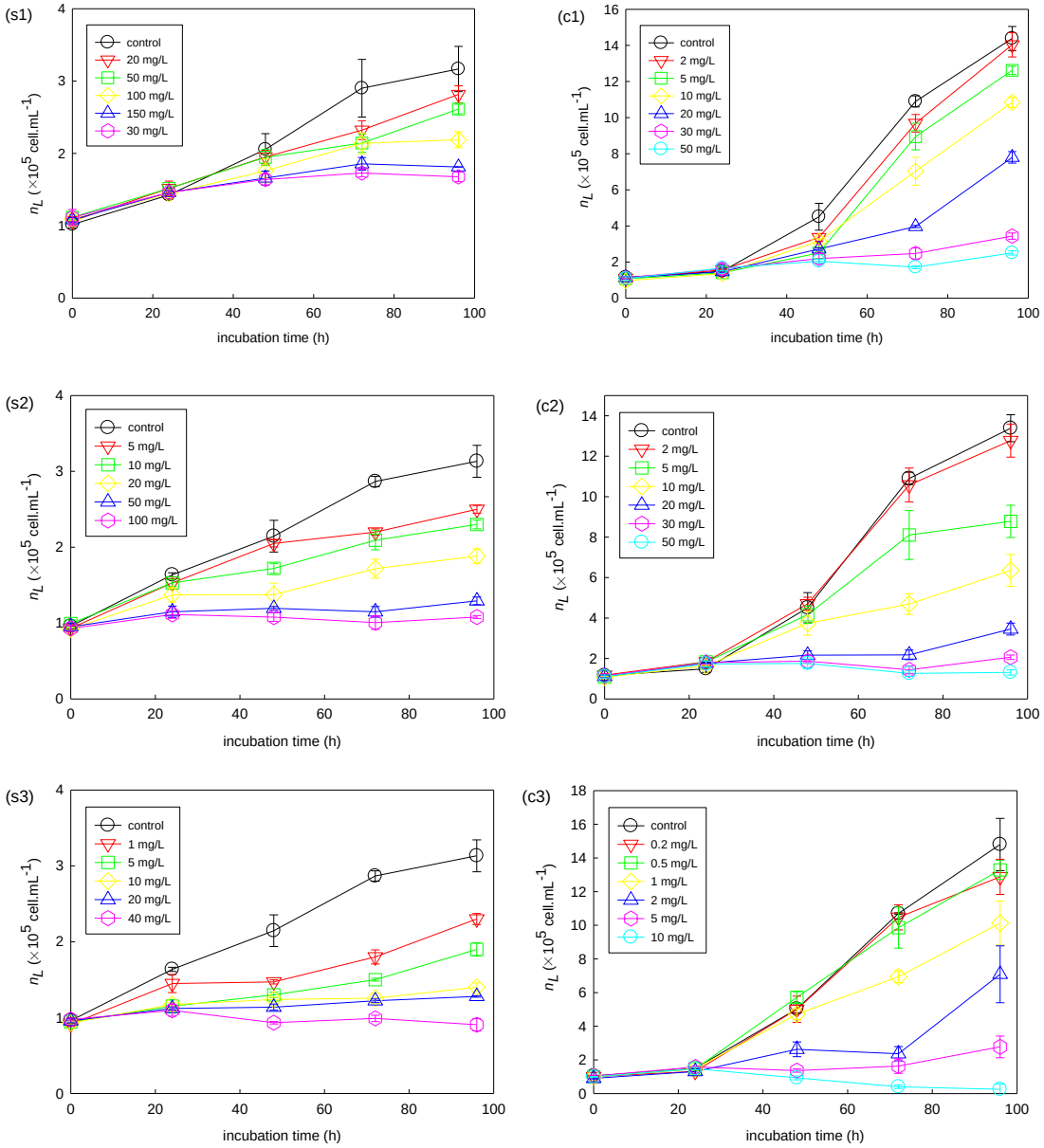

Fig. 1 Cell densities at different incubation time: (s1) *S quadricauda* exposed to [C_4mim][Cl]; (s2) *S quadricauda* exposed to [C_6mim][Cl]; (s3) *S quadricauda* exposed to [C_8mim][Cl]; (c1) *C. vulgaris* exposed to [C_4mim][Cl]; (c2) *C. vulgaris* exposed to [C_6mim][Cl]; (c3) *C. vulgaris* exposed to [C_8mim][Cl].

Table 2 The 96 h-ErC_{50} values (mg/L) of [$C_n$mim][Cl] (n=4,6,8) for *S.quadricauda* and *C.vulgaris.*

ILs	[C_4mim][Cl]	[C_6mim][Cl]	[C_8mim][Cl]
S.quadricauda	129	22.3	5.60
C.vulgaris	32	16.2	3.90

During the test period, the ErC_{50} values of each IL towards *C. vulgaris* were lower than those of *S. quadricauda*, suggesting that *C. vulgaris* is more sensitive than *S. quadricauda* (Table 2). Roubeix et al. (2011) also found that smaller species were more sensitive towards toxins. However, in another study, smaller species proved to be more tolerant (Ricart et al., 2009). This apparent paradox can be resolved if cell size is not, in fact, a determining factor in microalgal sensitivity.

[C_4mim][Cl] was used to study the toxic effect of [$C_n$mim] ILs on the morphology of cells (Fig 2). For both species, MFI from the forward scatter channel (MFI_{FSC}) and MFI from the side scatter channel (MFI_{SSC}) increased, suggesting that the particle size and fine structural complexity increased. This phenomenon usually occurs because of increased cell size or cell deformation (Franqueira et al., 2000; Franklin et al., 2001). However, an alternative explanation for this change in scattering intensity may be the formation of a mucilaginous sheath around the algal cells, as a protective barrier against the IL (Nam et al., 2018).

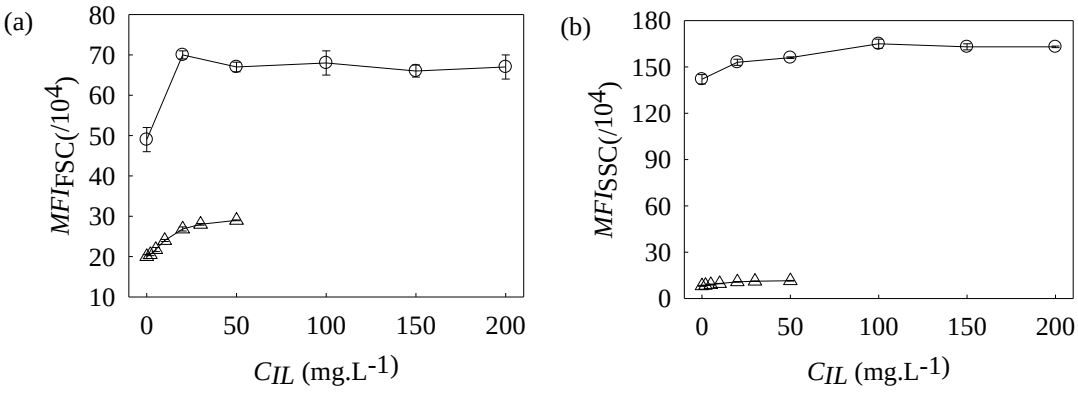

Fig 2 Effect of [C_4mim][Cl] on the morphology of algae after a 96 h exposure: ○, *S. quadricauda*; △, *C. vulgaris*.

When C_{IL} <50 mg/L, the MFIs increased generally with C_{IL}. At 50 mg/L, the MFI_{FSC} of exposed *S. quadricauda* and *C. vulgaris* were 9.86% and 40.24% higher than the control, whilst the MFI_{SSC} values were 36.73% and 45.00% higher, respectively. When C_{IL}s were higher than 50 mg/L, the MFIs did not increase further (P>0.05). These results suggest that both the cells' size and the complexity of their fine structure increased more for *C. vulgaris* than *S. quadricauda*. The disparity in cell size between the two algae became very obvious, with *C. vulgaris* cells enlarging by 4 times as much as those of *S. quadricauda* after exposure to the IL. The influence of [C_6mim][Cl] and [C_8mim][Cl] on the morphology of the cells showed similar tendencies, so no further investigation of these ILs was undertaken.

3.2. Membrane permeability

Because of its ability to penetrate damaged cellular membranes, the dye PI is frequently used to assess membrane integrity (cell viability) (Franklin et al., 2001). However, it is important to appreciate that uptake of PI is also observed in cells with intact membranes (healthy cells). Nevertheless, in a histogram of fluorescence intensity, healthy cells fluoresce in a region of lower fluorescence intensity, which is distinct and clearly separated from the region where dead cells fluoresce (Deng et al., 2015). In this study, we have used the MFI of cells stained with PI (MFI_{PI}) as an indicator of membrane permeability, as permeability is proportional to the quantity of

PI entering the cells.

From the results of ANOVA, after 96 h exposure, the MFI_{PI} for both species started to increase from a certain value, which we have referred to as the 'threshold concentration' (TC) of exposed IL (Table 3). At C_{IL} values lower than the TCs (Fig 3), the differences in membrane permeability for the samples were not statistically significant ($P>0.05$), compared with the control. At C_{IL} values higher than the TCs, MFI_{PI} increased in a concentration-dependent manner, indicating that membrane permeability had increased, and membrane integrity may have been compromised.

As shown in Table 3, *S. quadricauda* had a higher TC towards [C_4mim][Cl] than *C. vulgaris*. At 50 mg/L for [C_6mim][Cl] and at 10 mg/L for [C_8mim][Cl], MFI_{PI} increased by 51% and 42% for *S. quadricauda*, and by 210% and 220%, respectively for *C. vulgaris*, compared with the control. The superior membrane resistance of *S. quadricauda* is probably a major factor in its reduced sensitivity towards ILs, compared with *C. vulgaris*.

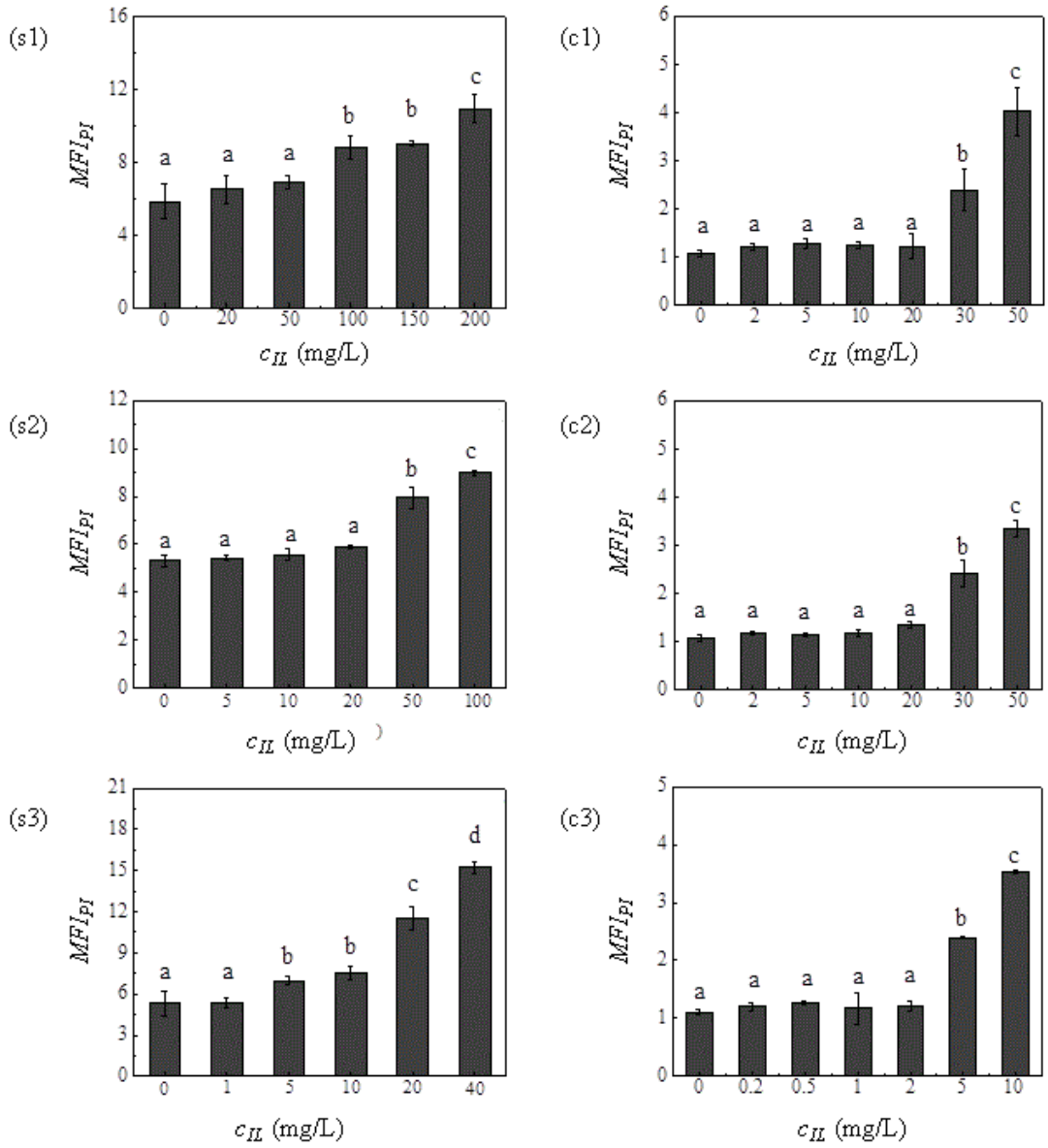

Fig. 3 MFI after staining with PI, reflecting cell permeability (graph numbers correspond to Fig. 1).

Note: The letters above the bars represent statistical differences: bars with the same letter have no statistical difference between them (P>0.05), whilst those with different letters exhibit statistical differences (P＜0.05).

Table 3 The threshold concentration (TC) for membrane damage (mg/L) of [$C_n$mim][Cl] (n=4,6,8) on *S. quadricauda* and *C. vulgaris.*

ILs	[C_4mim][Cl]	[C_6mim][Cl]	[C_8mim][Cl]
S.quadricauda	50-100	20-50	1-5
C.vulgaris	20-30	20-30	2-5

3.3. Chlorophyll *a* content (C_{Chl})

C_{Chl} is a good indicator of the physiological state of individual cells, as it provides information on metabolite absorption and distribution, as well as cellular energy use in preparation for photosynthesis (Hadjoudja et al., 2009; Cid et al., 1996). The MFI of chlorophyll *a* fluorescence (MFI_{Chl}) determined by FCM is proportional to the mean C_{Chl} of each cell.

After 96 h exposure, the MFI_{Chl} values of *S. quadricauda* exposed to [$C_n$mim][Cl] (n = 4, 6, 8) were generally reduced compared with the control (Fig 4). However, the inhibition effects were not obviously concentration-dependent, as the MFI_{Chl} of cells exposed to [C_4mim][Cl] at all concentrations were statistically the same ($P>0.05$), as were those of cells exposed to [C_6mim][Cl] at 5-20 mg/L. For [C_6mim][Cl] at 50-100 mg/L and [C_8mim][Cl] at all tested concentrations, the MFI_{Chl} of cells decreased in a concentration-dependent manner. This inhibition in C_{Chl} has been corroborated by several prior studies into the toxicity of [$C_n$mim]-based ILs towards microalgae (Liu et al., 2015 c) and is thought to be induced by inhibition of electron flow on the donor side of the photosystem II (PS II) reaction centre (Franqueira et al., 2000).

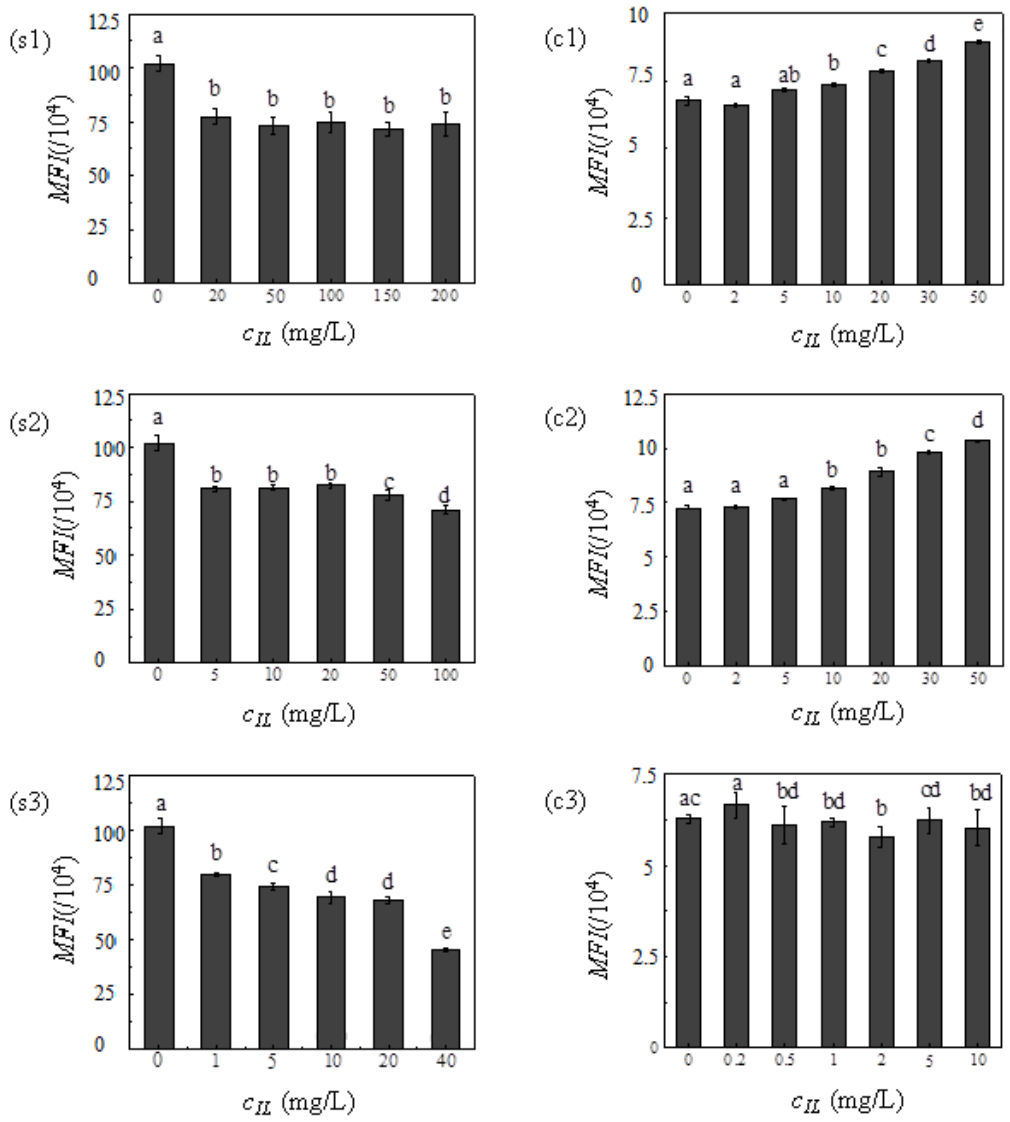

Fig. 4 Mean chlorophyll fluorescence of the samples exposed to different IL concentrations (graph numbers and notes correspond to Fig. 3).

The response of *C. vulgaris* towards the ILs was clearly different from that of *S. quadricauda*. For [C_4mim][Cl] and [C_6mim][Cl] at 2-5 mg/L and for [C_8mim][Cl] at 0.2 mg/L, the MFI_{Chl} values for the cells were statistically the same as for the control

(P>0.05). However, for [C_4mim][Cl] and [C_6mim][Cl] at 10-50 mg/L, the MFI_{Chl} of the cells increased with C_{IL} by 10.4%-62.9%, compared with the control. These results suggest that the ILs tested cannot be inhibiting Chl *a* synthesis in algal cells. When the [C_8mim][Cl] concentration was higher than 0.2 mg/L, the MFI_{Chl} was inhibited in a non-concentration-dependent manner, with a maximum inhibition of 5.4%.

Previous studies have demonstrated that the C_{Chl} of algae can actually increase when they are exposed to imidazolium-based ILs, metal ions (*eg*. vanadium, cobalt, copper) or other organic chemicals (*eg*. 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 2-benzoxazolinone, carbamazepine) (Tsarpali et al., 2016). This C_{Chl} increase can be explained by an inhibition of electron flow on the *acceptor* side of the PS II reaction centre (Franqueira et al., 2000).

It has even been suggested that an increase in C_{Chl} occurs as part of a protective mechanism during phototropic algal growth, although the exact mechanism remains unknown (Tsarpali et al., 2016). Some microalgae have been shown to secrete phytohormones when exposed to toxins (Debenest et al., 2010), and some exogenously applied phytohormones can stimulate C_{Chl} in *C. vulgaris* (Meisch et al., 1975). *C. vulgaris* exposed to [C_6mim][Cl] might also be expected to secrete phytohormones as a response to metabolic stress from an IL, resulting in a corresponding increase in C_{Chl}. However, in this study there was no indication to support such a hypothesis. On the contrary, *C. vulgaris* displayed a lower tolerance towards ILs (reflected in lower ErC_{50} values) than did *S. quadricauda*, suggesting that

no such protective response had occurred, or if it had, then its effect on sensitivity was negligible.

On the other hand, it has also been proposed that in certain photoautotrophic organisms, in the absence of cell division, chlorophyll synthesis is the major biochemical imperative (Hagen et al., 2001). In such organisms, an increase in C_{Chl} would naturally be expected to accompany any inhibition of cell division. As the cell division of *C. vulgaris* was inhibited more significantly than that of *S. quadricauda*, such a transformation of biochemical imperative might provide a possible explanation for the increased C_{Chl}.

3.4. **Esterase activity**

FDA is not itself a dye, but exhibits a green fluorescence after hydrolysis by nonspecific esterases within living cells. As a result, the MFI emitted by FDA stained cells (MFI_{FDA}) is commonly used as a rapid and sensitive technique to assess phytoplankton metabolic activity (Prado et al., 2012).

After 96 h exposure, $[C_4mim][Cl]$ at all concentrations, as well as $[C_6mim][Cl]$ at 5 mg/L-20 mg/L and $[C_8mim][Cl]$ at 1 mg/L-10 mg/L, respectively inhibited the MFI_{FDA} of *S. quadricauda* (Fig 5). Nevertheless, the relationship between the inhibition effects and C_{IL} was not as simple as might be expected. Among the cells exposed to different concentrations of $[C_4mim][Cl]$, the statistical differences were not significant ($P>0.05$). Moreover, the MFI_{FDA} of the *S. quadricauda* cells exposed to $[C_6mim][Cl]$ and especially $[C_8mim][Cl]$ increased with C_{IL}, instead of decreasing. At

50-100 mg/L exposure to [C_6mim][Cl] (an IL of relatively high lipophilicity) did not give rise to statistically significant differences compared with the control ($P>0.05$). However, at 40 mg/L the most lipophilic IL, [C_8mim][Cl] had a significant effect, enhancing MFI_{FDA} by 59.6% compared with the control.

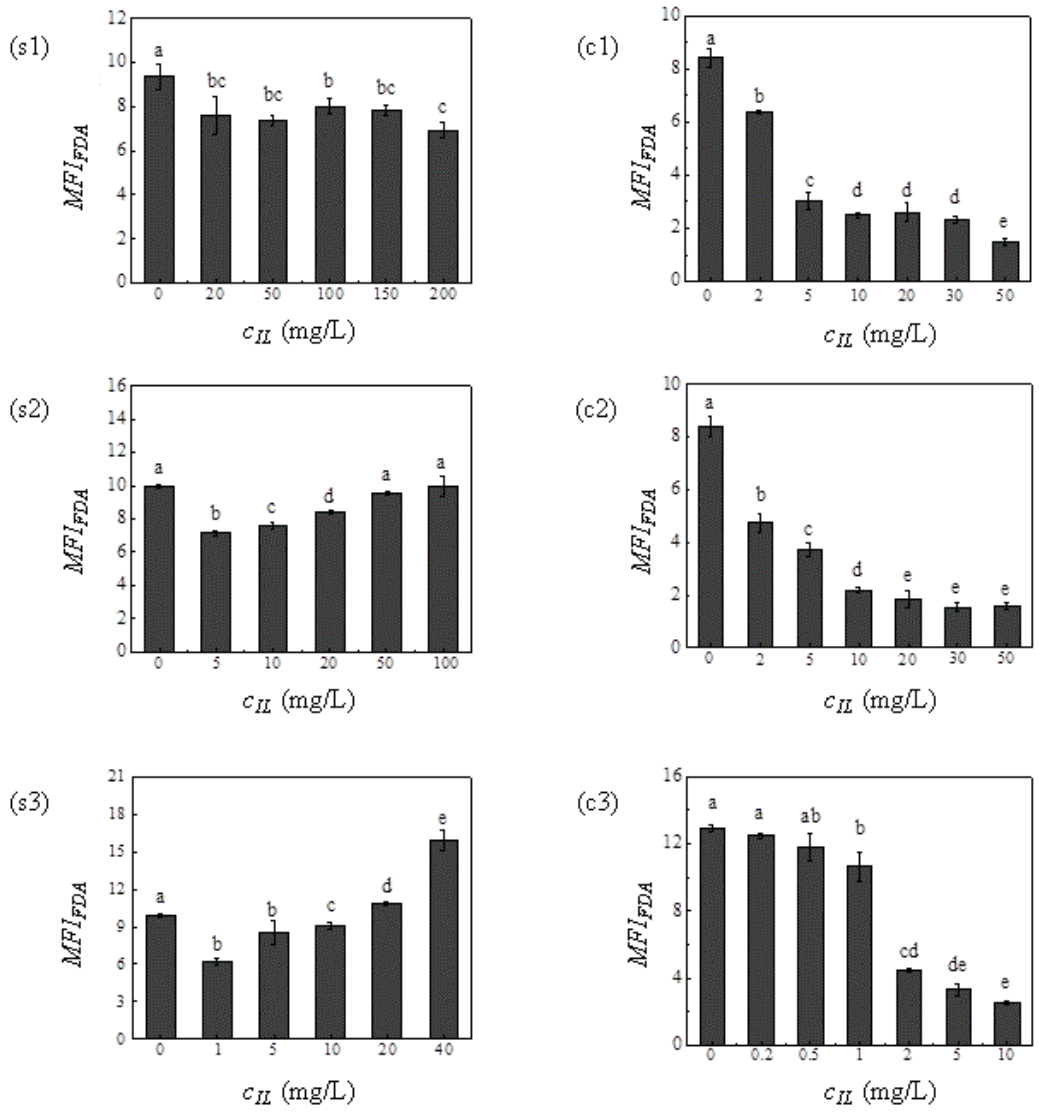

Fig. 5 MFI after staining with FDA, reflecting esterase activity (graph numbers and notes correspond to Fig. 3).

Most studies conclude that an increase in MFI_{FDA} is a result of greater esterase activity, which in turn reflects an activation and upregulation of detoxification

processes (Jamers et al., 2009; Sogorb et al., 2002). Many lipophilic toxins, such as herbicides and pesticides, are metabolized to give more polar molecules for easier expulsion in aqueous media. The most effective way to convert a lipophilic ester into water-soluble products is for it to undergo hydrolysis to the corresponding alcohol and carboxylic acid, catalyzed by an esterase enzyme. The resulting alcohol and acid metabolites are much more water-soluble as a result of effective hydrogen-bonding and often have lower toxicity. The biodegradation of [$C_n$mim] ILs, which do not contain esters, has been proposed to rely on oxidative metabolism, rather than hydrolysis (Docherty et al., 2007; Stolte et al., 2008). However, the marked increase in esterase activity with increasing C_{IL} may have occurred as part of a generalized metabolic response, which does not require the presence of an ester in the ILs.

Alternatively, the MFI_{FDA} increase may merely reflect an increase in FDA uptake, which is expected to occur as a result of greater membrane permeability (Hampel et al., 2001; Franklin et al., 2001), Fig 3 (s2 and s3). Moreover, an increase in intracellular pH (another defensive mechanism) mediated by intracellular ionic regulation may also play a part in the increase in MFI_{FDA} (Hadjoudja et al., 2009).

The response of *C. vulgaris* was more straightforward. Except in the case of [C_8mim][Cl], at 0.2 - 0.5 mg/L, *C. vulgaris* did not show any statistical differences from the control. However, for [C_4mim][Cl] and [C_6mim][Cl] at all other concentrations, as well as [C_8mim][Cl] at 1 mg/L-10 mg/L, the MFI_{FDA} had decreased, indicating that the metabolic activity of the cells was compromised. The esterase activities of cells exposed to [$C_n$mim][Cl] at 2 mg/L were 75.8%, 56.4% and 35.1%

compared with the control, for [$C_n$mim]s with n = 4, 6, 8 respectively. The inhibition increased in an IL-concentration dependent manner, reflecting the way *C. vulgaris* responds to toxic challenge by copper (Hadjoudja et al., 2009).

The presence of a more effective metabolic response, exemplified by increased esterase activity, may be a contributing factor in the greater tolerance of ILs exhibited by *S. quadricauda*, compared with *C. vulgaris.*

4. Conclusions

The toxic effects of [$C_n$mim][Cl] (n=4,6,8) toward the same algae follow similar trends. As expected, a decrease in the concentration of IL required to induce toxic effects is observed as the IL alkyl chain is extended. Although *C. vulgaris* and *S. quadricauda* are both freshwater green algae with cell walls, their biochemical responses towards the ILs, such as Chl *a* synthesis and esterase activity are different.

After exposure to the ILs, cell proliferation of both species was inhibited, and cell size, complexity, and membrane permeability increased. The toxic effects of the ILs toward *C. vulgaris* were more serious than those experienced by *S. quadricauda.* The ErC_{50} values towards *S. quadricauda* and *C. vulgaris* ranged from 5.60 - 129 mg/L and 3.90-32 mg/L, respectively. At the same C_{IL}, MFI_{PI} for *S. quadricauda* increased more than for *C. vulgaris*. Inhibition of Chl *a* synthesis was observed for *S. quadricauda*, whilst Chl *a* synthesis was induced in *C. vulgaris*, possibly from a reprioritisation of the biochemical imperative, accompanying inhibition of cell division. For *C. vulgaris*, the MFI_{FDA} results after exposure to most IL doses indicated

that esterase inhibition had occurred compared with the control, demonstrating that metabolic activity was compromised. The inhibitory effects in *C. vulgaris* occurred in an IL-concentration dependent manner, consistent with exposure to a lipophilic toxin. The effect of IL exposure on *S. quadricauda* metabolism was completely opposite, resulting in a notable induction of esterase activity with increasing C_{IL}, especially at higher doses.

In conclusion, evidence suggests that membrane resistance and a possible metabolic response resulting in enzyme induction, give rise to the lower sensitivity of *S. quadricauda* towards ILs, compared with *C. vulgaris*. This intriguing finding merits larger-scale studies (sample size ≥40) that will inform future ERA research into the environmental risk posed by ionic liquids in the environment.

Acknowledgements

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