The role of the cell wall in the toxicity of ionic liquids to the alga *Chlamydomonas reinhardtii*

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Room temperature ionic liquids (ILs) are non-volatile organic solvents that are considered environmentally-friendly alternatives to traditional industrial solvents. However, the evidence of IL toxicity is mounting, while the mechanisms of toxicity to freshwater organisms remain poorly understood. ILs have been shown to have a wide-ranging toxicity to different taxa of freshwater algae, and differences in algal cell wall composition have been posed as one possible explanation for this variation. The cell wall is known to play a critical role in mediating the transport of materials into and out of algal cells, including potential toxins. The objective of our study was to determine the role of the cell wall in the toxicity of ILs to the freshwater phytoplanktor *Chlamydomonas reinhardtii*. We exposed wild-type (having a cell wall) and mutant (lacking a cell wall) strains of *C. reinhardtii* to a range of concentrations of five structurally-different ILs in 96-h standard toxicity bioassays. Our results suggest that the cell wall is involved in determining the susceptibility of *C. reinhardtii* to some but not all ILs, indicating that other factors, such as the base cation of the IL, are also involved. The alkyl chain length of an IL, a key factor in previous IL toxicity bioassays, does not appear to influence the ability of the cell wall to mitigate IL toxicity. The results of this study have important implications for predicting the effects of ILs in aquatic ecosystems and for extrapolating the effects of ILs across organisms.

Introduction

Room temperature ionic liquids (ILs) are an emerging class of industrial solvents that can be customized to fit a number of process needs.[1] These ILs typically consist of a bulky, often asymmetrical, nitrogen- or phosphorus-containing cation (*e.g.*, imidazole, pyridine, ammonium or phosphonium) and an inorganic anion (*e.g.*, Br^- or Cl^-). Because of their non-volatility, ILs have been proposed as green replacements for traditional volatile organic solvents such as benzene and toluene. The beneficial properties of ILs include low vapor pressure and sufficient flexibility to allow chemicals to be designed, through modification of their constituent parts, for a specific industrial process.[2] Consequently, ILs are currently being designed for a variety of applications, including chemical synthesis, catalysis, extraction, biotechnology and electrochemistry.[3]

Unfortunately, many ILs have been shown to be very resistant to biodegradation,[4,5] and if used on a large scale, will most likely become a component of industrial effluent, move through waste water treatment systems and ultimately become pollutants.[6] ILs are also known to be water soluble,[7,8] and while certain aquatic sediments have been shown to have an affinity for binding ILs, their hydrologic transport is still likely.[9] Such qualities suggest that detailed information on IL toxicity to aquatic organisms is needed before an accurate evaluation of their potential environmental impact can be made. More specifically, information about the effects of ILs on algal primary producers is needed due to the importance of these organisms in aquatic food webs and nutrient cycling.[10]

Previous studies have assessed the effects of ILs on aquatic primary producers,[5,6,11–17] and several of these have suggested that differences in observed toxicities to test species might reflect differences in cell wall structure. The cell wall is known to play a critical role in the transport of materials into and out of algal cells, including toxins.[18] Latała *et al.*[17] hypothesized that interactions between ILs and algal cell walls were responsible for differences in IL toxicities, especially as influenced by the presence of excess chlorine anions, as present in marine environments. Kulacki and Lamberti[14] found that *Scenedesmus quadricauda*, which has a cell wall consisting primarily of cellulose,[19] was more sensitive to three imidazolium ILs than *Chlamydomonas reinhardtii*, whose cell wall consists primarily of layers of glycoprotein.[20]

Past studies of other toxicants have used a mutant strain of *C. reinhardtii* that lacks a cell wall to assess the importance and function of the cell wall. For example, such strains have been used to analyze the accumulation and biomethylation of arsenic,[21] to screen anticancer drugs[22] and to examine the cell wall's role as a barrier to intracellular delivery.[23] Macfie *et al.*[24] showed that the cell wall provides protection against the toxicity of Cd, Co, Cu and Ni. We used the same wall-less strain of

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C. reinhardtii that is the result of single gene mutation and, except for the lack of a cell wall, is considered to be biologically and physiologically identical to the wild-type form.[24,25] Using this strain, we investigated the importance of the cell wall of *C. reinhardtii* to IL toxicity by comparing the wild-type form of the alga to the mutant form.

The objectives of our study were to determine: (1) whether the presence of the cell wall influences the toxicity of ILs to freshwater algae, (2) if the responses of the two strains of *C. reinhardtii* differ for structurally different ILs and (3) whether the alkyl chain length of an IL alters the role of the cell wall in mitigating toxicity to *C. reinhardtii*. We sought to establish differences in toxicity by performing standard bioassays using ILs containing the same anion (bromide) but different cations (imidazolium, pyridinium and ammonium) with different alkyl chain lengths (ethyl, butyl and octyl, Fig. 1). We hypothesized that ILs would be less toxic to wild-type *C. reinhardtii* because of the presence of the cell wall. Furthermore, we hypothesized that the role of the cell wall in mitigating toxicity would be similar across IL cations and alkyl chain lengths, suggesting that the cell wall is not a factor in differences in IL toxicities identified by previous studies. Such information is critical for the development of environmentally benign ILs.

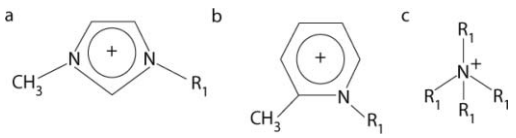

Fig. 1 The structures of the ILs used in this study. The (a) imidazolium, (b) pyridinium and (c) tetra-alkyl ammonium cations shown here were all paired with a bromide anion. R_1 represents alkyl side chains of varying lengths: butyl or octyl for imidazolium, butyl for pyridinium and ethyl or butyl for tetra-alkyl ammonium.

Results

Imidazolium-based ILs

Growth inhibition occurred for both strains with increasing concentration of imidazolium-based ILs (Friedman's test $p < 0.001$; Fig. 2; Table 1). The effect of these ILs did not differ significantly between the two strains, based on overlapping 95% confidence intervals (bmimBr $EC_{50\ wild\text{-}type} = 1150$ mg L^{-1}, $EC_{50\ mutant} = 1220$ mg L^{-1}; omimBr $EC_{50\ wild\text{-}type} = 18$ mg L^{-1}, $EC_{50\ mutant} = 16$ mg L^{-1}; Table 1) and Friedman's test ($p > 0.05$; Fig. 2; Table 1). For both ILs, growth rates declined quickly with increasing IL concentration until a zero or negative growth was observed, which represents algal death during the 96-h bioassay. Of these ILs, omimBr was two orders of magnitude more toxic to both strains than was bmimBr.

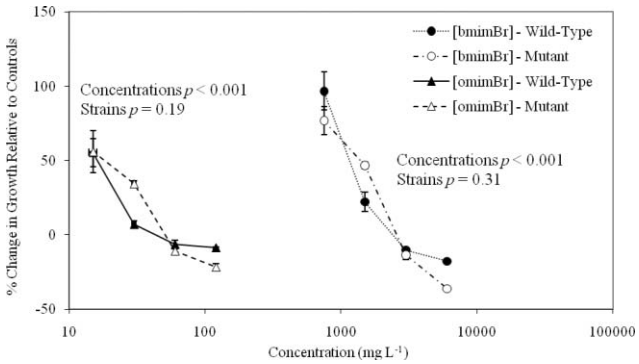

Fig. 2 The effect of bmimBr and omimBr on the average growth rates of wild-type and mutant *C. reinhardtii* relative to controls ($n = 4$; mean ± standard error). A 100% response indicates no difference between treatment and control; a 0% response indicates no growth. Outcomes of statistical tests of the factors of concentration and strain are given adjacent to each line.

Pyridinium-based ILs

Growth inhibition occurred for both strains with increasing concentration of the pyridinium-based IL (Friedman's test $p < 0.001$; Fig. 3; Table 1). The effect of this IL differed significantly between strains, as the wall-less mutant *C. reinhardtii* was more sensitive than the walled strain based on non-overlapping 95% confidence intervals (bmpyrBr $EC_{50\ wild\text{-}type} = 2543$ mg L^{-1}, $EC_{50\ mutant} = 930$ mg L^{-1}; Table 1) and Friedman's test ($p < 0.001$; Fig. 3; Table 1). The growth rate declined with increasing IL concentration for both strains, and a negative growth was observed for the mutant strain.

Table 1 Summary of growth EC_{50} values (mg L^{-1}), 95% confidence intervals (CI) and the outcome of Friedman's tests for all of the ILs tested with wild-type and mutant strains of *C. reinhardtii*

IL	Wild-type EC_{50}/ mg L^{-1} (CL)	Mutant EC_{50}/ mg L^{-1} (CL)	Difference between strains (p value)	Difference between concentrations (p value)
bmimBr	1150 (1010–1307)	1220 (1035–1423)	0.19	<0.001
omimBr	18.43 (15.14–21.39)	15.89 (15.25–16.49)	0.31	<0.001
bmpyrBr	2534 (1732–3995)	930 (658–1170)	<0.001	<0.001
$teNH_4Br$	(n/a)[a] (n/a)[a]	(n/a)[a] (n/a)[a]	0.38	0.35
$tbNH_4Br$	5203 (3690–8347)	9.39 (0.95–20.16)	(n/a)[b]	(n/a)[b]

[a] No growth inhibition exhibited. [b] A statistical analysis could not be performed.

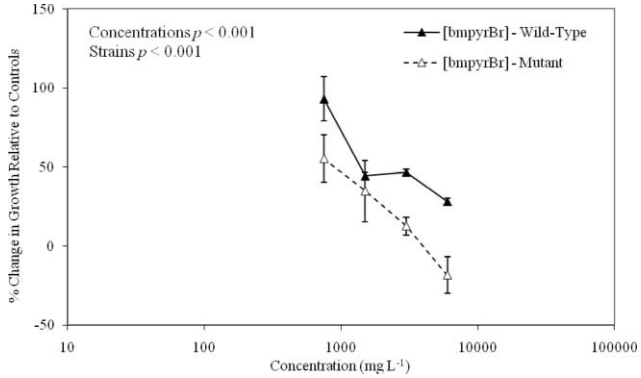

Fig. 3 The effect of bmpyrBr on the average growth rates of wild-type and mutant *C. reinhardtii* relative to controls ($n = 4$; mean ± standard error). A 100% response indicates no difference between treatment and control; a 0% response indicates no growth. Outcomes of statistical tests of the factors of concentration and strain are given adjacent to each line.

Ammonium-based ILs

Growth inhibition did not occur for either strain with increasing concentration of teNH$_4$Br (Friedman's test, $p > 0.05$; Fig. 4; Table 1). As a result, we were unable to compute EC$_{50}$ values for this shorter-chained ammonium-based IL. A Friedman's test showed that the effect of teNH$_4$Br did not differ significantly between the two strains ($p > 0.05$). While in this case the Friedman's test was not powerful enough to detect differences, inspection of the data suggests the growth of the wild-type strain decreased when exposed to 3000 mg L^{-1} teNH$_4$Br but remained steady with further increases in IL concentration. In addition, the growth of the mutant strain decreased when exposed to 1000 mg L^{-1} but remained steady with further increases in IL concentration. In contrast, growth inhibition occurred at all concentrations of tbNH$_4$Br (Fig. 5), and the effect of this IL differed significantly between the two strains based on non-overlapping 95% confidence intervals (tbNH$_4$Br EC$_{50\ \text{wild-type}}$ = 5203 mg L^{-1}, EC$_{50\ \text{mutant}}$ = 9.39 mg L^{-1}; Table 1). Furthermore, growth rates declined steadily with increasing tbNH$_4$Br concentration for both strains, and a negative growth was observed for the mutant strain.

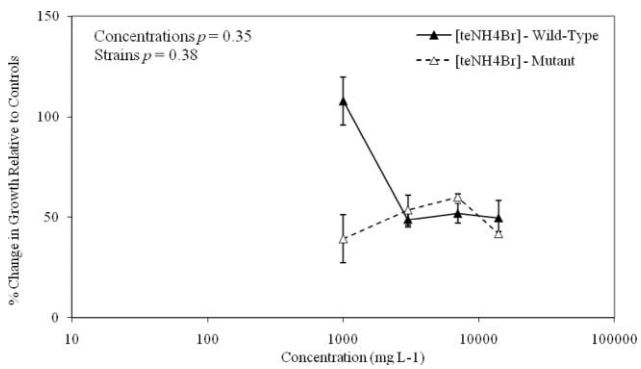

Fig. 4 Effect of teNH$_4$Br on the average growth rates of wild-type and mutant *C. reinhardtii*, relative to controls ($n = 4$; mean ± standard error). A 100% response indicates no difference between treatment and control; a 0% response indicates no growth. Outcomes of statistical tests of the factors of concentration and strain are given adjacent to each line.

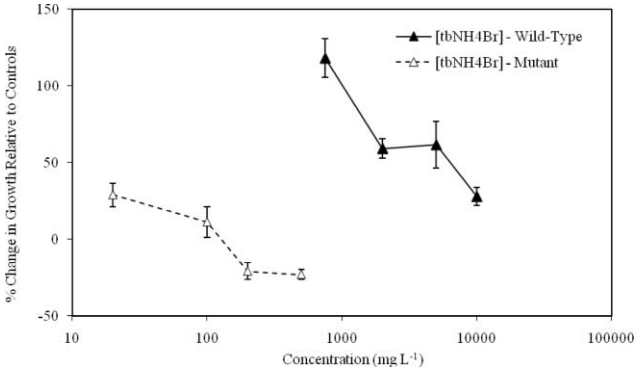

Fig. 5 Effect of tbNH$_4$Br on the average growth rates of wild-type and mutant *C. reinhardtii*, relative to controls ($n = 4$; mean ± standard error). A 100% response indicates no difference between treatment and control; a 0% response indicates no growth. Statistical tests were not possible.

Discussion

Observed differences in toxicity

We found evidence of differences between the two strains of *C. reinhardtii* in the toxicity of tbNH$_4$Br and bmpyrBr, indicating that the wild-type was less susceptible to IL toxicity than the mutant lacking a cell wall. However, the two imidazolium-based ILs, bmimBr and omimBr, had similar effects on the two strains, while teNH$_4$Br displayed no consistent pattern. These results suggest that the cell wall can be involved in determining the susceptibility of *C. reinhardtii* to some but not all ILs, and indicates that other factors are likely to be involved in determining IL toxicity. One such factor may be the base cation of the IL, which could change the efficacy of the cell wall to mitigate IL toxicity. Alkyl chain length, which strongly influences the toxicity of some ILs,[12,14,26] does not appear to be as important in influencing the ability of the cell wall to mitigate toxicity as the general toxicity of the IL itself. In other words, short-chained ILs such as teNH$_4$Br may be sufficiently non-toxic to freshwater algae that the cell wall plays no role.

Mechanisms of IL toxicity

Relatively few mechanisms have been suggested to explain the toxicity of ILs to freshwater primary producers,[6,14,15] but the most common is membrane disruption. ILs at concentrations several orders of magnitude higher than those used in our study have been shown to disrupt synthetic membranes.[27] Surfactants have a similar mode of chemical action and resemble the chemical structure of several ILs.[28] Cationic surfactants have also been shown to increase membrane permeability,[29] leading to cell narcosis.[12] The cell wall of primary producers may be able to mitigate membrane disruption by ILs by preventing the exposure to the cell's outer and internal plasma membranes (*e.g.*, mitochondria, chloroplasts). The cell wall of *C. reinhardtii* is unique compared to many other unicellular green algae as it lacks cellulose and instead is composed of several hydroxyproline-rich glycoprotein layers, which also occur in the extracellular matrix of many multicellular green algae and higher plants.[20] Primary producers with glycoprotein cell walls are thought to be less sensitive to ILs compared to those of other green algae,

diatoms and blue green algae whose cell walls are composed primarily of cellulose, silica or peptidoglycan, respectively.[6,14–16] However, further studies are needed to identify what specific components of the *C. reinhardtii* cell wall regulate the toxic effect of ILs.

Our findings suggest that the ability of the cell wall to mitigate the toxicity of a compound may vary depending upon the base cation of the IL, allowing some compounds greater access than others to the cellular membranes. The unique characteristics of the base cations of the ILs tested in our experiments suggest that if the alkyl side chains of ILs are the primary cause of toxicity *via* membrane disruption or permeability, then the base cation may modify toxicity by influencing the degree to which ILs interact with the cell membrane. Information on the chemical structures that have the ability to bypass the cellular defences provided by the cell wall may be important for the future design of environmentally benign ILs.

The role of the cell wall in mitigating IL toxicity

Differences among ILs in their toxicity to the two strains suggest that the cell wall may interact with ILs in unique ways when exposed to different base cations. Our results indicate that the cell wall may have prevented access to the cell membrane of pyridinium-based ILs, such as bmpyrBr, and consequently the mutant strain was more susceptible to the IL compared to the wild-type. Conversely, the cell wall may allow imidazolium-based ILs with the same side chain, such as bmimBr, access to the cell membrane, causing both strains to be equally sensitive. Imidazolium and pyridinium bases have sufficiently similar chemical structures and properties[30] but have differences that may allow us to better understand the unique roles of the cell wall upon IL exposure. Both bases are polar, aprotic salts of the heterocyclic aromatic organic compounds imidazole and pyridine, respectively, and are both excellent solvents.[30] However, one key difference is that the base of imidazolium, imidazole, also serves as the side chain of the amino acid histidine, which plays a vital role in many biological functions as a component of enzymes and proteins.[31] Furthermore, histidine has been shown to be vital to the uptake of nutrients by *C. reinhardtii*.[32] While the uptake of dissolved organic nitrogen compounds by phytoplankton is typically considered insignificant under normal conditions,[33] in nitrogen-limited environments, *C. reinhardtii* has been shown to use organic sources of nitrogen, including histidine.[32] In the presence of both histidine and ammonium, *C. reinhardtii* begins utilizing histidine when ammonium concentrations fall to minimal concentrations, at which point this alga removes both nitrogen sources simultaneously.[32] *C. reinhardtii* has also been shown to utilize a number of other organic compounds as nitrogen sources, such as urea, uric acid, glutamate, ornithine, acetamide, hypoxanthine, allantoin, allantoic acid, guanine and adenine.[34–37] The use of imidazolium ILs by *C. reinhardtii* as a nitrogen source, because of their resemblance to histidine, can only be shown by measuring the concentrations of IL and inorganic nitrogen throughout experiments. Such a mechanism, however, may explain the greater ability of bmimBr and omimBr to penetrate the cell wall and gain access to the plasma membrane of *C. reinhardtii*, and thus may be an area to be considered for future research.

The cell wall of *C. reinhardtii* also appears to have increased tolerance to ammonium-based IL toxicity, similar to that seen with pyridinium-based ILs. In the case of $tbNH_4Br$, the cell wall of *C. reinhardtii* may provide protection from $tbNH_4Br$ by simply providing a physical obstruction. In other words, the presence of the cell wall may in this case prevent contact between the IL and the phospholipid membrane of *C. reinhardtii*, thus preventing toxic action by $tbNH_4Br$. This role of the cell wall is supported by studies that have found $tbNH_4Br$ to be more toxic to the marine bacterium *Vibrio fischeri* than the shorter-chained ILs $tmNH_4Br$ and $teNH_4Br$ because of the higher lipophilicity of its cation, and therefore a greater ability to penetrate a cell membrane.[26] If the toxicity of ammonium-based ILs to *C. reinhardtii* is governed by such lipophilicity, the wild-type strain may exhibit more resistance to the mobility of $tbNH_4Br$ than its wall-less mutant. This protective mechanism offered by the *C. reinhardtii* cell wall could explain the significant differences we observed between the EC_{50} values of the two strains of this alga exposed to $tbNH_4Br$, along with the lack of significant differences when the same strain was exposed to bmimBr and omimBr.

Unlike the results observed with imidazolium-based ILs, we observed differences between strains in the toxicity of ammonium-based ILs with different alkyl chain lengths. For the shorter-chained IL $teNH_4Br$, since we were unable to calculate EC_{50} values, indicating that the toxicity was not quantifiable at the concentrations used in our study, the role of the cell wall is uncertain. Past studies have shown that $teNH_4Br$ is relatively non-toxic to the bacterium *V. fischeri* in comparison to imidazolium and pyridinium ILs, as well as to traditional solvents such as benzene and phenol.[4,26,38] Considering these results against the likely role of the cell wall in mitigating the known toxicity of $tbNH_4Br$, a minimum alkyl chain length may be required before ammonium-based ILs are toxic to *C. reinhardtii*. Further testing is needed using ammonium-based ILs with intermediate (propyl, $tpNH_4Br$) and longer alkyl chain lengths in order to assess how these ILs interact with the cell wall of *C. reinhardtii*.

Conclusions

We have demonstrated that algal mutant strains lacking cell walls can be useful as tools for studying toxicological mechanisms in primary producers (*cf.* Macfie *et al.*[24]). However, the variation we observed among IL cation classes, and among alkyl chain lengths within these classes, reveals challenges to predicting which ILs will not disrupt biological membranes. The fact that our study found that concentrations up to 10 g L^{-1} of $teNH_4Br$ did not halt growth in either algal strain reinforces the idea that certain ILs are likely to be more benign to aquatic organisms than other compounds. While the mechanisms by which *C. reinhardtii* can mitigate the toxic effects of ILs are likely to be complex, we have found evidence that the cell wall plays an important role and could provide protection against potential toxins, including ILs.

Understanding the mechanisms by which ILs exert toxicity is critical to understanding how these novel chemicals could impact the environment, especially when they are put into wider use and are more likely to contaminate aquatic systems. The use of mutant strains with different physiological or morphological

traits to study the mechanisms of IL toxicity could be a useful tool in such pro-active risk assessments. With more information on the morphological traits that influence the toxicity of ILs to primary producers, combined with existing knowledge of primary producer assemblages, the impacts of ILs released into an environment could be more easily predicted. However, given the enormous biodiversity of primary producers present in freshwater ecosystems,[10] the additional testing of ILs on a variety of algal taxa with different cell wall types will also be needed.

Experimental

Test organisms

Two strains of the unicellular green alga *C. reinhardtii* were obtained from the University of Toronto Culture Collection (UTCC): a wild-type that possessed a cell wall (UTCC #243) and a cell wall-less mutant (UTCC #12). The lack of cell wall is the only known difference between the two strains used in our experiments.[24,25] Both stock cultures were maintained in 800 mL of a liquid high-salt medium[39] held in 1 L Erlenmeyer flasks and kept in a solarium under natural light and an average temperature of 20 °C. For regular maintenance, culture flasks were decanted and refilled with fresh nutrient media weekly to maintain exponential population growth.

Test chemicals

The imidazolium-based ILs used in this study consisted of two forms of 1-alkyl-3-methylimidazolium bromide, where the alkyl chain was either four (butyl; bmimBr) or eight (octyl; omimBr) carbons long (Fig. 1). The ammonium-based ILs used in this study consisted of two forms of tetra-alkyl ammonium bromide, where the alkyl chains were either two (ethyl; teNH$_4$Br) or four (butyl; tbNH$_4$Br) carbons long. The pyridinium-based IL used in this study was 1-butyl-3-methylpyridinium bromide (bmpyrBr). Ammonium- and imidazolium-based ILs were purchased from Sigma-Aldrich (St. Louis, MO, USA); bmpyrBr was synthesized in the Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, IN, USA using established synthesis procedures.[40,41]

Test methods

We performed concurrent 96-h acute algal toxicity bioassays to examine the effects of ILs on the population growth rates of wild-type and mutant *C. reinhardtii* according to standard protocols.[42] For each bioassay, we tested four concentrations of IL along with a no-IL control; each concentration was replicated four times for a total of 40 experimental units. Range-finding tests were performed initially to determine concentrations for the actual experiment.

Bioassays took place in 500 mL Erlenmeyer flasks containing a 250 mL total of algal inoculum and high-salt media, which were randomly placed on a rotary shaker table (140 rpm) within a light- and temperature-controlled environmental chamber (photoperiod of 12 h). To measure algal growth, we sampled chlorophyll *a* during the initial and final hour of each bioassay by filtering a 20 mL sample through a glass fiber filter (1.0 μm pore size, Pall Corporation). The filtered material was extracted in methanol and analyzed for chlorophyll *a* using the fluorometric method[43] on a fluorometer (TD-700 Turner Designs, Sunnyvale, CA, USA). Algal growth rates [Δchl *a* (μg L^{-1} 24 h^{-1})] were determined for each flask from changes in chlorophyll *a* and then averaged across the replicates. Growth rates for each IL concentration were then compared to the controls for that test to determine the percentage response relative to the controls.

Statistical analyses

We determined EC_{50} values (the effective concentration of a toxicant that causes a 50% reduction in growth relative to a control) and associated 95% confidence intervals for the algal growth rates of each of the ILs tested. These values were established by fitting the dose–response curves to a logistic model by the maximum likelihood method[44] using SAS® (version 9.1) statistical software (SAS Institute, Cary, NC, USA). To test the two factors of strain (mutant and wild-type) and concentration, we used a Friedman's test, a non-parametric equivalent to a two-way analysis of variance ($\alpha = 0.05$), modified to incorporate multiple observations per cell.[45] A non-parametric test was used because the growth rate data failed to meet the parametric assumptions of normality and equal variance; unfortunately, an interaction between the two main factors cannot be tested with such a test. The data for tbNH$_4$Br was not considered because the range-finding test indicated that the two strains required different IL concentrations for EC_{50} value determination.

Acknowledgements

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