Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/greenchem

Green Chemistry

ARTICLE

Environmental safety of cholinium-based ionic liquids: assessing structure-ecotoxicity relationships

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

J.I. Santos[a,b], A. M. M. Gonçalves[a,c,d], J. L. Pereira[a,c], B. F. H. T. Figueiredo[b], F. A. e Silva[b], J. A. P. Coutinho[b], S. P. M. Ventura[b] and F. Gonçalves[a,c]

Ionic Liquids (ILs) are innovative solvents that can be tuned for their specific application through the selection, or functionalization, of the cation and the anion. Although the cation has been assumed as the main driver of toxicity, the importance of the anion must not be underestimated. This study considers a series of cholinium based ILs aiming at assessing the effects of the functionalization of the cation and the anion on their ecotoxicity. Those effects were assessed using three biological models, the microalgae *Raphidocelis subcapitata*, the macrophyte *Lemna minor* and the cladoceran *Daphnia magna*, representing aquatic ecosystems, a major putative recipient of ILs due to their high water solubility. Since the toxicity trends fluctuated depending on the biological model, the results were integrated with previous data through a species sensitivity distribution approach in an attempt to provide a useful safety variable for the design of eco-friendlier ILs. The results here reported challenge some heuristic rules previously proposed for the design of ILs, in particular in what concerns the side-chain effect for the cholinium ILs, and the notion that cholinium-based ILs are inherently safe and less environmentally hazardous than most conventional solvents. Moreover, it was confirmed that structural changes in the ILs promote differences in toxicity highlighting the importance of the role of the anion on the toxicity. Different biological systems yielded different toxicity trends across the IL series tested and also distinct from previous data retrieved with the bacteria *V. fischeri*; such a novel integration effort challenges the suitability of establishing structure-ecotoxicity relationships to assist cholinium-based IL design. Overall, this study reinforces the need to perform a complete ecotoxicological characterisation before assuming an IL as a suitable, environmentally compatible, alternative solvent.

Introduction

Ionic liquids (ILs) are poorly coordinated salts and therefore are liquid at or close to room temperature. The design of IL characteristics can be achieved by modifying either the cation or the anion by adding alkyl chains, functional groups (e.g cyano, ether, hydroxyl, among others), and/or aromatic rings, in order to meet a set of specific properties[1]. This results in virtually endless possibilities of tuning an IL to a specific application, which is the reason behind the assumption of their "designer solvent" character[2].

It has been found that nearly all ILs have very low vapour pressures (e.g. [3]), making them unlikely atmospheric pollutants. However, their ionic character makes most of them soluble in water[4,5], which can translate into an environmental problem if they happen to be toxic to the organisms inhabiting aquatic ecosystems. Experimental evidences have been collected in the recent past to allow the establishment of certain trends ruling ILs ecotoxicity. For example, ILs with longer cation alkyl side chains tend to be more ecotoxic ("side-chain effect"; e.g. [6,7]) until a certain threshold; regardless the number of carbons added, above this threshold there is no further increment in the IL toxicity ("cut-off effect"; e.g. [6]). There has been also an agreement on the fact that functionalized cations tend to produce less toxic ILs, when compared with non-functionalized counterparts as they are made more hydrophilic[8-10], and that the cation is the main driver of toxicity[11-13]. Although a number of researchers[14-16] have been showing that the anion moiety is also responsible for toxic effects of ILs by altering the hydrophobicity of the compounds[8], its role in the toxicity still tends to be underestimated. These assumptions, valid for most IL families already studied, tend to be assumed as heuristic rules defining the environmental-friendly development of new ILs[6].

Cholinium chloride (choline) is an essential nutrient[17] which has been the target of increasing interest from researchers designing ILs. This is based on the assumption that the supposed "biocompatibility" of the cholinium cation would translate into a lower environmental hazardous potential of cholinium-based ILs. Actually, and contributing to the 'biocompatibility' nomination, cholinium chloride has been argued to constitute a sustainable building block of ILs through its low toxicity[14,18,19] and considering

[a] Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal.
[b] CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.
[c] CESAM, University of Aveiro, 3810-193 Aveiro, Portugal.
[d] MARE & IMAR, Faculty of Sciences and Technology, University of Coimbra, 3004-517 Coimbra, Portugal
† J. I. Santos and A. M. M. Gonçalves contributed equally to this work.
Correspondence should be sent to J. L. Pereira – jpereira@ua.pt
Electronic Supplementary Information (ESI) available: nomenclature and chemical structure of the studied compounds plus numerical values for the determined ecotoxicity references. See DOI: 10.1039/x0xx00000x

that cholinium-based ILs are readily biodegradable[19-22]; it is also worth mentioning within this context that most ILs based on cations other than cholinium were not successful in biodegradation tests[20]. Several successful biotechnological applications are foreseen for these ILs, namely as solvents for biocatalysis, biopolymer science, as well as in separation and purification processes, in particular in aqueous biphasic systems[20,23-31]. The expected boost in the development of cholinium salts and their industrial application adds meaning to the study of their ecotoxicological properties for better compliance with regulatory demands (see e.g. the REACH framework; CE1907/2006). In fact, the ecotoxicological behaviour of the cholinium family is still poorly known. Previous studies[14,31,32] show that cholinium-based ILs are almost nontoxic to the bioluminescent bacteria *Vibrio fischeri*, but still their ecotoxicity is close to or higher than that of some common organic solvents. These scarce studies on the ecotoxicity of cholinium-based ILs demonstrate that, although structure-ecotoxicity relationships can be identified, the heuristic rules described above do not apply to this new family. Whether the trends found for the bacteria can be extended as a standard for cholinium ILs ecotoxicity needs confirmation, through testing with other biological systems. This constitutes the major goal of the present study, representing a significant add-on to the existent knowledge on the putative environmental effects of the cholinium family of ILs.

A secondary arena of this study regards the difficulties of assessing the environmental hazardous potential of ILs, which link directly to their designer solvent character. In fact, there is an endless list of alternatives to deal with within each IL family, tuneable to a specific application[33]. Given the relevance of environmental safety in the market regulation, a rational attitude is to select those ILs representing lower environmental hazardous potential yet keeping favourable functionality performance for further development and finally licensing. However, from a traditional environmental risk assessment perspective[34-36], this means dealing with an unmanageable amount of ecotoxicological assessments, to integrate with data from other lines of evidence, before a feasible indication can be given at early stages of technological development. In this context, the use of mathematical models relating key structural elements of ILs to their biological reactivity, (e.g., QSARs or QSPRs such as those explored by Alvarez-Guerra et al.[37], Couling et al.[11], Das and Roy[38], Ma et al.[39], Roy et al.[40], Torrecilla et al.[41] and Zhao et al.[42], can be of assistance in defining compounds of interest (e.g. [43]). Actually, such an approach within risk assessment of chemicals has been encouraged by different regulatory authorities[44-46] because it represents a cost-effective shortcut to an environmentally precautionary recommendation. Still, the success (accurate predictive ability) of such an approach has been hampered by the report of "outliers" in the expected quantitative relationships, for example in the link between lipophilicity and (eco)toxicity[8]. Qualitative alternatives may gain favour as successful, more informative models to address the safety of new chemicals (see e.g. the T-SAR approach by Jastorff et al.[47]). Besides internal features, a major external actor within this context is the focused biological system, i.e. (in)consistency in tendencies between structural changes and the biological responses have been found as different biological systems are tested (e.g. [8,48,49]). This rationale makes it mandatory to assess the consistency among responses of different organisms to structural variations within the cholinium IL family before assuming the possibility of feasibly relating chemical properties or molecular structure to ecotoxicity in general [8,13].

In this way, and as a follow-up to the work on cholinium ILs by Ventura et al.[14], the present study aimed at assessing the consistency in structure-ecotoxicity relationships among different biological systems. By using ten cholinium ionic structures (see Experimental for clarification on the abbreviations used here), several functionalization options were addressed to probe their ecotoxicological effects: (i) the introduction of hydroxyl groups ([Chol][Bic], [Chol][Bit] and [Chol][DHCit]); (ii) a range of anion hydrophobicities by varying the length of the alkyl chain ([Chol][Ac], [Chol][Prop] and [Chol][But]); (iii) the introduction of aromatic rings ([Chol][Sal]) and phosphate groups ([Chol][DHPhosp]) in the anion; and (iv) the introduction of an aromatic ring in the cation core ([Chol]Cl and [BzChol]Cl). Three standard ecotoxicological models[50-52] were selected for comparison with the data for the same IL series gathered with *Vibrio fischeri*[14]: the green microalgae *Raphidocelis subcapitata*, the macrophyte *Lemna minor* and the freshwater cladoceran *Daphnia magna*. This set of organisms covers main functional groups of the aquatic trophic web, but equally important is the fact that it considers different chemical uptake routes eventually constraining the magnitude of the toxic effect. Differences between the prokaryotic and eukaryotic cell walls, between systemic and surface contact absorption routes or ingestion were taken into account to address a hypothesised link to the variation in the organism's sensitivity to the ILs.

Experimental

Test chemicals

Ten cholinium-based chemicals were tested in this work (see Table S1 for details on their chemical structures): cholinium bicarbonate [Chol][Bic] (80wt%), cholinium bitartrate [Chol][Bit] (99wt%), and cholinium chloride [Chol]Cl (98wt%), all purchased from Sigma-Aldrich; cholinium acetate [Chol][Ac] (98wt%), cholinium dihydrogenophosphate [Chol][DHPhosp] (≥98wt%) and cholinium dihydrogenocitrate [Chol][DHCit] (98wt%), purchased from Iolitec (Ionic Liquid Technologies, Germany); benzyldimethyl (2-hydroxyethyl)ammonium chloride [BzChol]Cl (97wt%), acquired from Fluka; cholinium propanoate [Chol][Prop] (≥99wt%), cholinium salicylate [Chol][Sal] (95wt%) and cholinium butanoate [Chol][But] (99wt%) which were synthesized in our laboratory[18,53]. With the exception of [Chol][Bic] all the compounds were washed with ultrapure water before testing, and then dried under constant stirring at high vacuum and moderate temperature (≈353K) for a minimum of 48h. This treatment allows the removal of water and other volatile compounds. Cholinium bicarbonate was used without the drying step, being the initial water content considered in the preparation of the aqueous solution of this specific IL. The ILs purity was checked by 1H and 13C NMR. Ultrapure water, i.e. double distilled water, passed through a reverse osmosis system and further treated with a Milli-QPlus185 water purification apparatus, was used in all procedures described above.

Microalgae bioassays

R. subcapitata was maintained in the laboratory as a non-axenic culture in Woods Hole MBL medium, under 20 ± 2°C and $16h^{L}$:$8h^{D}$ photoperiod. Prior to the beginning of the test, an inoculum was harvested from the bulk culture and incubated for three days under 23 ± 1°C and permanent illumination (8000 lux). This inoculum fed the test, which was conducted following guidelines by OECD[52] adapted to the use of 24-well microplates[54]. Briefly, the inoculum cell density was determined microscopically using a Neubauer haemocytometer and its concentration was adjusted to deliver an initial test cell density of 10^4 cells mL^{-1}. The microalgae were then exposed to a geometric range of concentrations of each IL. All treatments included a MBL blank control, an algae control and three replicates of each tested IL concentration. The microplates were incubated for 72h as described for the inoculum. To prevent cell clumping and promote gas exchange, the algal suspension in each well was thoroughly mixed by repetitive pipetting twice a day. At the end of the test, the microalgae yield in each individual treatment was calculated as the difference between the cell densities (microscopic cell counting using a Neubauer haemocytometer) at the end and the beginning of the test. The growth rate was also calculated and addressed to add to the ecotoxicity database generated in the present study (see supplementary information).

Macrophyte bioassays

L. minor was maintained in Steinberg medium[50] at 23 ± 1°C and under permanent illumination. The growth inhibition test was performed under the same conditions as the culture, following OECD guidelines[50] adapted to the use of 6-well plates[55,56], where the macrophyte was exposed to a geometric range of concentrations of each IL. Individual wells held 10 mL of test solution plus three macrophyte colonies of three fronds each. Three replicated wells were established per concentration and each test included six plain-Steinberg control wells. At the beginning of each test, six replicates consisting in three colonies of three fronds were oven-dried for 24h at 60°C to obtain the initial dry weight. The test plates were incubated for 7 days under the same conditions as used for the culture. At the end of the test, the fronds present in each well were counted and oven-dried (at least 24h at 60°C) for dry weight records. Exposure-driven effects were discussed using yield, based on frond number records. Yield based on dry weight, as well as the growth rate based on both frond number and dry weight were also calculated and addressed to add to the ecotoxicity database generated in the present study (see supplementary information).

Cladoceran bioassays

D. magna was reared as a monoclonal bulk culture in synthetic ASTM hard water medium[57] with vitamins[58], supplemented with a standard organic additive[59], under 20 ± 2°C and $16h^{L}$:$8h^{D}$ photoperiod. The daphnids were fed with *R. subcapitata* (3×10^5 cells mL^{-1}) three times a week, right after medium renewal. Acute immobilisation tests were conducted following the OECD guideline 202[51], using neonates from the 3rd to the 5th broods and aged less than 24h. Tests were carried out in glass test tubes containing 25 mL of test solution. Geometric ranges of IL concentrations were established, and the culture medium was used as the negative control treatment. A static design was employed, using twenty animals randomly assigned into four replicates with five animals per treatment. The organisms were exposed for 48h under the same conditions as used for cultures; the number of immobilised daphnids was recorded at the end of the exposure period.

Data analysis

The records obtained from the bioassays with microalgae and the macrophyte were used to estimate concentrations promoting x% yield or growth inhibition (EC_x values, with x = 10, 20, 50) and corresponding 95% confidence intervals for each tested IL by non-linear regression, using the least-squares method to fit the data to the logistic equation. To estimate immobilisation EC_x from the data collected in the bioassays with the cladocerans, Probit analysis[60] was applied. Besides constituting standard ecotoxicological references, EC_x data were used to assess changes in toxicity promoted by structural variations in the cholinium compounds. Furthermore, as an integrated indicator for these trends, Species Sensitivity Distributions (SSDs[61]) were estimated using the U.S.EPA's Species Sensitivity Distribution Generator. After validating the quality of our EC_x estimates following widely recommended guidelines[62], the feeding of SSDs with the EC50 dataset was established, which generally holds the tightest associated confidence intervals, hence improving the overall feasibility of the derived curves. The four species used in the present study (*V.fischeri*, with data from Ventura et al.[14], *R. subcapitata*, *L. minor* and *D. magna*) are clearly insufficient to estimate an SSD able to produce feasible HC_p (Hazard Concentration for *p*% of the species) benchmarks for risk assessment purposes[61,63]. However, because they constitute an integrative parameter, these limited HC_p values can certainly be used as reference for exploiting the structure-ecotoxicity trends, particularly when there is no consistency between the responses given by the different biological systems tested.

Results and discussion

This study addressed the environmental toxicity of ten cholinium-based salts using adequate standard organisms for a screening of their hazardous potential. Two main avenues were explored. On the one hand, the way structural changes influence the toxicity (on the basis of the most feasible EC_{50} estimates) was assessed, and on the other hand, the consistency in these structure-ecotoxicity relationships between different biological systems was analysed.

Environmental hazardous potential of the cholinium-based ILs

Figure 1 provides an overall view on the toxicity variation as the structure of the ILs was functionalized. For a detailed view on absolute EC_x values and respective 95% confidence intervals considering all estimated endpoints, please refer to Table S2.

Concerns should be raised on the environmental hazardous potential of six out of the ten ILs tested: [Chol][Prop], [Chol][But], [Chol][Bit], [Chol][DHCit], [Chol]Cl and [BzChol]Cl. EC_{50} values below 100 mg L^{-1} regarding the biomass yield of *R. subcapitata* (Figure 1) assign the first five cholinium ILs to the category Acute 3 of the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS)[64], meaning that they are included in the group of the least severely hazardous substances, but should nevertheless be labelled as harmful to the aquatic environment. The effects of [BzChol]Cl in the growth of *L. minor* drives its inclusion also in this group, while [Chol][Ac], [Chol][Bic], [Chol][DHPhosp] and [Chol][Sal] are apparently of no significant environmental concern.

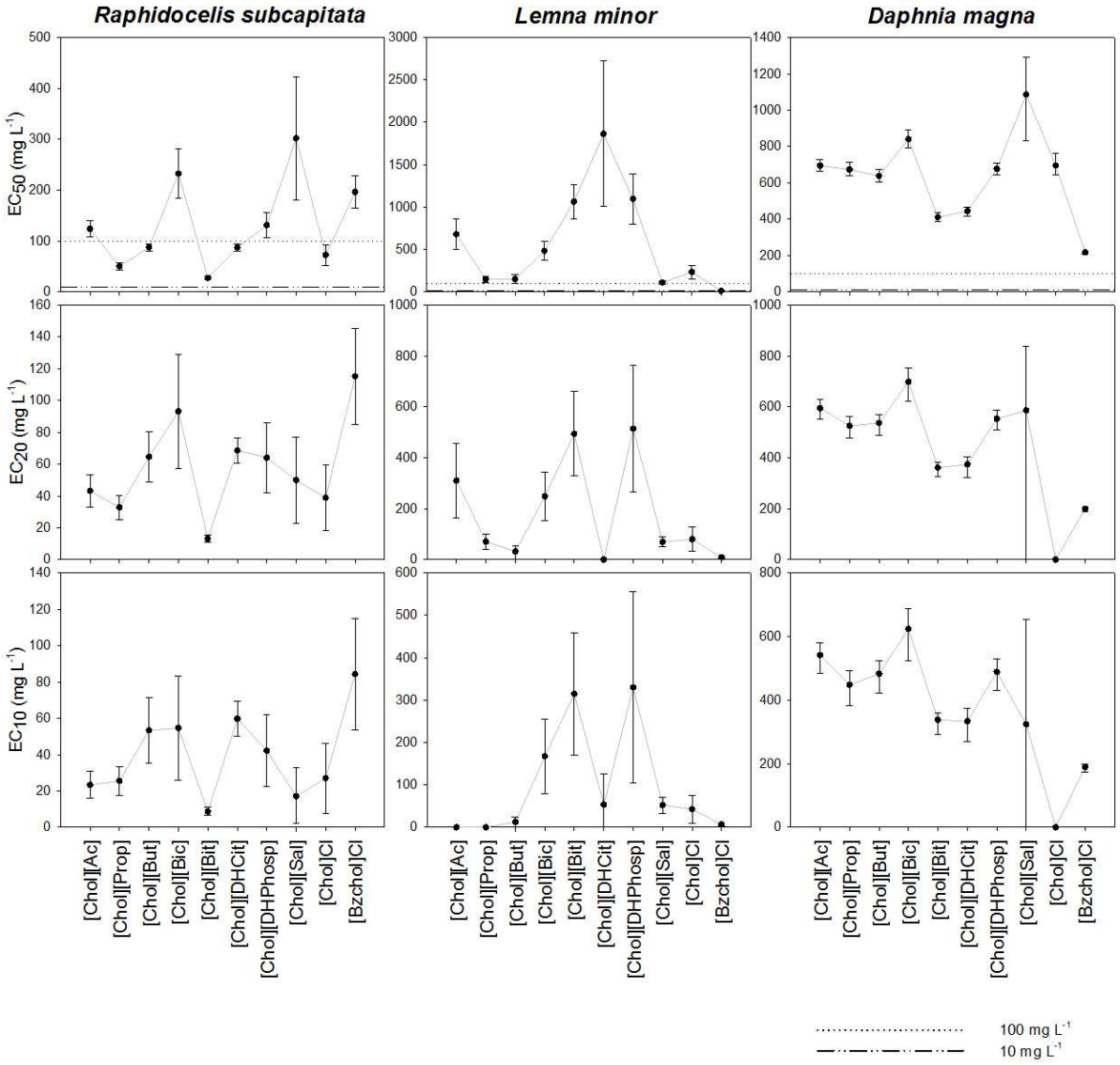


Fig.1 Trend plots representing the EC_x values (filled circles) and corresponding 95% confidence intervals (error bars) estimated through fitting of linear or nonlinear models to the experimental responses of *R. subcapitata* (biomass yield on the basis of cell density), *L. minor* (biomass yield on the basis of number of fronds) and *D. magna* (immobilisation), respectively, to the ten cholinium-based ILs tested. The grey line evidencing the trend of EC_x variation as the cholinium IL changes was added for clarity purposes and does not represent any adjusted model. The reference horizontal lines in the EC_{50} trend graphs represent environmental hazard benchmarks[64]: substances with EC_{50} values falling between 10 mg L^{-1} (dash-dot line) and 100 mg L^{-1} (dotted line) are considered harmful, while those with an EC_{50} value below 10 mg L^{-1} are deemed toxic to the aquatic life.

As to our knowledge, this study together with that by Ventura et al.[14] is pioneer in denoting a significant environmental hazardous potential of cholinium-based ILs. Petkovic et al.[18] found minimum inhibitory concentrations above 20 g L^{-1} after exposing fungi to [Chol]Cl, [Chol][Prop] and, [Chol][But], Ninomiya et al.[28] found specific growth rate EC_{50} values above

50 g L^{-1} following a 5-12 h exposure of *Saccharomyces cerevisiae*, while Hou et al.[19] recorded an EC_{50} value for acetylcholinesterase activity higher than 400 mg L^{-1} and minimum inhibitory concentrations for bacteria growth above 100 g L^{-1} following exposures to [Chol]Cl.

Our results support the rise of doubts about the assumed higher environmental friendliness of ILs containing the cholinium structure as the cation compared to conventional solvents, following previous authors[11,14,32]. Indeed, the EC_{50} values found in the literature for such solvents (Table 1) are generally much higher (frequently 1-2 orders of magnitude) than those determined in our study (Table S2). ILs were initially touted green compared to traditional solvents given their low vapour pressure and hence very low potential as air pollutants[65]. In the aquatic compartment, rapid volatilization can actually be an advantage, and should contribute to the lower toxicity generally found for the latter[6]. Still, the widespread non-volatile solvent dimethylsulfoxide shows EC_{50} values (Table 1) which are 1-3 orders of magnitude higher than the counterparts obtained here for the tested cholinium-based compounds (Figure 1; Table S2). Phenol is also poorly volatile and it was found very toxic to *D. magna* (Table S2) but its aromatic character should have been the main driver of toxicity in this case. On the other hand, the highly volatile trichloromethane was highly toxic to the microalgae but the corresponding bioassay was developed in closed systems[66].

Table 1 Summary of toxicity benchmarks for traditional organic solvents. The mean effective concentration (EC_{50} or LC_{50}) was focused to facilitate comparison with the present study. Whenever data yield from testing with the same species (*R. subcapitata*, *L. minor* and *D. magna*) could not be found in the literature, a note was added to the citation. Exposure periods and endpoints considered for the estimates are given for all values: $_y$ stands for biomass yield, $_g$ for growth and immobilisation (EC_{50} values) was invariably the endpoint used in *D. magna* alternatively to mortality (LC_{50} values). Data which are not quoted a citation (original manuscripts identified as table footnotes) were retrieved from the USEPA ECOTOXicology database[96]. Exceptional cases were conventional solvents seem more toxic than ionic liquids were marked bold.

	Microalgae		***Lemna* sp.**		***D. magna***	
	endpoint	mg L^{-1}	endpoint	mg L^{-1}	endpoint	mg L^{-1}
Acetone	2h-E_yC_{50}; 48h-E_gC_{50}	41121[96†];7270[97]	7d-E_yC_{50}	10978[98‡]	48h-LC_{50}	30849[99]; 9218[100]; 12667[101§]
Acetonitrile	2h-E_yC_{50}	34154[96†]	4d- E_gC_{50}	3685[102]	48h-LC_{50}	**7.6**[103]
Benzene	96h-E_gC_{50}	28.7[97]			48h-LC_{50}	200[103]; 426[101§]
Dimethylformamide	96h-E_gC_{50}; 2h-E_yC_{50}	751[107]; 152685[96†]	7d- I_gC_{50}	4900[105]	48h-LC_{50}	12324[99]
Dimethylsulfoxide	E_gC_{50}	22118[106¥]			48h-EC_{50}; 48h-LC_{50}	14500[107]; 24600[108]
Ethanol	2h-E_yC_{50}	40127[96†]	7d-E_yC_{50}	8265[98‡]	48h-LC_{50}	5680[109]; 9248[100]; 12340[110]
Isopropanol	96h-E_gC_{50}; 48h-E_gC_{50}; 2h-E_yC_{50}	11719[104]; 10500[97] 35399[96†]	7d-EC_{50}	1257*	96h-EC_{50}	10390[111]; 5732[111]
Methanol	96h-E_gC_{50}; 2h-E_yC_{50}	22683[104]; 82343[96†]	7d-EC_{50}	9880*	48h-LC_{50}; 96h-EC_{50}	3289[109]; 18260[111]
Phenol	96h-EC_{50}	46.42	7d-E_yC_{50}	247[98‡]	48h-LC_{50}	**13**[100]; **12**[103]
Triethylene glycol			96h- I_gC_{50}	47750[112]	48h-LC_{50}	39393[99]
Trichloromethane	72h-E_yC_{50}	**13.3**[66¤]	7d-E_yC_{50}	>1000[98‡]	48h-LC_{50}	353[100]
Toluene	48h-E_gC_{50}	26.3[97]			48h-LC_{50}	310[103]

† yield considering photosynthetic activity as the endpoint; ‡ Average of EC_{50} estimates on the basis of frond number yield with n = 4; § value with n=3; ¥ data *for Chlorella pyrenoidosa* with no test time period specified; ¤ data for *Chlamydomonas reinhardtii*; * Data for *Lemna gibba*.

Figure 2 shows clearly that the microalgae *R. subcapitata* was the most sensitive organism for seven out of ten tested compounds. It was replaced by either the macrophyte or the bacteria for [Chol][DHCit], [Chol][Sal] and [BzChol]Cl, but never by *D. magna*. The cladoceran was indeed one of the least sensitive species for about half of the tested ILs. It is worth noting here that, although the Microtox® test platform is an attractive and widely used time-effective methodology for environmental screening, it should be carefully selected depending on the ILs under scrutiny and the overall rationale behind each study. A good ion-pairing environment for cations should be provided by the high chloride burden of *V. fischeri* saltwater test media. Chloride is hence likely to compete for the IL cation cores with the negatively charged groups of cell walls and membranes, this reducing the permeability of the cations through the cell walls and ultimately reducing the toxicity[67].

Structure-ecotoxicity trends

Contrarily to the observations by Ventura et al.[14], an inconsistency of the trend in toxicity variation depending on whether EC_{50}, EC_{20} or EC_{10} is focused can be retrieved from the interpretation of Figure 1; see for example the peaking EC_{50} of [Chol][Sal] that does not reflect as trends in EC_{20} or EC_{10} values are inspected, as well as the peaking of [Chol][DHCit] expressed by the EC_{10} and EC_{20} values but not by the EC_{50} values. This is somewhat constraining within a general environmental risk assessment scenario. EC_{10} and EC_{20} values are generally understood as protective benchmarks informative of no and lowest observable effect concentrations, respectively (e.g. [46]), thus its use to address the environmental hazardous potential of developing substances would eventually refine the realism of the conclusions. However, the estimation of the EC_{50} is intrinsically the most robust because it is less susceptible to differences in the formulation of the fitted model; also, its position in the concentration-response curve is more likely to be covered by actual experimental data[52,62,68]. Although aware on the significance of lower effect level benchmarks, the benefit of the robustness of the EC_{50} estimates for further analysis of structure-toxicity tendencies was rather valued. In fact, here we focus on early-stage ecotoxicological screening of developing substances, aiming at comparatively signalling on the environmental safety of alternative IL structures, rather than on

establishing a firm conclusion on the environmental risk they represent.

The inexistence of common trends among species at the EC_{50} level is noteworthy as the relative effect of structural modification in toxicity is focused (Figure 1). Clarifying examples can be given for (i) [Chol][Bic], which is one of the least toxic ILs for microalgae and cladocerans, but its relative toxicity increases according to the macrophyte response; or for (ii) the introduction of an aromatic ring in the cation core of [Chol]Cl, which decreased the toxicity of [BzChol]Cl for the microalgae but boosted the IL toxicity for the remaining species. Following these observations, a refined analysis of the trends should include systematic comparisons between the tested cholinium compounds as they are featured with particular structural modifications.

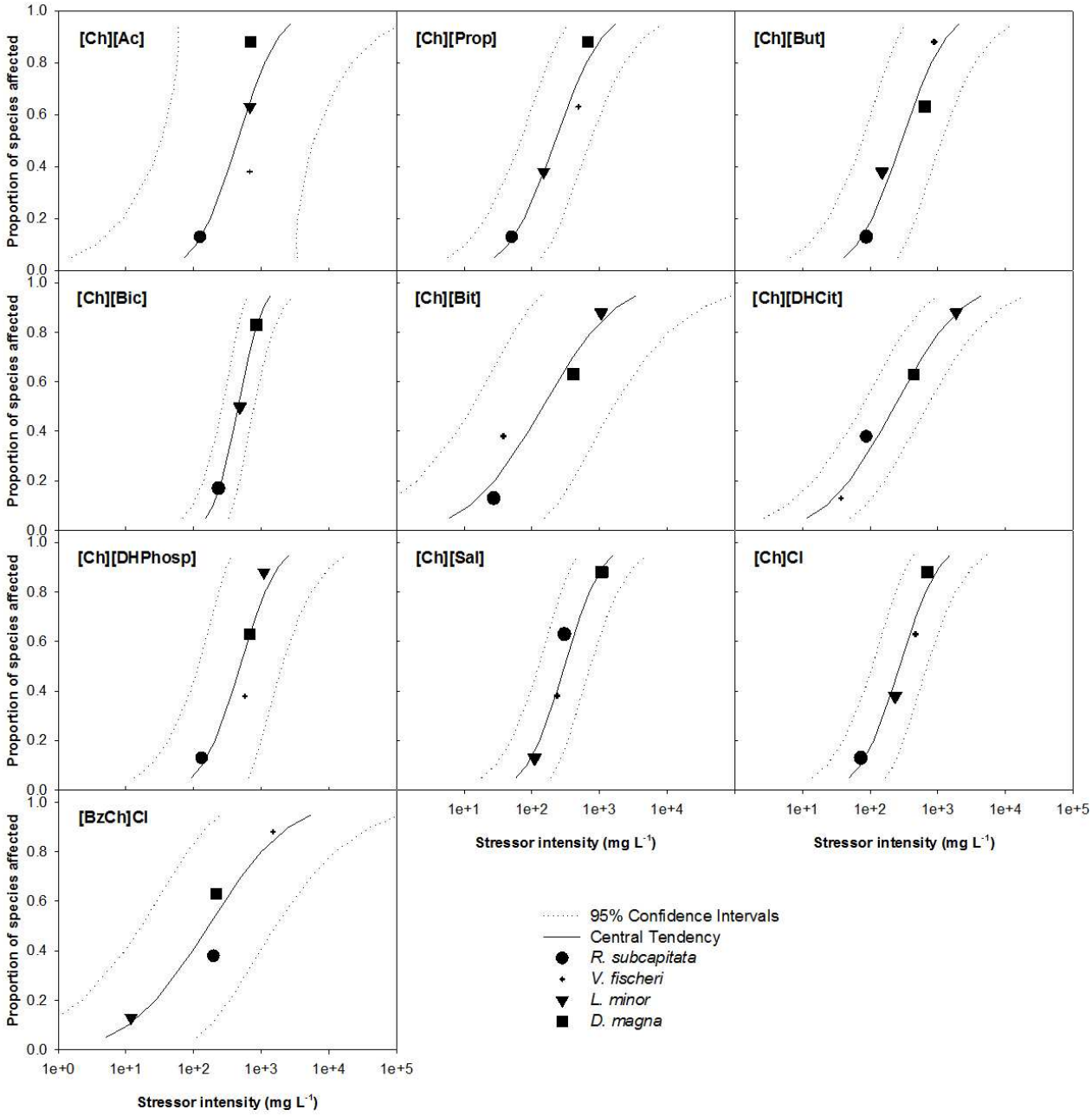


Fig. 2 Species sensitivity distribution plots for the cholinium ILs tested, build on the basis of the EC_{50} values estimated in the present study for *R. subcapitata*, *L. minor* and *D. magna* plus those estimated in a previous study by Ventura et al.[14] for *V. fischeri*.

The effect of the anion's alkyl side chain elongation. The side-chain effect is an heuristic rule typically applied to the cation core of ILs, which translates the observed increase in (eco)toxicity with the alkyl side chain elongation until a given threshold[6,49,69]. Such effect seems to be due to the increase in lipophilicity driven by the elongation of the alkyl chain, implying higher reactivity with biological membranes and embedded proteins[8,11,12,69-71]. Experimental evidences have been produced that confirm this rationale (e.g. [72-74]). Despite we rather focused the elongation of the alkyl chains of the anion moiety, the direct proportionality between lipophilicity and toxicity seems to still hold (see the correlations shown by Ventura et al.[14]) and hence increased reactivity with biological membranes was expected translating into decrease in EC50 values when sequentially following [Chol][Ac], [Chol][Prop] and [Chol][But]. This effect could be confirmed for *D. magna*, where the EC_{50} values monotonically decreased from 694.6 to 637.3 mg L^{-1} (Table S2; Figure 1), but not for the other biological models (microalgae, macrophytes, or bacteria as observed by Ventura et al.[14]). References in the literature denoting inconsistencies in the side-chain effect are very scarce, but were already reported for leukemia cells by Zhao et al.[42], for breast cancer cell lines by Muhammad et al.[75] and, regarding short alkyl chains, by e Silva et al.[25].

All test systems but the daphnids include a cell wall preventing a direct interaction between the toxic and the cell membranes. The cell wall may be the feature biasing the expected response trend following the elongation of the anion's alkyl chain, as supported by a comparative view of previous observations, e.g.: (i) on the yield of differential responses as bacteria with distinct cell wall organisation (Gram+ and Gram-) were challenged by the same IL[7,9,16,76]; (ii) on fungi cell wall damage by tetrabutylphosphonium chloride[72]; (iii) on the interaction of 1-butyl-3-methylimidazolium chloride with the siliceous valves of diatoms[77]; (iv) on the differential susceptibility of mutant (no cell wall) and a wild-type (with cell wall) strains of the microalgae *Chlamydomonas reinhardtii* to imidazolium, pyridinium and ammonium-based ILs[78]. In spite of this, the alkyl chain effect has actually been conspicuously observed for plant species[8,38,48,78,79], fungi[72,80] and bacteria[11,32,81], but in all cases the elongation of the cation rather than the anion was focused for cholinium ILs or derivatives and mostly for other IL families. The stronger influence of structural changes in the cation compared to the anion, particularly through the alkyl chain length, has been acknowledged in the literature[11,48]; still, the same mechanisms of toxic action have been suggested for the modifications in both moieties by several authors[8,82,83]. Furthermore, alkyl chains typically introduced in the cation for ecotoxicological assessment are larger than those introduced in the anion in the present study (e.g. C1-C18 vs C1-4, respectively). The polarity changes induced by the former and hence interaction with biological membranes[84] should be significantly stronger. Finally, a direct interaction of the cation rather than the anion with biological membranes is expected since these are negatively charged; any effects driven by structural variations in the anion should take place only at a secondary stage. The interplay of these features can confound the identification of an eventual side-chain effect as the anion is elongated, as reflected here for *V. fischeri*[14], *R. subcapitata*, *L. minor* or when the integrative HC_{50} estimates are focused (Table 2). Even for the daphnids, the overlapping of the confidence intervals of the HC_{50} estimates should be noticed, denoting the frailty of the recognised side-chain effect tendency.

Table 2 Summary of the Hazard Concentration for 5% and 50% of the species (HC_5 and HC_{50}, respectively) and corresponding 95% confidence intervals, obtained following SSD analysis on the basis of EC_{50} values (Fig. 2), for all the tested cholinium chemicals. CI – Confidence Interval.

Compound	HC_{50} (95% CI)	HC_5 (95% CI)
[Chol][DHCit]	227.8 (75.49-687.1)	11.67 (2.676-50.89)
[Chol]Cl	272.9 (107.6-692.1)	48.92 (14.03-170.6)
[Chol][Bit]	145.7 (13.34-1592)	5.999 (0.233-154.2)
[Chol][But]	293.4 (76.23-1129)	41.09 (6.640-254.2)
[Chol][Prop]	222.6 (68.04-728.4)	28.27 (5.756-139.1)
[Chol][DHPhosp]	485.7 (117.6-2006)	92.57 (12.28-654.2)
[Chol][Bic]	455.4 (265.6-780.6)	151.6 (69.53-330.5)
[Chol][Ac]	445.8 (33.38-5955)	73.11 (1.566-3412)
[Chol][Sal]	303.9 (126.6-729.4)	58.47 (18.06-189.3)
[Bzchol]Cl	165.9 (16.96; 1624)	5.027 (0.231-109.2)

Addition of hydroxyl groups in the anion. The effect of the functionalization by introduction of hydroxyl groups was studied by comparing [Chol][Bic], [Chol][Bit] and [Chol][DHCit]) with [Chol][Ac]. When a single hydroxyl group is introduced in the anion ([Chol][Ac] vs [Chol][Bic]), ecotoxicity either does not change significantly as occurred for the macrophyte (overlapping EC_{50} 95% confidence intervals) or noticeably decreases as observed for *V. fischeri*[14], microalgae and cladocerans. Conversely, the results showed that the introduction of three hydroxyl groups (see [Chol][Ac] vs [Chol][Bit] or [Chol][DHCit]) generally promoted a significant increase in toxicity (Figure 1). Hydroxyl groups can increase the hydrogen bonding strength[85], thus increasing the polarity of the compound. Polarity can be correlated with the octanol-water partition coefficient (*P*) by being inversely proportional to log *P*. This means the increase of the number of hydroxyl groups is expected to enhance the polarity, consequently making the compound more reactive against negatively charged biological membranes, which translates into a greater baseline toxicity of the focused solvent[86]. Our bulk results confirm this rationale for the oxygenation of the anion alkyl chain through the introduction of more than one hydroxyl group, except for *L. minor* (Figure 1); the higher toxicity driven by alkyl chain hydroxylation seems to be confirmed when integrated ecotoxicity is focused through HC_x estimates, but still these data should be held carefully given the large associated confidence intervals (Table 2). As a leave-floating macrophyte, *L. minor* presents two toxicant intake pathways, via surface contact and systemically. While the former is shared with microalgae, as well as with bacteria and cladocerans *sensu lato*, systemic uptake constitutes an additional pathway for the transport of ions from the roots to the fronds, similarly to terrestrial monocotyledonous (reviewed in Cedergreen and Madsen[87]). It is conceivable that the significant role of *Lemna* roots in nutrient

uptake[87] reflects in the uptake of toxicants including ILs, thus probably biasing the attempts to interpret validated relationships between polarity and membrane reactivity and likely leading to inconsistent responses compared to the other studied systems.

Furthermore, the data indicate that the oxygenation via carboxylic addition (CH_2CH_2OH group) can be meaningful to decrease the toxicity of cholinium ILs: [Chol][DHCit] tended indeed to yield higher EC_{50} values (Figure 1) than its counterpart [Chol][Bit]. This is apparently inconsistent with the slightly higher log *P* by [Chol][DHCit] compared to [Chol][Bit] (-1.32 vs -1.83; http://www.chemspider.com/; accessed by 01/04/2015), which theoretically corresponds to a better affinity to the lipid membranes and higher polarity hence higher toxicity. Still, the difference between these log *P* values is mild, and besides a major toxicity driver, one must consider other more specific mechanisms of toxic action involved in a complex biological response, such as interference in metabolic pathways or enzyme activity (e.g. [69,88]). Moreover, the literature is also inconsistent in this field: although a decrease of toxicity has been found for the carboxylic oxygenation of the imidazolium cation (e.g. [8,89]), the opposite was observed for some cation functionalization series in cholinium compounds by e Silva et al.[25].

Addition of a phosphate group in the anion. The effects of the introduction of a phosphate group in the anion were monitored by comparing [Chol][Bic] with [Chol][DHPhosp]. Higher toxicity of the [DHPhosp] anion was found for *V.fischeri*[14], *R. subcapitata* and *D. magna*, while it did not operate significant toxicity changes compared to the anion [Bic] in *L. minor*. Similar inconsistency was also found by Biczaket al.[90], who found that the phosphate anion induced the highest phytotoxicity of an imidazolium-based IL against the dicotyledonous *Raphanus sativus* but not against the monocotyledonous *Hordeum vulgare*. In line with our observations, Nancharaiah and Francis[91] found better ability of the dimethylphosphate anion to impair bacteria growth compared to the acetate anion, both coupled with the same cation1-ethyl-3-methylimidazolium. The interplay of different properties may contribute to the general toxicity increase following the phosphate insertion. [DHPhosp] holds one more hydrogen bond acceptor than [Bic] and its Kow is about three orders of magnitude lower (log *P* of -2.15 vs -0.81, respectively; http://www.chemspider.com/; assessed by 01/04/2015); in addition, [DHPhosp] holds one more hydroxyl group than [Bic], which is likely to contribute to increase its polarity (see above). These properties, along with the kosmotropic character of the phosphate anion strongly favour the interaction of [Chol][DHPhosp] with lipids and proteins in biological membranes, which supports the observed decrease in EC_{50} values considering a baseline toxicity (narcosis equivalent) mode of action. Furthermore, and assuming that these ILs find their way into the cells, the acidic character of the [DHPhosp] ion is noteworthy as a property that can negatively influence the catalytic activity of different enzymes (see the study by Curto et al.[92]) for lactase oxidase). In spite of these properties, and our experimental evidences, when the ecotoxicological data are integrated, HC_x estimates (Table 2) suggest that there should be no appreciable variation in the environmental friendliness of [Chol][Bic] and [Chol][DHPhosp]. Further loading of the SSD curves with data from other adequate testing systems should be considered in the future for a robust conclusion.

Addition of aromatic rings in both the anion and the cation. The introduction of a phenolic group in the anion of [Chol][Ac], originating [Chol][Sal], did not reflect on a clear toxicity tendency, with the results indicating an increase of toxicity for *V. fischeri*[14] and *L. minor* but a decrease for *R. subcapitata* and *D. magna* (Figure 1; Table S2). The effect of the introduction of a benzyl group in the cation was not clear as well, since decreased toxicity for *V. fischeri* and *R. subcapitata* or increased toxicity for *L. minor* and *D. magna* was found when comparing [BzChol]Cl with its non-aromatic counterpart [Chol]Cl. This is contrary to previous studies with traditional IL families (e.g. [81]) where aromatic propyl imidazolium and pyridinium were consistently more toxic than the non-aromatic piperidinium and pyrrolidinium equivalents, regardless the species tested. It is worth noticing that the aromatization of the cholinium cation produced changes in toxicity of larger magnitude (1 order of magnitude or more, except for *D. magna*) while the aromatization of the anion produced a markedly lower impact in the EC_{50} values (Table S2). Although this does not deter the role of the anion in triggering toxic effects, it indeed supports the traditional view on the higher relevance of the cation[8,11,48].

Benzene is a class 1 compound according to the widely accepted Verhaar classification[93], thus it does not interact with specific receptors but rather shows a baseline (or narcotic) toxicity mode of action through interaction with cellular membranes[93-95]. Therefore, it is conceivable that the introduction of a benzyl group in [Chol]Cl ultimately works as an elongation of the cation alkyl chain in an IL that theoretically acts through the same mode of baseline action. Our results were consistent with such a rationale for *D. magna* and *L. minor*; while the cladoceran represents a direct contact between the IL and the membranes, and the macrophyte evidences a systemically facilitated route for the cellular uptake of the toxicant, the cell walls of the bacteria and the microalgae may constitute a primary barrier biasing the expected response. On the other hand, non-aromatic ILs are of generally higher hydrophobicity (i.e. lower water solubility) than their aromatic counterparts (e.g. [81]). Assuming that hydrophobicity and lipophilicity positively correlate for the cholinium family, our findings on the decrease of toxicity with the aromatization of the cholinium cation for the bacteria and the algae could be easily explained by the lower affinity of [BzChol]Cl with biological membranes; and here the complexity of the multicellular test systems could be used to explain the inconsistency of the toxicity trend. Overall, the present study does not support previous conclusions by our team (see [14,81]), since the aromatization of the cation core of [Chol]Cl does not invariably increases its toxicity.

Conclusions

Two major lessons should be retrieved from the present study. First, the establishment by Ventura et al.[14] that cholinium ILs are

not devoid of toxicity was validated here, with most of the ten cholinium compounds tested being more toxic than common solvents. Second, while structural changes can indeed operate significant variation in the ecotoxicity of cholinium ILs, the trends of such a variation yield from testing with a single sensitive ecological receptor cannot be generalised. The absence of consistency among the responses of the biological models to the ILs precludes the establishment of feasible structure-ecotoxicity relationships for the cholinium family, unless previous comprehensive ecotoxicological characterisation allows adequate SSD modelling in order to provide integrated HC benchmarks as feeding variables. Still, at early stages in the development pipeline, the environmental safety of the variants should be taken into account as a design variable (see the introduction for the related rationale). In this context, and in line with the precautionary principle, microalgae were proven here to better adequate to the purposes than the widely used Microtox® testing platform, given their generally higher sensitivity. Actually, with the validated use of micro-sized biotests such as that applied here, the cost-effectiveness of the task becomes more favourable.

Under a wider scope, the present study enlightens on the importance of the anion as a driver of toxicity, generally supporting the arguments by Weaver et al.[83], also dedicated to the cholinium family. Furthermore, it challenges the validation of heuristic rules such as the "side-chain" effect, the increase in toxicity through oxygenation or its decrease through aromatization. As a final remark, it is worth to suggest the continuation of studies to better characterise the environmental hazardous potential of the cholinium family of ILs. Data on bioaccumulation and specific mechanisms of toxic action against the biota representative of distinct functional levels in aquatic ecosystems should contribute to an overall understanding of the potential of these ILs as alternative, environmentally compatible industrial solvents.

Acknowledgements

The authors are grateful for the financial support from FEDER funds through the program COMPETE and for national fund through the Portuguese Foundation for Science and Technology (FCT) for CESAM strategic programme Ref. UID/AMB/50017/2013,CICECO-Aveiro Institute of Materials Ref. FCT UID/CTM /50011/2013, and the research project PTDC/AAC-AMB/119172/2010. The authors also thank the financial support of FCT to the post-doctoral grants SFRH/BPD/97210/2013, SFRH/BPD/79263/2011 and SFRH/BPD/101971/2014 of A.M.M. Gonçalves, S.P.M. Ventura and J.L. Pereira, respectively, and the doctoral grant of F. A. e Silva (SFRH/BD/94901/2013).

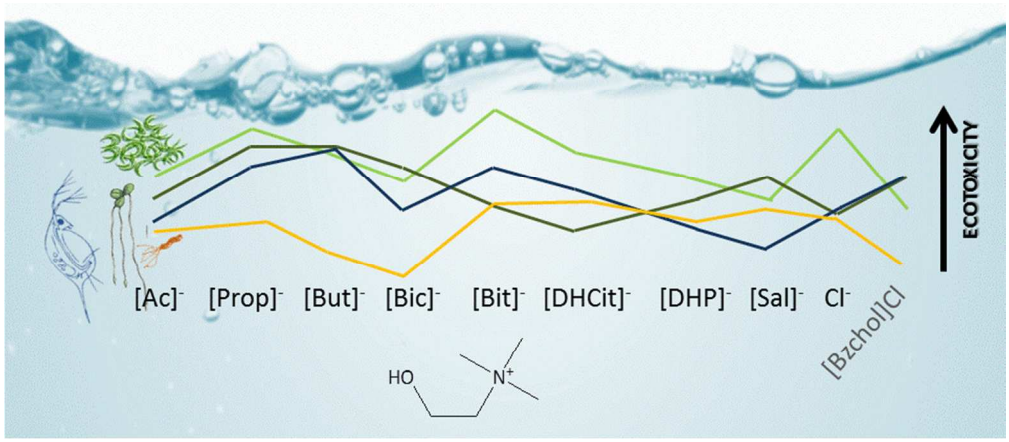
The authors declare that they have no conflict of interest.

References

1 W. L. Hough and R. D. Rogers, *Bull. Chem. Soc. Jpn.*, 2007, **80**, 2262–2269.
2 M. Freemantle, *Chem. Eng. News Arch.*, 1998, **76**, 32–37.
3 K. R. J. Lovelock, A. Deyko, P. Licence and R. G. Jones, *Phys. Chem. Chem. Phys.*, 2010, **12**, 8893–901.
4 J. L. Anthony, E. J. Maginn and J. F. Brennecke, *J. Phys. Chem. B*, 2001, **105**, 10942–10949.
5 M. G. Freire, L. M. N. B. F. Santos, A. M. Fernandes, J. A. P. Coutinho and I. M. Marrucho, *Fluid Phase Equilib.*, 2007, **261**, 449–454.
6 M. Matzke, J. Arning, J. Ranke, B. Jastorff and S. Stolte, in *Handbook of Green Chemistry*, ed. P. T. Anastas, Wiley-VCH Verlag GmbH & Co. KGaA, 2010, pp. 235–298.
7 S. P. M. Ventura, R. L. F. de Barros, T. Sintra, C. M. F. Soares, Á. S. Lima and J. A. P. Coutinho, *Ecotoxicol. Environ. Saf.*, 2012, **83**, 55–62.
8 S. Stolte, M. Matzke, J. Arning, A. Böschen, W.-R. Pitner, U. Welz-Biermann, B. Jastorff and J. Ranke, *Green Chem.*, 2007, **9**, 1170–1179.
9 S. Morrissey, B. Pegot, D. Coleman, M. T. Garcia, D. Ferguson, B. Quilty and N. Gathergood, *Green Chem.*, 2009, **11**, 475–483.
10 J. Arning, S. Stolte, A. Böschen, F. Stock, W.-R. Pitner, U. Welz-Biermann, B. Jastorff and J. Ranke, *Green Chem.*, 2008, **10**, 47–58.
11 D. J. Couling, R. J. Bernot, K. M. Docherty, J. K. Dixon and E. J. Maginn, *Green Chem.*, 2006, **8**, 82–90.
12 J. Ranke, K. Mölter, F. Stock, U. Bottin-Weber, J. Poczobutt, J. Hoffmann, B. Ondruschka, J. Filser and B. Jastorff, *Ecotoxicol. Environ. Saf.*, 2004, **58**, 396–404.
13 T. P. Thuy Pham, C. W. Cho and Y. S. Yun, *Water Res.*, 2010, **44**, 352–372.
14 S. P. M. Ventura, F. A. e Silva, A. M. M. Gonçalves, J. L. Pereira, F. Gonçalves and J. A. P. Coutinho, *Ecotoxicol. Environ. Saf.*, 2014, **102**, 48–54.
15 K. J. Kulacki and G. A. Lamberti, *Green Chem.*, 2008, **10**, 104–110.
16 S. P. M. Ventura, M. Gurbisz, M. Ghavre, F. M. M. Ferreira, F. Gonçalves, I. Beadham, B. Quilty, J. A. P. Coutinho and N. Gathergood, *ACS Sustain. Chem. Eng.*, 2013, **1**, 393–402.
17 S. H. Zeisel and K.-A. da Costa, *Nutr. Rev.*, 2009, **67**, 615–23.
18 M. Petkovic, J. L. Ferguson, H. Q. N. Gunaratne, R. Ferreira, M. C. Leitão, K. R. Seddon, L. P. N. Rebelo and C. S. Pereira, *Green Chem.*, 2010, **12**, 643–649.
19 X. D. Hou, Q. P. Liu, T. J. Smith, N. Li and M. H. Zong, *PLoS One*, 2013, **8**.
20 D. Coleman and N. Gathergood, *Chem. Soc. Rev.*, 2010, **39**, 600–637.
21 K. Radošević, M. Cvjetko Bubalo, V. Gaurina Srček, D. Grgas, T. Landeka Dragičević and I. Radojčić Redovniković, *Ecotoxicol. Environ. Saf.*, 2015, **112**, 46–53.
22 S. Stolte, S. Steudte, O. Areitioaurtena, F. Pagano, J. Thöming, P. Stepnowski and A. Igartua, *Chemosphere*, 2012, **89**, 1135–1141.
23 H. Garcia, R. Ferreira, M. Petkovic, J. L. Ferguson, M. C. Leitão, H. Q. N. Gunaratne, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, *Green Chem.*, 2010, **12**, 367–369.
24 Q.-P. Liu, X.-D. Hou, N. Li and M.-H. Zong, *Green Chem.*, 2012, **14**, 304–307.
25 A. Kokorin, Ed., *Ionic Liquids: Applications and Perspectives*, InTech, 2011.
26 J. Lu, F. Yan and J. Texter, *Prog. Polym. Sci.*, 2009, **34**, 431–448.

27 N. Jain, A. Kumar, S. Chauhan and S. M. S. Chauhan, *Tetrahedron*, 2005, **61**, 1015–1060.
28 K. Ninomiya, T. Yamauchi, M. Kobayashi, C. Ogino, N. Shimizu and K. Takahashi, *Biochem. Eng. J.*, 2013, **71**, 25–29.
29 S. Shahriari, L. C. Tomé, J. M. M. Araújo, L. P. N. Rebelo, J. A. P. Coutinho, I. M. Marrucho and M. G. Freire, *RSC Adv.*, 2013, **3**, 1835–1843.
30 J. F. B. Pereira, F. Vicente, V. C. Santos-Ebinuma, J. M. Araújo, A. Pessoa, M. G. Freire and J. A. P. Coutinho, *Process Biochem.*, 2013, **48**, 716–722.
31 M. Taha, M. R. Almeida, F. A. e Silva, P. Domingues, S. P. M. Ventura, J. A. P. Coutinho and M. G. Freire, *Chem. - A Eur. J.*, 2015, **21**, 4781–4788.
32 F. A. e Silva, F. Siopa, B. F. H. T. Figueiredo, A. M. M. Gonçalves, J. L. Pereira, F. Gonçalves, J. A. P. Coutinho, C. A. M. Afonso and S. P. M. Ventura, *Ecotoxicol. Environ. Saf.*, 2014, **108**, 302–10.
33 N. V. Plechkova and K. R. Seddon, in *Methods and Reagents for Green Chemistry: An Introduction*, eds. P. Tundo, A. Perosa and F. Zecchini, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2007.
34 G. W. SuterII, *Ecological Risk Assessment*, CRC Press, Second., 2006.
35 U.S.EPA, in *U.S. Environmental Protection Agency, Risk Assessment Forum*, Washington, DC, 1998.
36 EEA, *Environmental Risk Assessment - Approaches, Experiences and Information Sources*, Copenhagen, Denmark, 1998.
37 M. Alvarez-Guerra and A. Irabien, *Green Chem.*, 2011, **13**, 1507.
38 R. N. Das and K. Roy, *Chemosphere*, 2014, **104**, 170–176.
39 S. Ma, M. Lv, F. Deng, X. Zhang, H. Zhai and W. Lv, *J. Hazard. Mater.*, 2015, **283**, 591–598.
40 K. Roy, R. N. Das and P. L. A. Popelier, *Chemosphere*, 2014, **112**, 120–127.
41 J. S. Torrecilla, J. Palomar, J. Lemus and F. Rodríguez, *Green Chem.*, 2010, **12**, 123.
42 Y. Zhao, J. Zhao, Y. Huang, Q. Zhou, X. Zhang and S. Zhang, *J. Hazard. Mater.*, 2014, **278**, 320–329.
43 B. Jastorff, R. Störmann, J. Ranke, K. Mölter, F. Stock, B. Oberheitmann, W. Hoffmann, J. Hoffmann, M. Nüchter, B. Ondruschka and J. Filser, *Green Chem.*, 2003, **5**, 136–142.
44 S. Kar and K. Roy, *J. Indian Chem. Soc.*, 2010, **87**, 1455–1515.
45 The European Parliament and the Council of the European Union, *Off. J. Eur. Union*, 2006, **L396**, 1–894.
46 ECB, *Technical Guidance Document on Risk Assessment - Part III*, 2003.
47 B. Jastorff, R. Störmann and J. Ranke, *Clean - Soil, Air, Water*, 2007, **35**, 399–405.
48 M. Matzke, S. Stolte, K. Thiele, T. Juffernholz, J. Arning, J. Ranke, U. Welz-Biermann and B. Jastorff, *Green Chem.*, 2007, **9**, 1198–1207.
49 K. S. Egorova and V. P. Ananikov, *ChemSusChem*, 2014, **7**, 336–360.
50 OECD guideline 221, in *OECD Guidelines for the Testing of Chemicals, Section 2*, OECD Publishing, 2006.
51 OECD guideline 202, in *OECD Guidelines for the Testing of Chemicals, Section 2*, OECD Publishing, 2004.
52 OECD guideline 201, in *OECD Guidelines for the Testing of Chemicals, Section 2*, OECD Publishing, 2011.
53 Y. Yu, X. Lu, Q. Zhou, K. Dong, H. Yao and S. Zhang, *Chem. - A Eur. J.*, 2008, **14**, 11174–11182.
54 S. W. Geis, K. L. Leming, E. T. Korthals, G. Searle, L. Reynolds and D. A. Karner, *Environ. Toxicol. Chem.*, 2000, **19**, 36–41.
55 M. Kaza, J. Mankiewicz-Boczek, K. Izydorczyk and J. Sawicki, *Polish J. Environ. Stud.*, 2007, **16**, 81–89.
56 J. Kolasińska, M. Bielińska and G. Nałęcz-Jawecki, *Fresenius Environ. Bull.*, 2010, **19**, 1453–1457.
57 ASTM, *Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians*, American Society for Testing and Materials, Philadelphia, 1980.
58 B.-P. Elendt and W.-R. Bias, *Water Res.*, 1990, **24**, 1157–1167.
59 D. J. Baird, A. M. V. M. Soares, A. Girling, I. Barber, M. C. Bradley and P. Calow, in *Proceedings of the First European Conference on Ecotoxicology*, eds. H. Lokke, H. Tyle and F. Bro-Rasmussen, Lyngby, 1989, pp. 144–148.
60 D. J. Finney, *Probit Analysis*, Cambridge University Press, Cambridge, United Kingdom, 1971.
61 L. Posthuma, G. W. SuterII and T. P. Traas, Eds., *Species Sensitivity Distributions in Ecotoxicology*, Lewis Publishers, Boca Raton, Florida, USA, 2002.
62 EC, *Guidance Document on Statistical Methods for Environmental Toxicity Tests*, Ottawa, Ontario, Canada, 2007.
63 U.S.EPA, *Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses*, Springfield, VA, USA, 1985.
64 United Nations, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, New York and Geneva, Fourth rev., 2011.
65 P. T. Anastas and M. M. Kirchhoff, *Acc. Chem. Res.*, 2002, **35**, 686–694.
66 W. Brack and H. Rottler, *Environ. Sci. Pollut. Res.*, 1994, **1**, 223–228.
67 A. Latała, P. Stepnowski, M. Nędzi and W. Mrozik, *Aquat. Toxicol.*, 2005, **73**, 91–98.
68 J. Römbke, S. Jänsch, T. Junker, B. Pohl, A. Scheffczyk and H.-J. Schallnass, *Environ. Toxicol. Chem.*, 2006, **25**, 776–787.
69 M. C. Bubalo, K. Radošević, I. R. Redovniković, J. Halambek and V. G. Srček, *Ecotoxicol. Environ. Saf.*, 2014, **99**, 1–12.
70 J. Ranke, S. Stolte, R. Störmann, J. Arning and B. Jastorff, *Chem. Rev.*, 2007, **107**, 2183–2206.
71 K. O. Evans, *Colloids Surfaces A Physicochem. Eng. Asp.*, 2006, **274**, 11–17.
72 M. Petkovic, D. O. Hartmann, G. Adamová, K. R. Seddon, L. P. N. Rebelo and C. S. Pereira, *New J. Chem.*, 2012, **36**, 56–63.
73 K. Radošević, M. Cvjetko, N. Kopjar, R. Novak, J. Dumić and V. G. Srček, *Ecotoxicol. Environ. Saf.*, 2013, **92**, 112–118.
74 D. Rengstl, B. Kraus, M. Van Vorst, G. D. Elliott and W. Kunz, *Colloids Surfaces B: Biointerfaces*, 2014, **123**, 575–581.
75 N. Muhammad, M. I. Hossain, Z. Man, M. El-Harbawi, M. A. Bustam, Y. A. Noaman, N. B. Mohamed Alitheen, M. K. Ng, G. Hefter and C. Y. Yin, *J. Chem. Eng. Data*, 2012, **57**, 2191–2196.
76 K. M. Docherty and C. F. Kulpa, Jr., *Green Chem.*, 2005, **7**, 185–189.

77 C. Samorì, G. Sciutto, L. Pezzolesi, P. Galletti, F. Guerrini, R. Mazzeo, R. Pistocchi, S. Prati and E. Tagliavini, *Chem. Res. Toxicol.*, 2011, **24**, 392–401.
78 D. W. Sena, K. J. Kulacki, D. T. Chaloner and G. A. Lamberti, *Green Chem.*, 2010, **12**, 1066–1071.
79 J. H. Larson, P. C. Frost and G. A. Lamberti, *Environ. Toxicol. Chem.*, 2008, **27**, 676–681.
80 J. Pernak, K. Sobaszkiewicz and I. Mirska, *Green Chem.*, 2002, **5**, 52–56.
81 S. P. M. Ventura, A. M. M. Gonçalves, T. Sintra, J. L. Pereira, F. Gonçalves and J. A. P. Coutinho, *Ecotoxicology*, 2013, **22**, 1–12.
82 R. A. Kumar, N. Papaiconomou, J.-M. Lee, J. Salminen, D. S. Clark and J. M. Prausnitz, *Environ. Toxicol.*, 2009, **24**, 388–395.
83 K. D. Weaver, H. J. Kim, J. Sun, D. R. MacFarlane and G. D. Elliott, *Green Chem.*, 2010, **12**, 507.
84 S. Stolte, J. Arning, U. Bottin-Weber, A. Müller, W.-R. Pitner, U. Welz-Biermann, B. Jastorff and J. Ranke, *Green Chem.*, 2007, **9**, 760–767.
85 C. Reichardt, *Green Chem.*, 2005, **7**, 339.
86 C.-W. Cho, T. P. T. Pham, S. Kim, Y. R. Kim, Y. C. Jeon and Y. S. Yun, *J. Appl. Phycol.*, 2009, **21**, 683–689.
87 N. Cedergreen and T. V. Madsen, *New Phytol.*, 2002, **155**, 285–292.
88 D. Zhao, Y. Liao and Z. D. Zhang, *Clean - Soil, Air, Water*, 2007, **35**, 42–48.
89 C. Pretti, C. Chiappe, I. Baldetti, S. Brunini, G. Monni and L. Intorre, *Ecotoxicol. Environ. Saf.*, 2009, **72**, 1170–1176.
90 R. Biczak, B. Pawłowska, P. Bałczewski and P. Rychter, *J. Hazard. Mater.*, 2014, **274**, 181–190.
91 Y. V. Nancharaiah and A. J. Francis, *Bioresour. Technol.*, 2011, **102**, 6573–6578.
92 V. F. Curto, S. Scheuermann, R. M. Owens, V. Ranganathan, D. R. MacFarlane, F. Benito-Lopez and D. Diamond, *Phys. Chem. Chem. Phys.*, 2014, **16**, 1841–1849.
93 H. J. M. Verhaar, C. J. van Leeuwen and J. L. M. Hramens, *Chemosphere*, 1992, **25**, 471–491.
94 H. J. M. Verhaar, J. Solbé, J. Speksnijder, C. J. Van Leeuwen and J. L. M. Hermens, *Chemosphere*, 2000, **40**, 875–883.
95 S. J. Enoch, M. Hewitt, M. T. D. Cronin, S. Azam and J. C. Madden, *Chemosphere*, 2008, **73**, 243–248.
96 U.S.EPA, 2015, ECOTOX User Guide: ECOTOXicology Database System. Version 4.0. Available: http:/www.epa.gov/ecotox/.
97 S. H. Hsieh, K. P. Tsai and C. Y. Chen, *Water Res.*, 2006, **40**, 1957–1964.
98 U. M. Cowgill, D. P. Milazzo and B. D. Landenberger, *Res. J. Water Pollut. Control Fed.*, 1991, **63**, 991–998.
99 G. A. LeBlanc and D. C. Surprenant, *Arch. Environ. Contam. Toxicol.*, 1983, **12**, 305–310.
100 U. M. Cowgill and D. P. Milazzo, *Arch. Environ. Contam. Toxicol.*, 1991, **20**, 211–217.
101 J. H. Canton and D. M. M. Adema, *Hydrobiologia*, 1978, **59**, 135–140.
102 Z. Tong and J. Hongjun, *Environ. Pollut.*, 1997, **98**, 143–147.
103 G. A. LeBlanc, *Bull. Environ. Contam. Toxicol.*, 1980, **24**, 684–691.
104 C.-W. Cho, T. Phuong Thuy Pham, Y.-C. Jeon and Y.-S. Yun, *Green Chem.*, 2008, **10**, 67.
105 H. G. Peterson, N. Ruecker, K. Dennison and M. Moody, *Toxicity testing of the compound N,N-dimethylformamide to phytoplankton (green algae, diatoms, and cyanobacteria) and a vascular plant (duckweed).*, Saskatoon, Saskatchewan, Canada, 1997.
106 G. W. Stratton and T. M. Smith, *Bull. Environ. Contam. Toxicol.*, 1988, **40**, 736–742.
107 S. H. Poirier, M. L. Knuth, C. D. Anderson-Buchou, L. T. Brooke, A. R. Lima and P. J. Shubat, *Bull. Environ. Contam. Toxicol.*, 1986, **37**, 615–621.
108 I. R. Barbosa, R. M. Martins, M. L. Sá e Melo and A. M. V. M. Soares, *Bull. Environ. Contam. Toxicol.*, 2003, **70**, 1264–1268.
109 L. Guilhermino, T. Diamantino, M. C. Silva and A. M. Soares, *Ecotoxicol. Environ. Saf.*, 2000, **46**, 357–362.
110 I. T. Takahashi, U. M. Cowgill and P. G. Murphy, *Bull. Environ. Contam. Toxicol.*, 1987, **39**, 229–236.
111 N. Dom, M. Penninck, D. Knapen and R. Blust, *Chemosphere*, 2012, **87**, 742–749.
112 D. a. Pillard and D. L. DuFresne, *Arch. Environ. Contam. Toxicol.*, 1999, **37**, 29–35.



Ecotoxicity of cholinium-based ionic liquids