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Ionic liquids: a pathway to environmental acceptability

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Ionic liquids were initially proposed as replacements for conventional organic solvents; however, their chemistry has developed remarkably and offers unexpected opportunities in numerous fields, ranging from electrochemistry to biology. As a consequence of ionic liquids advancing towards potential and actual applications, a comprehensive determination of their environmental, health and safety impact is now required. This *critical review* aims to present an overview of the current understanding of the toxicity and environmental impact of the principal ionic liquid groups, and highlights some emerging concerns. Each cation type is considered separately, examining the significance of the biological data, and identifying the most critical questions, some yet unresolved. The need for more, and more detailed, studies is highlighted (176 references).

Introduction

Ionic liquids are increasingly attracting interest in both the academic and the industrial fora, as demonstrated in the continually growing number of publications and patents (currently >10 000 and >2000, respectively). Though their history effectively started in 1914, when the physical properties of ethylammonium nitrate ([EtNH₃][NO₃]; m.p. 13–14 °C) were first reported,¹ the accepted use of the term “ionic liquid” is quite recent, and as a major scientific discipline they have flourished only in the past decade.

Ionic liquids are salts, completely composed of ions, and generally are liquid below 100 °C.² Their negligible vapour pressure, conventional non-flammability, and outstanding solvation potential are the basis for them often being classified as “green” solvents.³ Their potential is further emphasised by the fact that their physical and chemical properties may be finely tuned by varying both the cation and the anion. This dual nature—as well as that due to the existence of two (high and low) electrically charged nanodomains⁴—is, relative to conventional molecular organic solvents, a remarkable advantage. Their generic (but not universal) properties are enabling rapid advances in numerous applications, with some extant processes at an industrial scale, e.g. BASF (BASIL,⁵ aluminium plating, cellulose dissolution),⁶ Institut Français du Pétrole (Difasol),⁷ Degussa (paint additives),⁶ Linde (hydraulic ionic liquid compressor),⁸ Pionics (batteries),⁹ and G24i (solar cells).¹⁰ Ionic liquids are also providing unexpected opportunities at the interface of chemistry with the life

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Marija Petkovic

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aspects of ionic liquids and their exploitation as co-solvents in fungal biocatalysis.



Kenneth R. Seddon

Kenneth Seddon was born in Liverpool in 1950, and graduated from Liverpool University with a first class BSc (Hons) and a PhD. In 1993, he was appointed to the Chair in Inorganic Chemistry at the Queen's University of Belfast, where he is also a co-director of QUILL (Queen's University Ionic Liquids Laboratories), an industrial-academic consortium which was awarded the 2006 Queen's Anniversary Prize for Higher and Further

Education. He is a Professor Catedrático Visitante at ITQB (New University of Lisbon), holds a Visiting Professorship of the Chinese Academy of Sciences, and is Associate editor of Australian Journal of Chemistry. He has published over 350 papers and patents, co-authored four books, and co-edited nine books.

sciences, *e.g.* acting as solvents in enzymatic¹¹ and whole-cell biocatalysis,^{12,13} and as protein stabilisation agents.¹⁴ In addition, their potential use as active pharmaceutical ingredients, though still rather exploratory, further highlights their potential in biochemical studies.¹⁵

Globally, there is a growing awareness of the environmental impact of man-made chemicals, which consequently results in more severe legislation and restrictions. The conscious design of chemicals and the use of structure–activity relationships are essential tools to deliver safer chemicals with enhanced technical performance. These are embedded in the Twelve Principles of Green Chemistry,¹⁶ which endeavour to prevent hazard generation, and challenge and encourage the development of creative solutions to improve old or to create novel processes.¹⁷ Examples are spread across a broad diversity of fields, from catalysis and alternative solvents to renewable feedstocks.¹⁸ The potential of ionic liquids to conceptually fulfil the requirements of environmental sustainability is remarkable. However, they are still fairly innovative (neoteric) solvents comprising a very heterogeneous group of fluids that cannot, *a priori*, be considered benign, especially because precise knowledge is still nebulous. Their lack of vapour pressure is, relative to traditional volatile molecular solvents, a significant advantage, yet true “greenness” should incorporate a sustainable synthesis,¹⁹ low toxicity, and limited environmental persistence (Green Chemistry Principles 2, 3 and 10, respectively).¹⁶ These aspects are yet to be comprehensively considered for the majority of ionic liquids.

The understanding of ionic liquids (their core chemistry, syntheses and purification methods) has advanced significantly over the past decade, and is currently set on solid ground, opening doors to the design of biocompatible ionic liquids,²⁰ incorporating (*inter alia*) amino acids,²¹ carboxylic acids,²² non-nutritive sweeteners,²³ or glucose.²⁴ Our current understanding of the issues of purity, and the influence of impurities on both physicochemical and toxicological studies, now allows

the design of precise and reproducible syntheses, and the collection of viable and accurate data.²⁵

Up to now, a broad range of testing models—bacteria, fungi, crustaceans, algae, plants, mammalian cell lines and animals (representing the five Kingdoms in the classification of living organisms)²⁶—has been used to evaluate the ecotoxicity of ionic liquids. The organisms within each Kingdom carry a set of general, yet specific, characteristics (*e.g.* multi- or unicellular, carrying or not a cell wall), and in the presence of ionic liquids will show very distinct behaviours. The toxicity tests are often carried out under dissimilar conditions, *e.g.* incubation periods and end points, therefore providing uncorrelated information on lethal or sub-lethal effects.

The environmental fate of ionic liquids is a complex situation which crosses numerous unknown abiotic and biotic factors. A better structure-based understanding of this is critical, and only recently have their major abiotic mechanisms been analysed, *e.g.* their sorption in soils.^{27,28} Jastorff and coworkers have proposed a multidimensional risk analysis, correlating five distinct indicators, namely release, spatiotemporal range, bioaccumulation, biological activity, and uncertainty, which can be used for predicting the environmental impact of chemicals, *e.g.* antifouling biocides²⁹ and ionic liquids.³⁰ Though a multidimensional analysis is important for the risk assessment of any chemical, for most ionic liquids the proposed indicators are yet to be comprehensively addressed. With the expanding number of studies on the toxicity and biodegradability of ionic liquids, certain trends are becoming apparent and, though data interpretation and comparison should be made very cautiously, their predictive value is unquestionable. Nonetheless, there are yet major questions to be resolved, such as their modes of toxicity, biodegradation pathways, and behaviour concerning biosorption.

Some recent initiatives, such as the BATIL (Biodegradability and Toxicity of Ionic Liquids) meetings (Berlin, 2007 and Frankfurt, 2009)³¹ and the online available UFT/Merck Ionic



Luís Paulo N. Rebelo

Luís Paulo N. Rebelo was born in 1960 in Lisbon, Portugal. In 1989 he received his PhD in physical chemistry from the Universidade Nova de Lisboa (UNL) and became Assistant Professor of its Faculty of Science and Technology. He was a Research Associate at the University of Tennessee (1990, 1996) and Princeton University (1997). He joined the Instituto de Tecnologia Química e Biológica (ITQB) in 2000 where he is currently Full

Professor and Vice-Dean. His research interests are centered on molecular thermodynamics of liquids and liquid solutions, in particular, isotope effects, ionic liquids, and metastability.



Cristina Silva Pereira

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group (10+) in Applied and Environmental Mycology. Her research interests are centered on the understanding of fungal response to critical anthropogenic stresses, such as ionic liquids, and their potential utility for designing novel and environmentally friendly biotechnological processes.

Liquids Biological Effects Database,³² are valuable contributions to the exchange of multidisciplinary data, promoting a more articulate research strategy. The present review provides a critical outlook on the current understanding of the toxicity and environmental impact of the most commonly encountered ionic liquids, and highlights some emerging concerns. It examines the most relevant toxicity data, structured by the head group of the cations. Each bioassay has been explained so as to make it accessible to the reader who is not so familiar with the different tested organisms, the observed behaviours, and ultimately, their significance in the context of ecotoxicity. Though these subjects have been already reviewed,^{33,34} we aim here to achieve overall a higher degree of systematisation of the data, and obviously higher clarity, especially for chemists. The major correlations between the chemical structure of the ions and their observed toxicities have been highlighted, despite the fact that, mostly due to their commercial availability, the great majority of the extant studies have focused on the imidazolium family. It should be made clear, from the outset, that the common generalisations that ionic liquids are either “green” or “toxic” solvents should be avoided: both extremes are totally misleading.

Toxicity of ionic liquids containing aromatic head groups in the cation

Imidazolium-based ionic liquids

The imidazolium-based ionic liquids are the most commonly investigated group, for synthesis, in physical chemistry, and for environmental studies: they were one of the first to find application on an industrial scale.⁶

In the context of the toxicity of ionic liquids, there are numerous studies that may assist their advanced design, in order to deliver either biocompatible and/or biodegradable materials, or novel biocides. A pioneering study was performed more than ten years ago by Davis and coworkers, where for the first time imidazolium ionic liquids based on a biologically active molecule, namely miconazole ($C_{18}H_{14}Cl_4N_2O$) (Fig. 1a) were synthesised and characterised.³⁵ Some years later, Pernak and coworkers developed new cationic surfactants: 1-alkoxymethyl-(3-nicotinylaminomethyl)benzimidazolium chlorides (m.p. 110 to 155 °C) (Fig. 1b), and observed that their antimicrobial properties, defined by minimal inhibitory and bactericidal concentrations

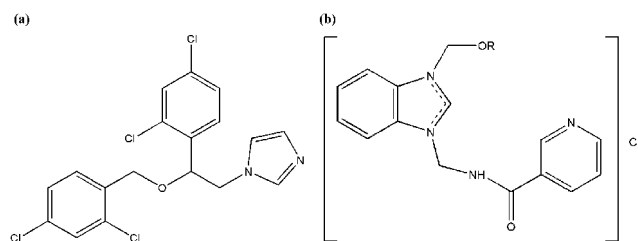


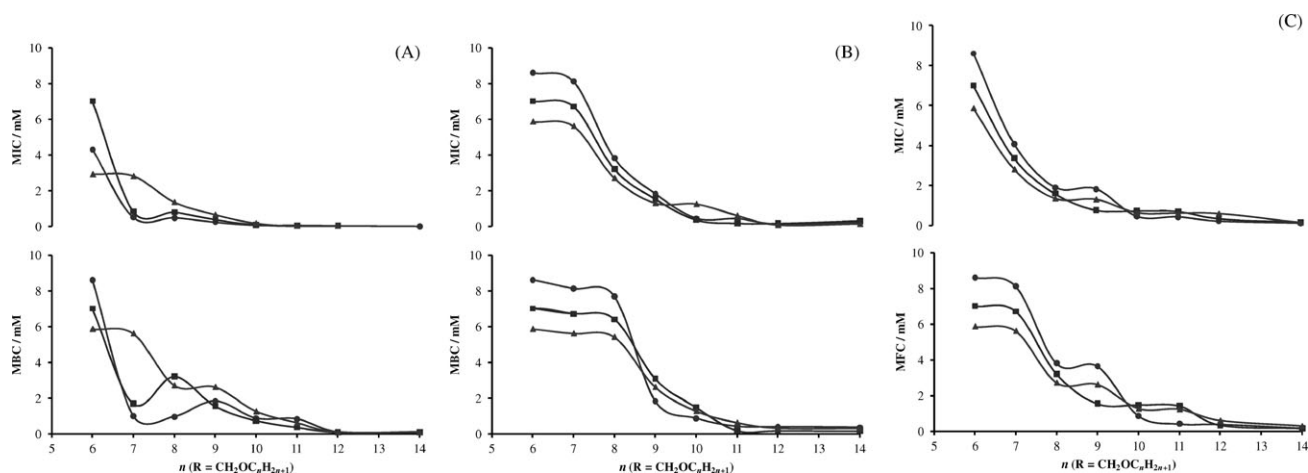
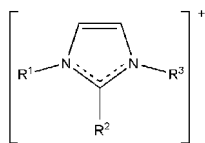
Fig. 1 (a) The chemical structure of miconazole ($C_{18}H_{14}Cl_4N_2O$), a biologically active molecule used as a base for the synthesis of imidazolium ionic liquids by Davis *et al.*,³⁵ (b) the chemical structure of 1-alkoxymethyl-(3-nicotinylaminomethyl)benzimidazolium chlorides, cationic surfactants developed by Pernak and coworkers.³⁶

(MIC and MBC, distinguishing between growth inhibition and death, respectively) against microbial strains relevant for human health, increased with the length of the alkoxy chain (between 2 to 12 carbon atoms: 1.4 mM > MIC > 0.034 mM, respectively).³⁶ These data constituted one of the first systematic studies on the ecotoxicity of ionic liquids. The same methodology was subsequently applied to investigate several novel imidazolium ionic liquids, comprising compounds with slight modifications in the substituted chain of the cation and different anions (Table 1), namely 1-alkyl-3-methylimidazolium chlorides and bromides; 1-alkyl-3-hydroxyethyl-2-methylimidazolium chlorides;³⁷ 1-alkoxymethyl-3-methylimidazolium chlorides, tetrafluoroborates ($[BF_4]^-$) and hexafluorophosphates ($[PF_6]^-$);³⁸ 1,3-dialkoxymethylimidazolium chlorides;³⁹ and 1-alkyl- and 1-alkoxymethylimidazolium lactates (DL and L).⁴⁰ The length of the alkyl or the alkoxy side chains varied between one and sixteen carbon atoms, as depicted in Table 1. These studies demonstrated high data consistency and a clear trend towards a stronger toxic effect with the increase in length of the side chain. This effect was constrained, however, since further elongation of the side chains, in position R^1 (>10–12)³⁸ or symmetrical chains in positions R^1 and R^3 (>7–9)³⁹ resulted in lower antimicrobial activities, probably due to steric effects which may limit interaction with the cell surface. In some cases, the growth media have shown eye-gauged turbidity, suggesting that the ionic liquid solubility limit was reached.³⁸

Overall, it becomes apparent that the effect of the tested anions was secondary to the effect of the cations on the observed toxicities (this was most evident for the less toxic cations), yet their broad diversity (often chemically unrelated) does not yet allow a conclusive analysis of their effect. In Fig. 2, the MIC values of several 1-alkoxymethyl-3-methylimidazolium cations combined with Cl^- , $[BF_4]^-$ and $[PF_6]^-$ for three different microbial species are presented.³⁸ The L-lactate salts were more toxic than DL-lactate salts,⁴⁰ thus agreeing with previous observations.⁴¹ These data have inspired some innovative applications, *viz.* 1-alkoxymethyl-3-methylimidazolium tetrafluoroborate, as a formalin substitute in embalming and tissue preservation,⁴² and as a wood preservative.⁴³ It was underlined that toxicity is correlated with the lipophilicity of the cation, suggesting that interaction with the surface of the microbial cells plays a major role. This assumption was validated by the observation that Gram-positive bacterial strains (*e.g.* *Staphylococcus aureus*) were more susceptible than Gram-negative strains (*e.g.* *Escherichia coli*). The classification of Gram-positive and Gram-negative bacteria is based on the chemical and physical properties of their cell walls (defined by an empirical staining method). Gram-positive bacteria have thicker and more hydrophobic cell walls,⁴⁴ and a much higher peptidoglycan content (~90%); the cell walls of Gram-negative bacteria are chemically more complex and have an additional outer membrane mostly composed of lipopolysaccharides.⁴⁵ The latter is often associated with the higher resistance of Gram-negative bacteria to biocides.⁴⁶ Moreover, the methicillin-resistant *S. aureus* (MRSA) strains possess MICs similar to Gram-negative bacteria,³⁸ probably due to their generally thickened cell walls and chemically altered

Table 1 Structural formulations of imidazolium ionic liquids screened in the antimicrobial activity tests

R_1	R_2	R_3	Anion	Reference
C_nH_{2n+1} , $n = 8, 10, 12, 14, 16$	H	CH_3	Br^- or Cl^-	37
C_nH_{2n+1} , $n = 14, 16$	CH_3	C_2H_4OH	Cl^-	37
C_nH_{2n+1} , $n = 1-12$	H	H	DL- or L-lactate	40
$CH_2OC_nH_{2n+1}$, $n = 4-12$	H	H	DL- or L-lactate	40
$CH_2OC_nH_{2n+1}$, $n = 3-12, 14, 16$	H	CH_3	Cl^- , $[BF_4]^-$, or $[PF_6]^-$	38
$CH_2OC_nH_{2n+1}$, $n = 3-12$	H	$CH_2OC_nH_{2n+1}$, $R^3 = R^1$	Cl^-	39

**Fig. 2** The influence of the length of the alkoxy side chain and of the anion on the antimicrobial activity of 1-alkoxymethyl-3-methylimidazolium ionic liquids {where $R = CH_2OC_nH_{2n+1}$, $n = 6-12$, and Cl^- (●), $[BF_4]^-$ (■) and $[PF_6]^-$ (▲) are the anions} towards (A) *Staphylococcus aureus* (Gram-positive bacteria), (B) *Escherichia coli* (Gram-negative bacteria), and (C) *Candida albicans* (yeast). For each species, minimal inhibitory and bactericidal/fungicidal concentrations (MIC and MBC/MFC, respectively) were obtained from the study by Pernak *et al.*³⁸

peptidoglycan.⁴⁶ Docherty *et al.* noticed that while assessing the antimicrobial activity of $[C_n\text{mim}]\text{Br}$, which was correlated with the number of colony forming units (throughout 8 h, 2 h intervals), both the most and the least resistant strains were Gram-positive.⁴⁷ Based on this, the authors opposed the current opinion, suggesting that the structure of the bacterial cell wall was not determinant for the observed behaviour. The characteristics of the microbial strains could partially explain their atypical behaviour (*e.g.* antibiotic resistance); however, this information was not included, and their conclusions were probably confuted by the use of a single concentration and a shorter exposure time compared with those commonly used to determine MICs (*e.g.* 24 h).³⁸

As initially proposed by Gathergood *et al.*,⁴⁸ incorporation of an oxygen atom in the side chains of the imidazolium cation reduced significantly toxicity and enhanced primary biodegradability.⁴⁹ In addition, incorporation of a methyl group in the 1-position,^{37,49} or a 2-hydroxyethyl group in 3-position of the ring,³⁷ has not consistently affected toxicity. This emphasises the complexity of comparing independent studies. Furthermore, amongst the tested anions, Br^- , $[BF_4]^-$, $[PF_6]^-$, $[N(CN)_2]^-$, $[NTf_2]^-$ and $[C_8SO_4]^-$, the last two were contributing more to the overall toxicity, but

in most cases the inhibitory end points were not reached (MICs > 70 mM).⁴⁹

More recently, the Agar Diffusion Test using a model Gram-positive bacteria (*Clostridium butyricum*) was suggested as a high throughput screening method for the biocompatibility of several $[C_n\text{mim}]^+$ ionic liquids.⁵⁰ Briefly, a disc soaked with the testing substance is placed on a lawn of a microbial culture, and after incubation the diameter of the inhibition zone is measured. However, no correlation between the inhibition zone diameters and the EC_{50} values (effective concentration scale based on a 50% response) could be defined, suggesting that the former cannot be used quantitatively.⁵⁰ Agar Diffusion Tests assume that the chemical diffuses freely, and does not aggregate or interact with the solid nutrient medium.⁵¹ Bearing in mind the high viscosities and densities of many ionic liquids, these assumptions are somewhat questionable. While the selection of a single microbial species to determine endpoints can be extremely useful, generalisation and extrapolation of results should be strongly avoided. Generally, studies on biotransformations in ionic liquids are often based on a single species and the bioassay is adjusted to the purpose of the study. Though, from an ecotoxicological perspective, they lack a quantitative

significance, some might present valuable additional data, namely by defining lethal endpoints.^{52,53} Some of these studies focused on water-immiscible ionic liquids, yet (in our opinion) an optimal methodology for these is yet to be presented; the current approaches lead to contradictory observations, e.g. the cellular membrane of *E. coli* was, after exposure to [C₄mim][NTf₂] (20% v/v), undamaged^{13,54} or severely disrupted.¹²

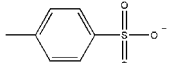
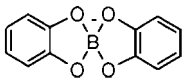
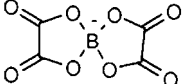
Microorganisms may form a biofilm where they are enclosed in a protective extracellular polymeric matrix, which usually confers higher resistance to antimicrobial agents. Though this is different from cell immobilisation, the latter was observed to increase *Saccharomyces cerevisiae* tolerance to [C_nmim][PF₆], yet it has not altered the toxicity mode of action, since the longest alkyl chains ($n = 4, 5, 6, \text{ or } 8$) were the most toxic.⁵⁵ Carson *et al.* evaluated for the first time the antimicrobial and antibiofilm activities of [C_nmim]Cl ($n = 6, 8, 10, 12, \text{ or } 14$) testing e.g. clinical MRSA strains and biofilm forming *S. epidermidis* strains.⁵⁶ The ionic liquids with alkyl chains of length 12 or 14 were proposed as surface biocides, reporting the lowest Minimum Biofilm Eradication Concentrations (MBEC), e.g. varying for [C₁₄mim]Cl from 124 to 1984 μM for *S. aureus* and *Proteus mirabilis* biofilms, respectively: longer or shorter chains were less effective. Their potential is undeniable, especially regarding the increasing number of multi-antibiotic-resistant clinical strains, yet some critical questions, facing either regulatory demands (e.g. environmental persistence) or practical aspects (e.g. antibiofilm activity against mixed communities) need to be addressed. Based on current knowledge, especially our recent discovery of biocompatible ionic liquids able to dissolve complex biopolymers,⁵⁷ their use in combination with antibiotics should be encouraged.

Though some of the aforementioned ecotoxicological studies on ionic liquids have already included some microorganisms belonging to the Fungi Kingdom (unicellular yeasts), filamentous fungi were studied for the first time by our group.⁵⁸ Fungi are ubiquitous in all environmental compartments and are critical soil colonisers, playing a major role in the biotic decay of pollutants, especially by virtue of their high diversity of species, broad enzymatic capacities (playing a central role in the carbon cycle), extensive hyphae (i.e. long branching filamentous cells, collectively called a mycelium) reach, and high surface-to-cell ratio.⁵⁹ *Ascomycota* fungal strains were able to tolerate very high concentrations of ionic liquids (0.05 M) with a range of cations: the imidazolium ones were the most toxic, followed by the groups of pyridinium, pyrrolidinium, and piperidinium ionic liquids; cholinium salts were the most benign. The anion effect was less significant and, as often reported, less predictable.⁵⁸ Molecular 1-methylimidazole leads to complete inhibition of growth in all the tested fungal isolates, thus exhibiting a more toxic effect than the imidazolium ionic liquids (e.g. [C₄mim]Cl inhibited only four of the ten tested fungal strains). This contradicts previous observations (two different studies: cell lines and *V. fischeri*) where the toxicity of the free base was lower than that of [C₄mim]Cl.^{47,60} The reasons for this discrepancy are not clear: deviations may arise from the use of distant model organisms, different cultivation media, testing concentrations (higher in fungi by one order of magnitude)

and alternate sources of 1-methylimidazole. One major breakthrough in the fungal study⁵⁸ was the suggestion of a high degree of correlation between the phylogenetic origin of the strains and their response to the ionic liquid environment, which may allow rationalisation of future toxicological assessments.⁵⁸ It also became obvious that sub-lethal concentrations of these ionic liquids have ubiquitously caused metabolic alterations (i.e. metabolomics) and that the [C₂mim]⁺ cation, whilst being toxic and non-biodegradable, was the most effective.

Following the recommendations of regulatory agencies (e.g. Organisation for Economic Co-operation and Development, OECD), the cytotoxicity of novel chemicals is commonly analysed by measuring enzyme activities which may be correlated with cell proliferation and viability (period of exposure 24, 48 or 72 h).⁶¹ Ranke *et al.* were the first to propose the use of rat cell lines, namely leukaemia IPC-81 and/or C6 glioma, to evaluate the cytotoxicity of ionic liquids.^{62,63} Cellular sorption (i.e. adsorption to the membrane surface and uptake into the cell) was reported to be dose-dependent and amongst the tested ionic liquids, [C_nmim][BF₄] ($n = 4, 6, \text{ or } 8$), the longest alkyl chain showed, as expected, the highest affinity and cytotoxicity.⁶³ The use of gradient centrifugation of the lysated cells proved unsuitable to monitor the ionic liquid distribution in the membrane, nuclei and cytoplasm. Generally, the cytotoxicity of [C₄mim]X was much higher than their corresponding Na⁺ or Li⁺ salts, indicating a major contribution from the cation.⁶⁴ However, as summarised in Table 2, the physical and chemical characteristics of the anion greatly influenced its intrinsic cytotoxicity, and very lipophilic and/or unstable anions (e.g. some fluorinated ones), were reported to play a major role in the cytotoxicity of the ionic liquids. The higher cytotoxicity of [CF₃SO₃]⁻ (i.e. [OTf]⁻), relative to [CH₃SO₃]⁻, emphasised the major role of the anion lipophilicity; the higher cytotoxicity of [SbF₆]⁻, relative to [BF₄]⁻ or [PF₆]⁻, could be due to its higher vulnerability to hydrolysis (i.e. low chemical stability), forming HF. This was partially substantiated in a later study where, after nine days, the hydrolysis rates of some ionic liquids containing [PF₆]⁻, [BF₄]⁻ and [SbF₆]⁻ anions were reported to be null, moderate and extremely high, respectively.⁶⁵ Despite the apparent high chemical stability of Na[PF₆], it was suggested that the formation of ion pairs might explain its higher cytotoxicity, two and ten times more than [C₄mim][PF₆] in IPC-81,⁶² and HeLa cells,⁶⁶ respectively. These observations clearly indicate that cytotoxicity of the ionic liquids may be influenced by side-reactions, strongly suggesting the need for integration of complementary chemical analyses. Stolte *et al.* demonstrated that the model of concentration addition, which assumes that single substances of a mixture display a similar mode of toxic action and at the same target sites, could reasonably estimate the EC₅₀ values of ionic liquids.⁶⁴ [C₄mim][NTf₂] constituted an exception, exhibiting three times higher cytotoxicity than estimated from the EC₅₀ values of the cations and the anions corresponding salts. This deviant behaviour was suggested to involve the formation of ion pairs in the aqueous media of cation and anion moiety,⁶⁴ but no direct observation for ion pairing in water exists. The significant contribution of [NTf₂]⁻ to the cytotoxicity of

Table 2 Influence of the anion on the cytotoxicity of [C₄mim]X (IPC-81 cell line). Values, given as EC₅₀, were adapted from the UFT/Merck database³² (except for the methylpoly(oxy-1,2-ethanediyl)sulfates)⁶⁴ and log₁₀(K_{ow}) of the anions were predicted using algorithms available on the ChemSpider website¹⁷⁶

Anion structure	Name	EC ₅₀ /μM	log ₁₀ (K _{ow})
Cl ⁻	Chloride	3850	0.00
Br ⁻	Bromide	2670	0.00
I ⁻	Iodide	3030	0.00
[Co(CO) ₄] ⁻	Tetracarbonylcobaltate(-1)	277	—
[SCN] ⁻	Thiocyanate	2610	0.58
[N(CN) ₂] ⁻	Dicyanamide	1420	-0.67
[HSO ₄] ⁻	Hydrogen sulfate	1940	-1.03
[C ₁ SO ₄] ⁻	Methylsulfate	1630	-0.595 ± 0.4
[C ₈ SO ₄] ⁻	Octylsulfate	1680	3.27
[H ₃ CO(CH ₂) ₂ O(CH ₂) ₂ OSO ₃] ⁻	2-(2-Methoxyethoxy)ethylsulfate	1440	-0.80
[H ₃ C(OCH ₂ CH ₂) _n OSO ₃] ⁻	Methylpoly(oxy-1,2-ethanediyl)sulfate	1100	—
	4-Methylbenzenesulfonate	1950	0.93
[CH ₃ SO ₃] ⁻	Methanesulfonate	3250	-1.89
[OTf] ⁻ (i.e. [CF ₃ SO ₃] ⁻)	Trifluoromethanesulfonate	1050	-0.37
[BF ₄] ⁻	Tetrafluoroborate	1030	—
[PF ₆] ⁻	Hexafluorophosphate	1250	—
[SbF ₆] ⁻	Hexafluoroantimonate	180	—
[N(CF ₃) ₂] ⁻	Bis(trifluoromethyl)amide	154	3.37
[NTf ₂] ⁻ (i.e. [N(SO ₂ CF ₃) ₂] ⁻)	Bis{(trifluoromethyl)sulfonyl}amide	481	1.49
[(C ₂ F ₅) ₃ PF ₃] ⁻	Tris(pentafluoroethyl)trifluorophosphate	23.7	—
	Bis[1,2-benzenediolato(2-)]borate	10 ([C ₂ mim] ⁺)	—
	Bis[oxalato(2-)]borate	860 ([C ₂ mim] ⁺)	—

several imidazolium ionic liquids was reinforced in other studies, *e.g.* in IPC-81 cells⁶⁷ and MCF7 human breast cancer cells.⁶⁸ In the latter study, the authors have used saturated solutions of the hydrophobic [C_nmim][NTf₂] (*n* = 3 or 6), thus avoiding the addition to the aqueous media of an organic solvent, *e.g.* dimethyl sulfoxide,⁶² that despite being used below its toxicity threshold may lead to slight over-estimation of cytotoxicity.⁶⁸ The introduction of a terminal hydroxyl or nitrile group, or ether functions in the substituted chain of the imidazolium cation, decreased the ionic liquid cytotoxicity, independently of the anion ([NTf₂]⁻ or halide).⁶⁷ The effect of ether functions has been previously reported in bacteria,⁴⁸ yet Stolte *et al.* demonstrated that its effect was strongly dependent on its position in the side chain.⁶⁷

Despite the fact that generally elongation of the alkyl chain in the imidazolium ring leads to a regular increase of cytotoxicity, some exceptions have been reported, *e.g.* while increasing the length of the alkyl chain in position R¹ or R³.⁶⁶ This cannot be attributed to the cell line (HeLa tumour cells), since in a later study with the same cells the effect of the substituted alkyl, allyl and benzyl chains exhibited the expected trends of cytotoxicity, with [NTf₂]⁻ as the most toxic anion.⁶⁹ Ranke and co-authors collated their systematic data on ionic liquids cytotoxicity (IPC-81 cells)³³ which, when taken with a complementary study,⁷⁰ established that the cytotoxicity of the halides is strongly correlated with the lipophilicity of the imidazolium cation, a further characteristic

of a mode of toxicity which involves disruption of the cell membrane. In addition, [C₂mim][BF₄] was observed to increase the production of reactive oxygen species and the intracellular calcium concentration, while reducing the mitochondrial membrane potential, suggesting that its cytotoxicity involves membrane damage, correlated to cellular apoptosis (*i.e.* programmed cell death) and, to a less extent, necrosis (*i.e.* cell death due to injury or trauma).⁶⁹ The high consistency of these data is further highlighted by comparing the EC₅₀ values after 48 h reported by Garcia-Lorenzo *et al.*⁷¹ and Ranke *et al.*,⁷⁰ even though different cell lines were used (CaCo-2 and IPC-81, respectively). The most different source of data (human colon carcinoma cell lines, HT-29 and CaCo-2)^{72,73} exhibited much higher EC₅₀ values, most probably due to the very short exposure time imposed to the cell lines (4 h instead of the commonly used 24–72 h). Despite that, the typical toxicity trends were generally maintained, underlining the significant role of the exposure time in the assessment of dose response relationships.

In some of the aforementioned studies, several ionic liquids were observed to induce a sub-lethal stimulus for short exposure times,^{66,67,74} a phenomenon (known as the hormetic effect), that is well described for toxic compounds,⁷⁵ though, up to now, its rationale remains unclear. In the studies by Frade *et al.*, some ionic liquids, *e.g.* 1-benzyl-3-methylimidazolium bis{(trifluoromethyl)sulfonyl}amide, have, at the selected temporal end point (4 h), significantly increased

CaCo-2 cell viability (>40%) and were classified as “unsafe”.⁷³ The ranges of doses tested (~0.5 to 17 mM) have only covered the stimulatory effect of the ‘U’-shaped curves, though it is likely that this effect was hormesis: solely based on these data, the classification must be considered speculative.

Recently, some imidazolium ionic liquids were suggested to display a potential as future anticancer drugs, since they caused high cytostatic (*i.e.* inhibition of cell proliferation) and low cytotoxic (*i.e.* cell death) effects in several tumour cell lines, including MCF7 and HT-29.⁷⁶ These values were very heterogeneous, reinforcing the need of further standardisation while evaluating the cytotoxicity of ionic liquids.

It is unquestionable that, due to their general properties, the vast majority of ionic liquids do not present any risk of atmospheric contamination; however, considering their industrial exploitation,⁶ they may present ecotoxicological risks to both aquatic and soil environments, *e.g.* due to accidental discharges. Consequently, it becomes critical to screen the ecotoxicity of ionic liquids on selected aquatic or terrestrial model organisms. One fast screening method, commonly used, is the standard ecotoxicological bioassay (ISO 11348),⁷⁷ which correlates the reduction of luminescence in cultures of *Vibrio fischeri* (Gram-negative bacteria) with cellular toxicity defining *e.g.* EC₅₀ values. The EC₅₀ data for [C_{*n*}mim]⁺ (*n* = 3 to 10) ionic liquids (exposure time of 30 min) reinforced that their toxicity, higher than that of selected conventional organic solvents (*e.g.* methanol or propanone), was controlled by the cation lipophilicity (*i.e.* alkyl chain length), yet incorporating a minor contribution from the anion (Cl⁻, Br⁻, [BF₄]⁻ and [PF₆]⁻).⁶² The indisputable correlation between the toxicity of [C_{*n*}mim]⁺ ionic liquids and the alkyl chain length was reinforced afterwards in a study that also highlighted the consistency of the *V. fischeri* bioassay,⁷⁸ as judged by comparison of the EC₅₀ values reported in different studies, even despite the use of divergent exposure periods.^{47,62,79} *Daphnia magna*, an aquatic crustacean, is also commonly used in ecotoxicity tests (OECD chronic assays) and recognised as an adequate model organism because of its rapid reproduction rate and sensitivity to changing environmental conditions.⁸⁰ The crustacean’s high susceptibility to [C₄mim]⁺ ionic liquids was demonstrated by a dose-response analysis (lethal effect, LC₅₀), with the cation and the anions playing either a major (*i.e.* the corresponding simple salts of the anions showed lower toxicities) or a secondary role in toxicity, respectively.⁸¹ Amongst the tested anions, namely Cl⁻, [BF₄]⁻, Br⁻ and [PF₆]⁻, the last two were the most and the least toxic, respectively, and at sub-lethal concentrations,

[C₄mim]⁺ combined with either Br⁻ or [BF₄]⁻ caused the most dramatic chronic effects, decreasing the number of first-brood neonates, total number of neonates, and average brood size.⁸⁰ A comparison between the acute effects of some imidazolium ionic liquids on *D. magna* (LC₅₀ after 24 or 48 h) and *V. fischeri* (EC₅₀ after 30 or 15 min) reinforced the higher susceptibility of the crustacean, by at least one order of magnitude, independent of the anions (Cl⁻, Br⁻, [BF₄]⁻, [PF₆]⁻, or [N(CN)₂]⁻).^{79,82} In addition, in both organisms, the alkyl chain length in the cation played a deciding role in toxicity, and the halides were the least toxic anions towards *V. fischeri*, yet in *D. magna*, the bromide was, as reported before,⁸¹ the most toxic. The EC₅₀ values of different studies were in good agreement; however, in the *V. fischeri* bioassay, different exposure periods lead to profound discrepancies, as noticed by comparison of EC₅₀ values after 15,⁸² and 30 min.^{62,79} Interestingly, in *D. magna*, the activity of key antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and S-transferase) increased at ionic liquid concentrations close to the EC₅₀ values, suggesting that oxidative stress plays an important role in their toxicity mechanism.⁸³

Still in the context of ecotoxicological risks of ionic liquids in aquatic environments, algae appear as a large and diverse group of eukaryotic aquatic photosynthetic organisms classified in different phyla (groups), *e.g.* green algae and diatoms. The blue-green algae have been misplaced, but based on their characteristics, they have been re-grouped with other prokaryotes in the kingdom Monera, and renamed cyanobacteria.⁸⁴ These groups display very particular characteristics, some of which will be discussed in correlation with their susceptibilities to ionic liquids. The acute effect of several imidazolium ionic liquids to green algae has been systematically investigated, focusing on the effect of the side chain length and substitution, and testing several anions, namely on *Oocystis submarina*,⁸⁵ *Pseudokirchneriella subcapitata*,^{65,74,86–88} *Scenedesmus vacuolatus*,⁸⁹ and *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii*.⁹⁰ A direct comparison between the EC₅₀ values of several imidazolium halides on *S. vacuolatus*,⁸⁹ *S. quadricauda*,⁹⁰ *P. subcapitata*⁷⁴ (formerly known as *S. capricornutum*) and *C. reinhardtii*⁹⁰ is depicted in Table 3. The high data heterogeneity was probably due to the use of dissimilar methods, *e.g.* measures of cell density by electrical conductance, fluorometry, or optical density. Amongst these variables, the exposure time played a major role in the dose-response behaviour of *P. subcapitata* towards ionic liquids, particularly evident in the less toxic ones, *e.g.* [C₄mim]Br (Table 3).⁷⁴ This partially justifies the significant difference

Table 3 Comparison of toxicological data of three independent studies (different methods and incubation times) using green algae. The inhibitory effect of [C_{*n*}mim]Br (*n* = 4, 6 and 8) is given as log₁₀(EC₅₀) (EC₅₀ in μM)

Algae	<i>Selenastrum capricornutum</i> ^a			<i>Scenedesmus vacuolatus</i> ^b	<i>Scenedesmus quadricauda</i> ^c	<i>Chlamydomonas reinhardtii</i> ^b
Incubation time/h	48	72	96	24	96	96
log ₁₀ (EC ₅₀)						
[C ₄ mim]Br	3.46	3.36	3.02	2.25	1.34	3.69
[C ₆ mim]Br	2.57	2.54	2.46	0.08	-0.5	3.02
[C ₈ mim]Br	1.65	1.63	1.57	-2.67	-1.74	1.17

^a Values obtained from Cho *et al.*⁷⁴ ^b Values obtained from Stolte *et al.*⁸⁹ ^c Values obtained from Kulacki *et al.*⁹⁰

between the EC₅₀ values defined by dioxygen evolution and growth measurements, after short (2 h)⁸⁷ or long (24–96 h)^{74,89} exposure times, respectively. Nevertheless, the determining effect of the cation lipophilicity to the overall toxicity was, in all these studies, apparent, since the elongation of the side chain increased considerably the ionic liquids toxicities. The imidazolium ionic liquids, especially those carrying long side-chains, may interact and disrupt the biological membranes, as demonstrated by the increased membrane/water partition coefficients,⁸⁹ strongly corroborating that their mode of toxic action is baseline toxicity.^{91–93} In the very lipophilic compounds, e.g. alkyl chains carrying 14 or 16 carbons atoms, a cut-off effect has been reported, on algae and also on bacteria,^{38,86,89} which may be related to their slow uptake due to steric effects. In addition, decreasing the cation lipophilicity by introducing hydroxyl, ether, nitrile, or chlorine in the side chains,^{89,94} or by substitution of a methyl group with hydrogen in the 1-position of the ring,⁹⁴ has consistently reduced the ionic liquids toxicity to the green algae. By combining [C₄mim]⁺ with numerous anions (including some rarely studied), their individual contribution was systematically investigated, as depicted in Table 4.^{65,95} Due to the chemical diversity of the anions, their direct comparison was difficult, yet their contribution to the observed toxicity becomes more significant for the most hydrolytically unstable, namely some of the fluorinated species.^{65,95,96} The over-additive effect to toxicity of the stable anion [NTf₂]⁻ has been observed in other studies,⁶⁴ which may be related to its lipophilic (i.e. hydrophobic) nature as suggested by its ability to cause disruption of an artificial phospholipid bilayer.⁹⁷ The high toxicity of [NTf₂]⁻ and [PF₆]⁻, comparing to Cl⁻, against other green algae, namely *S. vacuolatus*⁸⁹ and *S. capricornutum*,⁸⁶ respectively, has been also reported.

As reported before in cell lines,⁶² low doses of some ionic liquids were observed to cause a stimulatory effect on algae growth (the hormetic effect).^{74,89,90} Kulacki and coworkers suggested that the cell wall structure of *S. quadricauda* and *C. reinhardtii*, mostly composed of cellulose and glycoprotein, respectively, could possibly explain the higher resistance of the latter (EC₅₀ values higher by several orders of magnitude) to [C_nmim]Br (*n* = 4, 6 or 8) (Table 3).⁹⁰ This hypothesis was not supported while comparing wild-type (having a cell wall) and mutant (lacking a cell wall) strains of *C. reinhardtii*, which have demonstrated similar susceptibility to [C_nmim]Br (*n* = 4 or 8) and [C₄py]Br. Even so, the significant role of the cell wall in

the algae susceptibility to tetraalkylammonium ionic liquids was also evident, with the mutant strain presenting values of EC₅₀ which are lower by two orders of magnitude.⁹⁸ The cell wall composition might also explain the higher resistance of diatoms (one of the most common types of phytoplankton), relative to green algae, e.g. *Cyclotella meneghiniana*,^{85,96} *Bacillaria paxillifer*⁹⁹ and *Skeletonema marinoi*,⁹⁶ thought to be related to their silica coating,¹⁰⁰ and their more negatively charged cell walls.

The media composition was found to strongly influence the cultures' susceptibility to some imidazolium halides, e.g. lower toxicities were observed at high nutrient availability⁹⁰ or media salinity,^{85,101} reinforcing the importance of testing under realistic environmental conditions. The high density of chloride anions in the high saline regime, may have favoured ion pairing or complexation of the imidazolium cations, yet this remains poorly investigated.

Additionally, ecotoxicity tests on monocotyledonous and dicotyledonous plants are often recommended in regulation of hazard assessments, such as lesser duckweed (*Lemna minor*) and wheat (*Triticum aestivum*), and cress (*Lepidium sativum*), respectively. Addition of [C_nmim][BF₄] (*n* = 4 or 8)^{60,102} or [C_nmim]Cl (*n* = 2, 4 or 8)¹⁰³ affected the growth of these plants, and as expected, the imidazolium cations with the longest alkyl chains displayed the highest toxicities (doses tested varied between 10–100 mg l⁻¹ and 10–1000 mg kg⁻¹ in aqueous and soil media, respectively). Apparently [C₂mim]Cl was similarly¹⁰² or more toxic¹⁰³ than [C₄mim]Cl, thought to be related to its weaker sorption to soils. Addition of organic matter or smectite (with high cation exchange capacity) reduced the bioavailability of the ionic liquids, since lower toxicities (and in some cases even hormesis) were observed.¹⁰² Amongst the tested anions, [NTf₂]⁻ was shown to be the most toxic to wheat, independent of the soil composition.¹⁰⁴ Exposure of wheat seedlings to increasing concentrations of [C₄mim][BF₄] caused major alterations in several growth parameters, perceived by an increased soluble proteins content and peroxidase activities, and a decrease of amylase activities in seeds and chlorophyll content (also reported in cress¹⁰³).¹⁰⁵

Some studies have ascertained that different organisms exhibit fairly diverse susceptibilities to ionic liquids, yet the high consistency of the toxicity trends defined suggest a similar

Table 4 Comparison of the anion influence on the overall toxicity of [C₄mim]X toward four different species of green algae. All values are given as EC₅₀ (in μM)

X ⁻	EC ₅₀ /μM			
	<i>Scenedesmus vacuolatus</i> ^a (72 h)	<i>Selenastrum capricornutum</i> ^b (96 h)	<i>Chlorella vulgaris</i> ^c (72 h)	<i>Oocystis submarina</i> ^c (72 h)
Cl ⁻	140	2884	1026	2224
Br ⁻	—	2137	—	—
[BF ₄] ⁻	130	2512	425	708
[PF ₆] ⁻	—	1318	—	—
[SbF ₆] ⁻	—	135	—	—
[OTf] ⁻ (i.e. [CF ₃ SO ₃] ⁻)	—	2188	1417	1690
[N(CF ₃) ₂] ⁻	840	—	—	—
[NTf ₂] ⁻ (i.e. [N(SO ₂ CF ₃) ₂] ⁻)	50	—	—	—
[C ₈ SO ₄] ⁻	60	2239	—	—

^a Values obtained from Matzke *et al.*⁹⁵ ^b Values obtained from Cho *et al.*⁶⁵ ^c Values obtained from Latała *et al.*⁹⁶

mode of toxic action. Stolte *et al.* demonstrated a very consistent response of *V. fischeri* (bacteria) and *S. vacuolatus* (green algae), both for the cation (following several structural alterations, alkyl side chain length, and substitution) and the anion, even though the former is generally more resistant. The water plant *L. minor* was more susceptible to [C₄mim]Cl than *V. fischeri*; however, [C₄mim][NTf₂], which was significantly more toxic to the luminescent bacteria and green algae, at the doses tested have stimulated plant growth (hormetic effect).⁸⁹ *Daphnia magna* (crustacean) was reported to be more susceptible to the tested ionic liquids than *P. subcapitata* (green algae),⁸⁶ and both were much more susceptible than *Danio rerio* (fish).⁹⁴ Matzke *et al.* reported a rather thorough study on the toxicity of some imidazolium ionic liquids, focusing on very different trophic levels (*i.e.* position that an organism occupies on the food chain), including marine bacteria, green algae, plants, and a soil invertebrate.⁹⁵ The contribution of the cation to the overall toxicity was consistent in all organisms, with longer alkyl side chains exhibiting higher toxicities, while the anion effect varied in different species. Nevertheless, an overview of the anion effects suggest that Cl⁻ and [BF₄]⁻ contributed in a similar way to the overall toxicity; [C₈SO₄]⁻ was more toxic to marine bacterium and algae, and the fluorine-based anions: [NTf₂]⁻ (*i.e.* [N(SO₂CF₃)₂]⁻) and [N(CF₃)₂]⁻ led, despite the differences in the organisms, to higher toxicities. The significantly higher toxicity of [C₁₄mim][NTf₂] compared with both [C₁₄mim]Cl and Li[NTf₂] (for an inferred mixture toxicity),⁹⁵ imply that the toxicity mode of some ionic liquids cannot be solely explained by the model of concentration addition (as previously observed for [C₄mim][NTf₂]),⁶⁴ and may reinforce that ion-pairing and/or clustering, or some other synergistic effect, may be playing a major role.

Though not very frequently, other organisms have also been used to better understand the toxicity of ionic liquids, yet, despite the fact their insight relies predominantly on the type of bioassay used, a rapid helicopter view is proposed in this review. The dose-response curves of several [C_{*n*}mim]⁺ (*n* = 4, 8 or 14) ionic liquids on *Caenorhabditis elegans* (nematode),¹⁰⁶ on *Physa acuta* (freshwater snail),¹⁰⁷ and on *Dreissena polymorpha* (zebra mussel)¹⁰⁸ reinforced the determining role of the alkyl chain length in toxicity. The snail feeding rates were significantly reduced when exposed to sub-lethal concentrations of the ionic liquids, *e.g.* close to the LC₅₀ for [C₄mim]Br and [C₆mim]Br (1 and 0.22 mM, respectively).¹⁰⁷ This should emphasise that though mortality data are valuable, they project a one-sided aspect that often underestimates the severe effects caused by sub-lethal concentrations of the toxic, *e.g.* as those observed for fungi,⁵⁸ further highlighting the importance of measuring different endpoints in toxicity assessment.

Exposure of rats and/or mice to [C₄mim]Cl led to slight irritation of the skin and the eye, and an acute oral LD₅₀ of 550 mg kg⁻¹ of body weight.¹⁰⁹ Moreover, after intravenous or oral administration of sub-lethal concentrations, the ionic liquid was readily absorbed and ultimately eliminated in urine, and hence no direct evidence supports its metabolism.¹¹⁰ In addition, under a sub-lethal dose (225 mg kg⁻¹ d⁻¹), [C₄mim]Cl and [C₁₀mim]Cl have apparently caused, in mice,

loss of maternal weight and mortality, and despite an unaltered number of viable foetuses, their average weight was lower and the number of total malformations increased (although not statistically significant, it *might* suggest a teratogenic potential).^{111,112} Interestingly, decreasing the length of the alkyl side chain, from 4 to 2 carbon atoms reduced substantially the toxicity (foetal weight was conserved).¹¹² In addition, the developmental toxicity of some imidazolium ionic liquids depends on the concentration (dose) and is stage-sensitive, *e.g.* [C₈mim]Br exhibits increased embryonic mortality and caused morphological malformations in the frog *Rana nigromaculata*¹¹³ and in the goldfish *Carassius auratus*.¹¹⁴

Toxic compounds can have a deleterious action on the genetic material of the cells, affecting their integrity (genotoxicity) and increasing the frequency of mutation above the natural background level (mutagenicity). No genotoxicity (Sister Chromatid Exchange test) was detected at [C₄mim][BF₄] doses up to 20 μM, while [C₁₀mim][BF₄] showed a dose dependent trend at a much lower dose (10 μM).⁶⁰ In addition [C_{*n*}mim]Br (*n* = 4, 6 or 8) {and also [C_{*n*}mβpy]Br (*n* = 4, 6 or 8) and [N_{*n*}Br (*n* = 1, 2, 4 or 6)} cannot be classified as mutagenic (Ames Test evaluates the ability of defective mutants of *Salmonella typhimurium* to reverse back the mutation).¹¹⁵ Obviously any extrapolation of these initial results to higher organisms is questionable.

Importantly, the model of mixture toxicity (*i.e.* the individual toxics will exhibit similar or different modes of action, leading to a concentration addition or an independent effect, respectively) has again underestimated the toxic effect of different ionic liquid mixtures ([C₄mim][BF₄], [C₈mim][BF₄] and [C₁₄mim][NTf₂]) on green algae (*S. vacuolatus*) and wheat (*T. aestivum*), further suggesting that interactions between the compounds or the environment have occurred.¹¹⁶ The anion [NTf₂]⁻ may initiate the observed divergence, since, as previously reported,^{64,95} it significantly contributes to the ionic liquid toxicity. Interestingly, cadmium¹¹⁶ and pesticides¹¹⁷ acted either antagonistically or synergistically on the ionic liquid toxicity, respectively. These findings imply that to better understand ionic liquids ecotoxicological risks, complex scenarios of mixture toxicity and pre-pollution need to be accounted for.

Pyridinium-based ionic liquids

Ionic liquids containing a pyridinium head group constitute a rather important and well-studied group, as underlined recently by Madaan *et al.* in a review on their synthesis, accenting the limitless pool of structural variations, properties and applications, especially as surfactants and antimicrobial agents.¹¹⁸ Their usability as biocides was initially considered by Pernak *et al.* in studies focusing on the antimicrobial properties of some very uncommon and structurally interesting salts (m.p. ranging from 65 to 175 °C), as depicted in Fig. 3, namely 1-alkoxymethyl-3-carbamoylpyridinium, 1-alkoxymethyl-3-(1-benzimidazolmethylamino)pyridinium, 1-alkoxymethyl-3-[1-(benzotriazol-1-yl)methylamino]pyridinium and 1,3-bis[3-(1-alkoxymethyl)pyridinyl]-1,3-diazapropane dichloride.^{36,119} The latter group, with alkoxy chain lengths

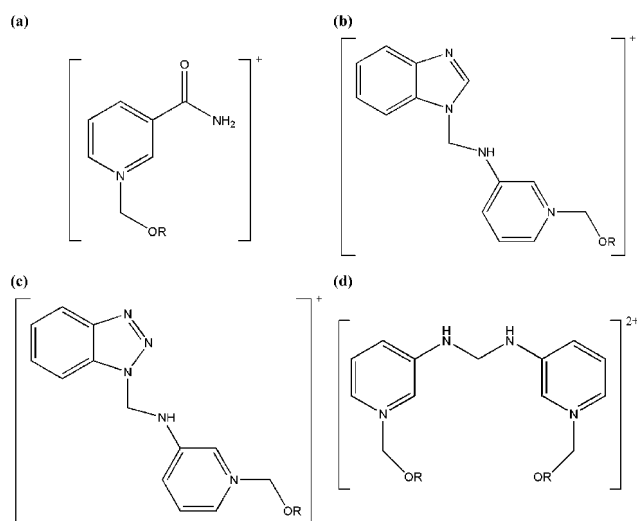


Fig. 3 Chemical structures of some interesting pyridinium-based cations investigated by Pernak *et al.*: (a) 1-alkoxymethyl-3-carbamoylpyridinium, (b) 1-alkoxymethyl-3-(1-benzimidazolmethylamino)pyridinium, (c) 1-alkoxymethyl-3-[1-(benzotriazol-1-yl)methylamino]pyridinium and (d) 1,3-bis[3-(1-alkoxymethyl)pyridinyl]-1,3-diazapropane dichloride.^{36,119}

of 9 to 12 carbon atoms and a melting point ranging from 68–73 °C, showed high biocidal potential and was generally more efficient against Gram-positive bacteria than Gram-negative.³⁶ As was found for the imidazolium ionic liquids, the length of the alkoxyethyl substituent in the pyridinium cation played a major role in toxicity. In a subsequent study, these authors investigated 1-alkoxymethyl-3-hydroxypyridinium and 1-alkoxymethyl-3-dimethylaminopyridinium chlorides (Table 5), which reported null/weak and strong biocidal effects, respectively.¹²⁰ In both groups, the alkoxy chain lengths (3 to 18 carbon atoms) played a major role, following the typical trend of increased toxicity with higher lipophilicity, yet in the latter group this effect was not unlimited and a threshold was detected above fourteen carbon atoms. Incorporating different substituents in position R³ in the pyridine ring (1-dodecyloxyethyl-3-R-pyridinium chloride), strongly influenced the toxicity, *e.g.* hydroxyl and bromide lead to the lowest and highest antimicrobial activity, respectively.^{120,121} Interestingly, the size of the anion also played a role in toxicity;¹¹⁹ however no explanation was given.

Table 5 Structural variations of pyridinium salts, 1-alkoxymethyl-3-hydroxypyridinium and 1-alkoxymethyl-3-(dimethylamino)pyridinium chlorides screened in antimicrobial activity tests by Pernak *et al.*¹²⁰

	Cl ⁻
$R^1 = C_nH_{2n+1}$	R^2
$n = 3-12, 14, 16, \text{ or } 18$	OH
$n = 3-12, 14, 16, \text{ or } 18$	$N(CH_3)_2$

The ecotoxicity of [C_nm₆py]Br has been analysed using several organisms, namely the luminescent bacteria *V. fischeri*,⁴⁷ the freshwater snail *P. acuta*,¹⁰⁷ the zebra mussel *Dreissena polymorpha*,¹⁰⁸ and the green algae *P. subcapitata*,⁸⁷ focusing on the effect of the alkyl side chain on the pyridinium cation ($n = 4, 6$ or 8). As for the imidazolium-based salts, toxicity was observed to increase with the length of the chain, and a correlation between toxicity and log₁₀(K_{ow}) (where K_{ow} is the 1-octanol/water partition coefficient) of the cation was proposed.⁴⁷ Docherty *et al.* have noted that methylation of the pyridinium ring (R³/R⁵) increased toxicity, possibly due to the more hydrophobic character of the cation.⁴⁷ The toxicity of methylpyridine was slightly higher than [C₄m₆py]Br, suggesting that even though the alkyl chain is contributing to toxicity, the ionic liquid may have a *greener* character than its synthetic precursors.⁴⁷

The cytotoxicity of pyridinium ionic liquids to several cell lines was analysed mostly focusing on the effect of alkyl⁶⁹ or alkoxyethyl¹²² side chains, or methylation⁶⁸ of the pyridinium ring, and, though less significantly, most of these studies have also covered the anion effect. As expected, increasing the length of the side chain, both alkyl and alkoxyethyl, increased toxicity, *e.g.* [C_npy]⁺ ($n = 4, 6, \text{ or } 8$) (HeLa)⁶⁹ and [C_nOC₁py]⁺ (1-alkoxymethylpyridinium cation) ($n = 3$ to 11) (IPC-81).¹²² The incorporation of methyl groups in the pyridinium ring alters their toxicity,^{121,122} but as previously observed in the imidazolium ionic liquids, the data do not allow a conclusive discussion. For example, Ranke *et al.* reported that an additional methyl group in positions (R² or R³) or R⁴ resulted in reduced and increased toxicity (IPC-81 cells), respectively,⁷⁰ whereas Kumar *et al.* observed that methylation in any position lead to a minor reduction of toxicity (MCF7 cells).⁶⁸ In agreement with previous observations, the effect of the anion becomes more pronounced when combined with less toxic cations, *e.g.* Br⁻ and [NTf₂]⁻, exhibited apparently the same effect when combined with [C₈m₆py]⁺,⁶⁸ but the latter was significantly more toxic with [C_npy]⁺ ($n = 2$ or 4).⁶⁹ Decisively, the selection of benign anions such as saccharinate or acesulfamate (chemical structures presented in Fig. 4) may constitute an advance towards a more conscious design of pyridinium ionic liquids.^{121,122} Their cytotoxicity was much lower than that of chlorides with the same alkoxyethyl chain length ($n = 3$), yet for longer chains ($n \geq 7$) their effect was less obvious. Interestingly, the anions were demonstrated to play a minor effect on the ionic liquid molecular toxicity (inhibition of acetylcholinesterase activity).¹²²

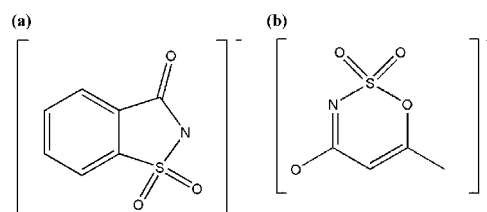


Fig. 4 Chemical structures of “benign” anions used in ionic liquid chemistry: (a) saccharinate (1,1-dioxo-1,2-benzothiazol-3-one) and (b) acesulfamate (6-methyl-2,2-dioxo-1,2,3-oxathiazin-4-olate).^{121,122}

Comparison of the toxicities displayed by the pyridinium and the imidazolium cations carrying same-length alkyl side chains does not allow a consistent conclusion, e.g. $[\text{C}_4\text{m}_\beta\text{py}]\text{Br}$ was, relative to $[\text{C}_4\text{mim}]\text{Br}$, observed to be more toxic against *V. fischeri*,⁴⁷ while less toxic to *P. acuta*.¹⁰⁷ There are numerous examples substantiating these discrepancies within several model organisms, e.g. in *C. vulgaris* and *O. submarina*,⁹⁶ *P. subcapitata*,^{87,123} and *D. polymorpha*.¹⁰⁸ $[\text{C}_4\text{py}]\text{Cl}$ was, relative to $[\text{C}_4\text{mim}]\text{Cl}$, less toxic (*D. magna*),⁸⁶ but exchanging Cl^- by $[\text{NTf}_2]^-$ ⁹⁴ inverted the toxicity ranking of the two groups. The anion has also been observed to influence toxicity even in the very resistant *Penicillium* strains (fungi), e.g. 50 mM of $[\text{C}_4\text{py}]\text{Cl}$, $[\text{C}_2\text{py}][\text{O}_2\text{CMe}]$ and $[\text{C}_2\text{py}][\text{lactate}]$ inhibited growth in 30%, 20% and 0% of the tested strains, respectively,⁵⁸ while in bacteria $[\text{C}_4\text{py}]^+$ combined with Cl^- , Br^- or $[\text{N}(\text{CN})_2]^-$ exhibited toxicities between 2.6 to 2.0 mM.⁴⁷ Couling *et al.* proposed a theoretical model for toxicity prediction which correlates several molecular descriptors with the EC_{50} values of several ionic liquids (*V. fischeri* and *D. magna* bioassays).⁸² Despite the fact that lower toxicity of pyridinium salts was proposed, the deviant behaviours observed experimentally exposed some of the model weaknesses, yet to be resolved. Nevertheless, it highlighted the importance of considering multiple molecular descriptors while predicting toxicity, such as the number of nitrogen or electronegative atoms in the cation.

The effect of the head group on the toxicity/cytotoxicity of ionic liquids has been well addressed by Stolte and coworkers, since comparison of seven different cationic cores against *V. fischeri* (bacteria), *L. minor* (plant) and *S. vacuolatus* (algae) clearly demonstrated the higher toxicity of the aromatic cations (containing imidazolium and pyridinium rings), relative to the alicyclic (containing morpholinium, piperidinium, or pyrrolidinium) and quaternary ammonium cations.⁸⁹ Their toxicity could be reasonably explained by the lipophilicity of the cations (measured by chromatography, $\log_{10}(K_{\text{ow}})$ values between 0.18 to 0.68). Irrespective of the head group, these ionic liquids (carrying a butyl side chain) showed relatively low cytotoxicity to IPC-81 cells, with the exception of 1-alkyl-4-(dimethylamino)pyridinium salts. The latter exhibited much higher cytotoxicity than that predicted by its lipophilicity, probably suggesting a specific mode of toxic action in this cells.⁶⁷

Quinolinium-based ionic liquids

Quinolinium ionic liquids are still rarely studied (Fig. 5); even so their cytotoxicity potential (IPC-81) and molecular toxicity (inhibition of acetylcholinesterase) was observed to be extremely high, and to increase with the elongation of the substituted alkyl chain.³³ For example $[\text{C}_n\text{quin}]^+$ was more toxic than $[\text{C}_n\text{mim}]^+$ ($n \geq 4$) by at least one order of

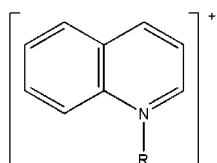


Fig. 5 The structure of the 1-alkylquinolinium cation.

magnitude. Therefore, it is not surprising that they exhibited lower MBEC values, demonstrating higher potential than $[\text{C}_n\text{mim}]\text{Cl}$,⁵⁶ to eradicate bacterial biofilms.¹²⁴

Toxicity of ionic liquids containing alicyclic head groups in the cation

Pyrrolidinium-, piperidinium- and morpholinium-based ionic liquids

The ecotoxicity of ionic liquids with nitrogen-containing alicyclic cations, namely pyrrolidinium, piperidinium and morpholinium (Fig. 6) is yet to be comprehensively investigated. Pretti and coworkers focused for the first time on their aquatic toxicity, testing, *inter alia*, several cations and anions, and reported that, for example, exposure to 0.24 μM of $[\text{C}_4\text{mpyr}][\text{NTf}_2]$ did not cause, after 96 h, lethal effects in the zebrafish (*D. rerio*).¹²⁵ More recently, their cytotoxicity in IPC-81⁶⁷ and MCF7⁶⁸ cell lines was investigated, focusing on the effect of the substituted chains on the cation, and of the anions. That extensive set of data can be found in the review by Ranke *et al.*,³³ and in the UFT/Merck Ionic Liquids Biological Effects Database.³² The data suggested that increasing the number of carbon atoms in the alicyclic ring generally increases toxicity, e.g. the piperidinium cation was more toxic than the pyrrolidinium cation (six and five member rings, respectively).⁶⁸ Moreover, these non-aromatic head groups were generally less toxic than their aromatic analogues.⁶⁷ This is highlighted by comparing the toxicities of ionic liquids with aromatic pyridinium and non-aromatic piperidinium rings, e.g. $[\text{C}_8\text{m}_\beta\text{py}]\text{Br}$ was 23-fold more toxic than $[\text{C}_8\text{mpip}]\text{Br}$, though the most toxic anions may lead to an exception, e.g. $[\text{C}_8\text{m}_\beta\text{py}][\text{NTf}_2]$ was slightly less toxic than $[\text{C}_8\text{mpip}][\text{NTf}_2]$.⁶⁸ As reported before, the length of the alkyl chains played a major role, as cytotoxicity increased with lipophilicity, e.g. $[\text{C}_n\text{mpyr}]^+$ ($n = 3, 4, 6$ or 8)^{68,70} and $[\text{C}_n\text{mpip}]^+$ ($n = 3, 4$ or 8).⁶⁸ Also as expected, incorporation of two hexyl chains instead of one on the pyrrolidinium ring increased cytotoxicity significantly,⁷⁰ and the incorporation of an oxygen atom in the alkyl side chain reduced toxicity.⁶⁷ The anion effect on the ionic liquids' cytotoxicity, even accounting for their lipophilicity and chemical stability, was unpredictable, as observed while ranking their contribution, e.g. in $[\text{C}_4\text{mpyr}]\text{X}$, $\text{Cl}^- < \text{Br}^- < [\text{NTf}_2]^- < [\text{BF}_4]^-$,³³ and in $[\text{C}_8\text{mpip}]\text{X}$, $[\text{N}(\text{CN})_2]^- < [\text{BF}_4]^- < [\text{OTf}]^- < \text{Br}^- < [\text{NTf}_2]^-$.⁶⁸

To date, the ecotoxicity of these alicyclic rings has seldom been studied; nevertheless, their lower toxicity, relative to the corresponding aromatic rings, was generally evident in the bioassays with *V. fischeri*, *S. vacuolatus* and *L. minor*.⁸⁹ Further evidence for this can be highlighted, e.g. $[\text{C}_4\text{mpyr}]^+$ combined with different anions ($[\text{N}(\text{CN})_2]^-$ and lactate)^{50,58} and $[\text{C}_4\text{mpip}][\text{O}_2\text{CMe}]$ ⁵⁸ were less toxic than the corresponding imidazolium ionic liquids. However, other organisms, namely the green algae *P. subcapitata*, showed quite similar susceptibilities with $[\text{C}_4\text{mpyr}]\text{Br}$ and $[\text{C}_4\text{mim}]\text{Br}$.¹²³ The morpholinium ionic liquids, due to the incorporation of an oxygen atom in the ring, were the least toxic, e.g. $[\text{C}_2\text{mmor}]\text{Br}$ and $[\text{C}_4\text{emor}]\text{Br}$ were apparently nontoxic to *P. subcapitata*

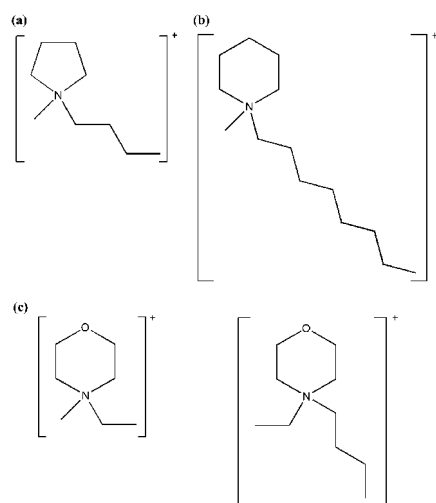


Fig. 6 Structures of common alicyclic cations used to form ionic liquids: (a) 1-butyl-1-methylpyrrolidinium ($[C_4\text{mpyr}]^+$), (b) 1-methyl-1-octylpiperidinium ($[C_8\text{mpip}]^+$), and (c) 4-ethyl-4-methylmorpholinium ($[C_2\text{mmor}]^+$) and 4-butyl-4-ethylmorpholinium ($[C_4\text{emor}]^+$).

and *D. magna*.⁹⁴ The significant effect of the $[\text{NTf}_2]^-$ anion was again observed, since substituting the halide in $[C_4\text{mmor}]\text{X}$ by $[\text{NTf}_2]^-$ increased, up to one hundred times, its toxicity against *V. fischeri* and *S. vacuolatus*.⁸⁹

Due to the apparent low toxicity of ionic liquids with nitrogen-containing alicyclic cations, there are no doubts that they will be in the heart of a more conscious design, and will certainly be harnessed more in the near future.

Quaternary ammonium ionic liquids

Quaternary ammonium salts (often referred to just as quats) are well known and widely used chemicals in numerous applications, as disinfectants, surfactants, antistatic agents, catalysts, *etc.*¹²⁶ Their properties depend on the chain length and functional groups, and on the anion.¹²⁷ Pernak and co-authors were the firsts to consider their antimicrobial activity, focusing on $[\text{N}_{11}(\text{CH}_2\text{OC}_n\text{H}_{2n+1})(\text{CH}_2\text{R})]\text{Cl}$ ($\text{R} = \text{CH}_2\text{OH}$, $\text{CH}_2\text{CH}_2\text{OH}$, $\text{CH}(\text{CH}_3)\text{OH}$, $\text{CH}_2\text{OC}(\text{O})\text{C}_9\text{H}_{19}$, or $\text{CH}_2\text{OC}(\text{O})\text{Ph}$)¹²⁸ and some very uncommon chiral ionic liquids, namely trialkyl[(1*R*,2*S*,5*R*)-(-)-menthoxyethyl]-ammonium chlorides,¹²⁹ as depicted in Table 6. Their antimicrobial activity was, as expected, governed by the length of the alkoxy and alkyl side chain (R^1), and was generally higher against Gram-positive bacteria, with *Pseudomonas aeruginosa* (Gram-negative) as the most resistant one (able to tolerate benzalkonium chloride (BAC) up to 23 μM).¹²⁸ Usually, the most toxic ionic liquids carried the longest alkoxyethyl chains, yet this effect was not monotonic and a threshold (as previously discussed for the imidazolium³⁸ and pyridinium¹²⁰

Table 6 Structural variations of substituted tetraalkylammonium ionic liquids screened in antimicrobial activity tests

Cation structure	R^1	R^2	R^3	Anion	References
	$\text{C}_n\text{H}_{2n+1}$, $n = 3-18$	HOCH_2 HOC_2H_4 $\text{HO}(\text{CH}_3)\text{CH}$ $\text{C}_9\text{H}_{19}\text{COOCH}_2$ PhCOOCH_2	—	Cl^-	128
	$\text{C}_n\text{H}_{2n+1}$, $n = 2-12$ CH_2Ph	CH_3 C_2H_5	CH_3 C_2H_5	Cl^-	129
	—	—	—	Cl^- $[\text{NO}_3]^-$ $[\text{NO}_2]^-$ $[\text{BF}_4]^-$ $[\text{NTf}_2]^-$	132
	—	—	—	DL- or L-lactate	133
	—	—	—	Acesulfamate (see Fig. 4) Saccharinate (see Fig. 4)	121
	mixture of $\text{R} = \text{C}_n\text{H}_{2n+1}$, $n = 8-18$ (mostly $n = 12$)	—	—	Cl^- $[\text{NO}_3]^-$ $[\text{NO}_2]^-$ $[\text{BF}_4]^-$	132
	—	—	—	DL- or L-lactate	133
	—	—	—	Acesulfamate Saccharinate	121

ionic liquids) was detected above 10, 14 or 16 carbon atoms, depending on the functional group. The alcohol derivatives $[N_{11}(\text{CH}_2\text{OC}_n\text{H}_{2n+1})(\text{CH}_2\text{R})]\text{Cl}$ ($\text{R} = \text{CH}_2\text{OH}$, $\text{CH}_2\text{CH}_2\text{OH}$, or $\text{CH}(\text{CH}_3)\text{OH}$) were slightly less toxic than those of the esters $[N_{11}(\text{CH}_2\text{OC}_n\text{H}_{2n+1})(\text{CH}_2\text{R})]\text{Cl}$ ($\text{R} = \text{CH}_2\text{OC}(\text{O})\text{C}_9\text{H}_{19}$, or $\text{CH}_2\text{OC}(\text{O})\text{Ph}$).¹²⁸ A high biocompatibility was observed for the $[N_{2,4}(\text{C}_2\text{OH})_2]^+$ {butylethyldi(hydroxyethyl)ammonium} and $[N_{1,1,2,4}]^+$ (butylethyldimethylammonium) cations, combined with $[\text{C}_2\text{SO}_4]^-$ and $[\text{N}(\text{CN})_2]^-$ anions, against *C. sporogenes*, either by determining EC_{50} or inhibition zone diameters.⁵⁰ The antimicrobial activity of several $[N_{4,4,4,4}]^+$ carboxylates was screened solely by using the Agar diffusion test, however (mostly due to the method weaknesses), the uneven data cannot be compared with other studies.¹³⁰ As previously reported for the pyridinium ionic liquids,^{121,122} the selection of the benign anion acesulfamate (Fig. 4b) resulted in potent antimicrobial activity, e.g. alkoxyethyl(2-hydroxyethyl)dimethylammonium acesulfamates carrying long alkoxyethyl chains (but not too long).¹³¹ The effect of other anions on the antimicrobial activity of several $[N_{1,1,10,10}]^+$ or benzalkonium (predominantly $[N_{1,1,12}(\text{CH}_2\text{C}_6\text{H}_5)]^+$, but also contains C_{14} and C_{16} homologues) salts, further highlighted their potential use as disinfectants and wood preservatives, especially of nitrites and nitrates,¹³² lactates (DL-lactate and L-lactate),¹³³ acesulfamates, and saccharinates.¹²¹ On the other hand, some of these ionic liquids should be handled carefully, since they may be regarded as harmful chemicals, displaying acute oral toxicity in rats (300 to 2000 mg kg⁻¹ of body weight)^{121,132} and a high potential for skin irritation.¹²¹

The toxicity of ammonium ionic liquids against *V. fischeri* (bacteria);⁸² *D. magna* (crustacean) and *P. subcapitata* (algae),^{86,94} *D. rerio* (fish),⁹⁴ and IPC-81 cells (leukæmia cells)⁷⁰ has been also considered. Previously described toxicity trends were generally demonstrated by the data, and hence the highest toxicity was observed for those carrying the longest alkyl chains, namely of $[N_{1,8,8,8}][\text{NTf}_2]$ and EcoEng 500 (Peg-5 cocomonium methosulfate; see Fig. 7),⁸⁶ and of AmmoEng 100 and 130 (Fig. 7).⁹⁴ The tetraalkylammonium ionic liquids have shown low potential as anti-tumour drugs, since longer alkyl chains lead simultaneously to higher cytostatic and cytotoxic activity towards several human tumour cell lines ($[N_{1,8,8,8}]^+$ cation), while shorter ones (up to 6 carbon atoms) were observed to be rather inactive.¹³⁴

The cytotoxicity of $[N_{1,1,2,4}]^+$ ionic liquids (IPC-81 cells) reinforced the lower toxicity of halides when compared to $[\text{NTf}_2]^-$.⁶⁷ Despite the lower cytotoxicity of $[N_{1,1,2,4}]\text{Cl}$ ^{33,67} and its “safeness” towards bacteria (*V. fischeri*) and algae (*S. vacuolatus*), it was highly toxic toward duckweed (*L. minor*).⁸⁹ In addition, the higher antimicrobial activity of $[N_{1,8,8,8}]\text{Cl}$ against bacteria, relative to $[N_{1,8,8,8}][\text{NTf}_2]$, was attributed to its faster absorption by the cells, which rapidly caused cell death and lysis.¹³⁵ The accumulation of the cation in the cells was detected after 3 h of exposure to $[N_{1,8,8,8}]\text{Cl}$ by Fourier transform infrared spectroscopy (FTIR); however, the analysis was strongly influenced by the degree of cell lysis, and the bioaccumulated and the bioadsorbed fractions were equally accounted for. In addition, the ability to disrupt liposomes (model for a cell membrane) and toxicity/cytotoxicity of some potassium and lithium

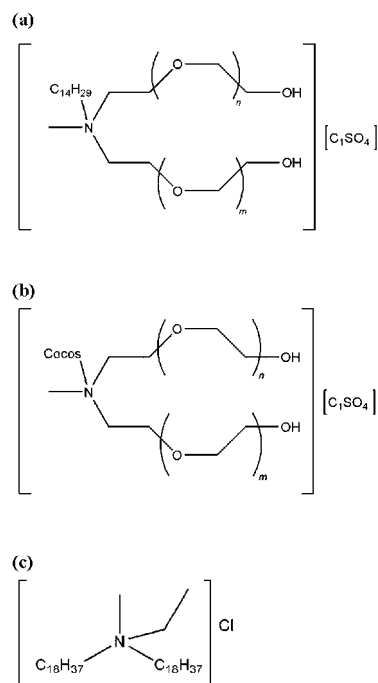


Fig. 7 Structures of (a) EcoEng 500 (Peg-5 cocomonium methosulfate), (b) AmmoEng 100 and (c) AmmoEng 130.

N,N,N-trialkylammoniododecaborates could be well correlated with the anion lipophilicity (alkyl chains carrying 1 to 6 carbon atoms).¹³⁶

In the search for ammonium ionic liquids of higher biocompatibility (and potential biodegradability), some of the most interesting groups are those containing the 2-hydroxyethyltrimethylammonium cation (henceforward described as cholinium). The selection of a benign cation, such as cholinium (cholinium chloride is known as choline, and is part of the vitamin B complex), combined with benign anions constituted a major breakthrough in the conscious design of ionic liquids.^{131,137} The low toxicity of several cholinium ionic liquids has been widely demonstrated, e.g. saccharinate and acesulfamate (*D. magna*)¹³⁸; dimethylphosphate (*C. sporogenes*)⁵²; lactates (DL-lactate) and alkanooates (*Penicillium sp.*)⁵⁸. The last of these were analysed by our group for the first time, and included a range of linear alkanooate anions ($[\text{C}_n\text{H}_{2n+1}\text{CO}_2]^-$, $n = 1$ to 9), as well as two structural isomers (for $n = 3$ or 4).⁵⁸ From the data, it became apparent that the toxicity increased with the chain length of the anion and that the branched isomers were of lower toxicity than the corresponding linear ones with the same number of carbon atoms. Additionally, the high biodegradability potential of cholinium alkanooates⁵⁸ and the tremendous solvent ability of some of them, either towards suberin⁵⁷ or stearic acid,¹³⁹ has been proven, reinforcing their major utility and interest.

The lower toxicity of quaternary ammonium ionic liquids was predicted by computational modelling of the toxicity of ionic liquids, using quantitative structure–activity relationships (QSAR) defined by the data retrieved in two bioassays (*V. fischeri* and *D. magna*), which have correlated low toxicity with the lack of a nitrogen-containing ring and, in the case of

cholinium, with the presence of the oxygen atom.⁸² This is supported by the extant data, e.g. in *P. acuta*¹⁰⁷ and in HeLa cells.⁶⁹

Quaternary phosphonium ionic liquids

The ecotoxicity of phosphonium ionic liquids, despite their high interest, is still seldom investigated. Some halides, e.g. $[P_{4444}]^+$ (tetrabutylphosphonium cation), exhibited similar levels of acute toxicity to the freshwater snail *P. acuta* as those of the imidazolium salts carrying the same chain length.¹⁰⁷ The antimicrobial properties of several $[P_{666n}]^+$ ($n = 2$ to 16, even numbers) halides (Fig. 8) demonstrated that their toxicity was very selective (e.g. MIC of $[P_{6666}]Cl$ was 2.5 μM in *E. coli* and 152 μM in *Candida albicans*).¹⁴⁰ The authors suggested that the structure of the cation plays a significant role in toxicity since the antimicrobial activity of the phosphonium chlorides was observed to decrease for the longest alkyl chains (carrying 8 to 14 carbon atoms). Interestingly, exchange of the halide by other anions, e.g. $[NTf_2]^-$, $[OTf]^-$, $[NO_3]^-$, $[N(CN)_2]^-$, $[BF_4]^-$, or $[PF_6]^-$, has resulted in a loss of antimicrobial activity and electrostatic properties,¹⁴⁰ thus rendering higher interest in these phosphonium ionic liquids. The important contribution of the anion has been demonstrated in other studies, e.g. $[P_{66614}]^+$ combined with chloride and $[NTf_2]^-$ led to high and low toxicity against *E. coli*.¹³⁵ Based on the ATR-FTIR analyses of the cells, the halide was suggested to be more rapidly biosorbed (i.e. adsorption onto the cell surface plus bioaccumulation); nevertheless, the data (as discussed above) were strongly influenced by the degree of cell lysis. Despite the fact that cross-contamination of the cytoplasmic and the membrane sub-cellular fractions may have occurred, $[P_{66614}][NTf_2]$ was found mainly in the cell membrane;¹³⁵ unfortunately, the authors have not also monitored $[P_{66614}]Cl$ distribution in the cells. As previously described in the imidazolium ionic liquids section, some water-immiscible phosphonium ionic liquids have generated high interest for developing novel biotransformation processes, e.g. xenobiotics-degradation.¹⁴¹

The apparently high toxicity of other phosphonium halides (namely $[P_{4444}]Br$, $[P_{66614}]Br$ and $[P_{66614}]Cl$) against *V. fischeri* and *D. magna*,^{82,86} and *P. subcapitata*,^{86,123} was also demonstrated. Interestingly, though substantially less effective than the halides, $[P_{2444}][(EtO)_2PO_2]$ was reported to be toxic to *V. fischeri* (0.07 mM),⁸² and *P. subcapitata* (0.016 mM).⁸⁶

In addition, their usually high cytotoxicity, despite the anions, was demonstrated (HeLa and IPC-81 cells) after analysing $[P_{44414}]^+$ and $[P_{66614}]^+$ combined with $[NTf_2]^-$ ⁶⁹ or $[BF_4]^-$,⁷⁰ and $[P_{4444}]Br$.⁷⁰ The lack of systematisation in these studies does not permit a conclusive rationalisation.

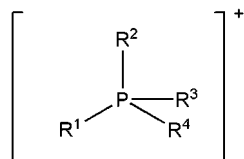


Fig. 8 Structure of quaternary phosphonium cation.

Nonetheless, $[P_{66614}]^+$ combined with different anions, namely $[PF_6]^-$ and $[NTf_2]^-$, displayed high and low cytotoxic activities against a broad diversity of human cancer cell lines, respectively.¹³⁴ The same cation combined with $[BF_4]^-$ exhibited very inconsistent behaviour, thought to be related to its low solubility in water (and therefore lower membrane permeability potential).

Biodegradability of ionic liquids

The biodegradability of ionic liquids plays an important role in evaluating their environmental impact. This subject has been recently discussed in an excellent review by Coleman and Gathergood;¹⁴² hence only selected key relevant facts will be discussed here. Most biodegradability assays are defined under static laboratory conditions and, despite their importance, are usually unrealistic, failing to reproduce the numerous abiotic and biotic processes occurring in the environment. The biodegradability potential of a chemical is often discussed accordingly to the OECD guidelines, meaning that its rapid and complete mineralisation indicates that the chemical is readily biodegradable, while its weak or extensive molecular cleavage suggests primary or ultimate biodegradability potential, respectively.

The conscious design of chemicals and the rules of thumb for delivering biodegradable chemicals, including ionic liquids, were excellently reviewed by Boethling *et al.*¹⁴³ Generalisations should be made cautiously, yet it is generally accepted that several molecular features strongly enhance biodegradability (not necessarily resulting in intermediate chemicals of lower toxicity), such as the presence of esters, amides, hydroxyl, aldehyde, carboxylic acid groups, or linear alkyl chains. Some examples can be found in the available literature on ionic liquids, e.g. the incorporation of an ester group in the side chain of several imidazolium^{79,144} or pyridinium¹⁴⁵ ionic liquids has significantly improved the primary biodegradability of the cation (i.e. weak molecular cleavage). Those carrying alkyl chains of four carbon atoms have been observed to be poorly biodegradable,^{78,79,146,147} even when unsaturated groups (allyl or vinyl) or a sulfonate group were incorporated.¹⁴⁸ Pham *et al.* proposed a degradation pathway for $[C_4m\beta py]Br$ through hydroxylation of the side chain, even though that the intermediate chemical product (3-methylpyridine) could not be conclusively identified.¹⁴⁹ Nevertheless, pyridinium ionic liquids have, relative to the corresponding imidazolium ones, higher biodegradability potential.¹⁵⁰ For example, $[C_n\text{mim}]Br$ and $[C_n\text{m}\beta py]Br$ ($n = 6$ or 8) were both degraded by an activated sludge microbial community, with the longer alkyl chains reaching, after over 40 days, partial and total mineralisation, respectively; however, after 25 days only $[C_8m\beta py]Br$ was shown to be fully mineralised (i.e. readily biodegradable).¹⁴⁷ More recently, the degradation pathway of $[C_n\text{m}\beta py]Br$ ($n = 4, 6$ or 8) was reported to involve unsaturation of the alkyl side chain and hydroxylation of the aromatic ring,¹⁵¹ thus opposing that previously suggested by Pham *et al.*¹⁴⁹ Taken together, these studies suggested that there are different possible degradation pathways that need to be considered, which are ultimately defined by the metabolic capacities of the

microbial community and stand for a “regional” impact of the data.

Additionally, some phosphonium ionic liquids, namely tricyclohexylphosphine- and trihexylphosphine-derived cations with various ester side chains, and combined with different anions, were observed to be very resistant to microbial attack.¹⁵² On the other hand, the cholinium cation was reported to be readily biodegradable,¹⁴³ and more recently, our group demonstrated the high biodegradability potential of several cholinium alkanooates.²²

The complexity in designing more reliable bioassays for determining the biodegradability potential of ionic liquids is partially due to critical knowledge gaps on their potential uses and disposal methodologies, their contamination levels and fate, and/or bioaccumulation factors in the environment. Ionic liquids sorption onto sediments and soil will have a major influence on their transport, reactivity (*e.g.* oxidation, hydrolysis and photolysis), bioavailability and ultimately, biodegradability. Even if still poorly investigated, there is an increasing interest in the behaviour of ionic liquids in soils; however, up to now, most studies have focused on the imidazolium family, further demonstrating their high persistence and ecotoxicological risk.¹⁵³ Their sorption and desorption in soil were observed to be ruled by ionic interactions;^{27,154} nonetheless, their lipophilicity, the length of the side chains, and Coulombic interactions may greatly influence their mobility.²⁸ The first biodegradability assessment in soil was conducted using several imidazolium-based ionic liquids, monitoring solely CO₂ evolution, which is not sufficiently informative to judge toxicity and biodegradability.¹⁵⁵ Nevertheless, mostly based on the high capacity of several environmental fungal strains (which are commonly found in soil) to tolerate high concentrations of ionic liquids,^{22,58} the role of filamentous fungi in their biodegradation in soil is expected to be high.

The prevailing opinion is that the cation and anion will undergo completely distinct fate mechanisms; however ion-pairs possibly formed between them and/or with other ions ubiquitously present in that environment may be involved. The faster biodegradability of certain anions relative to the cations has been demonstrated, *e.g.* alkylsulfates,^{48,79} saccharinate and acesulfamates,¹²² and alkanooates,²² further suggesting that their degradation followed distinctive pathways. This also stresses the importance of considering both the cation and the anion for successful conscious design of ionic liquids.

Molecular toxicity of ionic liquids

Some general considerations on molecular toxicity of ionic liquids will now be analysed. While molecular toxicity falls under the umbrella of toxicity, a direct extrapolation of any toxic behaviour based on *in vitro* analyses of single molecules/reactions may lead to an erroneous conclusion. Chemicals (or their biotransformation products) can display many modes of molecular toxicity (*i.e.* molecular mechanisms whereby chemicals cause toxicity) through interaction with specific biologic macromolecules, such as proteins and DNA.¹⁵⁶ Encouraged by this, Jastorff and coworkers have included

the concept of molecular toxicity as a standard component of their ecotoxicity test battery, selecting the acetylcholinesterase assay.^{95,110,157,158} Preference was given to this enzyme (already mentioned in the sections for pyridinium¹²² and quinolinium³³ ionic liquids) because it plays an essential role in the nervous system of almost all higher organisms, and the enzyme-active site is highly conserved amongst organisms. By the extensive set of IC₅₀ data based on this assay (effective concentration scale based on a 50% inhibition response) presented by Ranke *et al.*, it became apparent that the chain length of the cation strongly influenced the activity of the enzyme, probably due to the binding to the lipophilic active site; however, the effects of additional methyl groups in the ring and of the anions were unclear.³³ Even so, the fluorinated anions displayed consistently higher molecular toxicity,³³ probably due to their higher chemical instability. In addition, the introduction of polar hydroxyl or ether functions in the side chains induces reduced toxicity, relative to the alkyl chain.¹⁵⁸ The introduction of an additional hydroxyl group in position R³ in the pyridinium ring may have prevented the interaction of the quaternary nitrogen with the active site of the enzyme,¹²² since a reduction, by two orders of magnitude, was observed, *e.g.* while comparing 1-alkoxymethyl-3-hydroxypyridinium¹²² and 1-alkyl-3-methylpyridinium ionic liquids.¹⁵⁷ The head groups in the cation played a deciding role, mostly due to their ability to bind to the active site of the enzyme or its gorge.^{33,158} Essentially, the large aromatic systems of 1-alkyl-4-(dimethylamino)pyridinium and quinolinium, and the non-aromatic morpholinium and tetrabutylammonium cations, were responsible for leading to strong and weak inhibitions, respectively.¹⁵⁸ In addition, the sterically bulky structure and positive charged moiety shielded by the long alkyl chains of the tetraalkylphosphonium and tetraalkylammonium ionic liquids have probably reduced the interaction with the active centre of the enzyme, leading to weak inhibition, yet the benzyl aromatic residue in the benzyldecyl-dimethylammonium cation rationalises the deviant behaviour of high toxicity.

There are only a few studies on the molecular toxicity of ionic liquids focusing on, instead of the acetylcholinesterase, other systems such as the adenosine monophosphate (AMP) deaminase,¹⁵⁹ or the cytochrome P₄₅₀ assay.^{160,161} The activity of AMP deaminase in eukaryotes constitutes a primary step in the regulation of intracellular adenine nucleotide pools,¹⁶² and has therefore been proposed as a model enzyme for assessment of chemical risk.¹⁶³ Likewise, cytochrome P₄₅₀, a family of monooxygenases ubiquitously present in organisms, uses a wide spectrum of substrates, and are known to play an important role in xenobiotic metabolism, catalysing hydroxylation of C–H bonds.¹⁶⁴ The activity of AMP deaminase was inhibited by several [C₄mim]⁺ ionic liquids and, amongst the tested anions, the chloride and 4-tosylate exhibited slightly lower molecular toxicity than [BF₄]⁻ and [PF₆]⁻, with IC₅₀ values of 10 and 5 μM, respectively.¹⁵⁹ Likewise, inhibition of cytochrome P₄₅₀ BM-3 activity in the presence of [C_{*n*}mim]Cl (*n* = 4, 6 or 8) was stronger for the longer alkyl side chains.¹⁶⁰ This agrees with that previously observed while using the acetylcholinesterase assay,³³ though higher resistance of cytochrome P₄₅₀ was noticed with IC₅₀ values higher by at

least one order of magnitude. Surprisingly, the head group of the cation, and especially its aromaticity, apparently has played a minor role in the ionic liquid molecular toxicity against cytochrome P₄₅₀, since imidazolium, pyridinium and pyrrolidinium chlorides exhibited IC₅₀ values of 148, 195 and 175 mM, respectively.¹⁶⁰ In addition, the activity of the purified human enzyme cytochrome P₄₅₀ 3A4 was significantly inhibited in the presence of [C_nmim][BF₄] (*n* = 1 or 4) and [C₄py][BF₄].¹⁶¹ On the other hand, imidazolium ionic liquids apparently cannot be used as substrates by cytochrome P₄₅₀ BM-3, since the enzyme could not hydrolyse any of the tested [C_nmim]Cl (monitored by NADPH consumption).¹⁶⁰

Modes of toxicity of ionic liquids

The toxic effect of any chemical is an expression of the disorder of particular metabolic pathways, and their potential modes of action include disruption of membranes, mostly affecting membrane permeability and transport proteins, enzyme inhibition, and DNA damage. A better knowledge of chemical toxicity, *e.g.* in biocidal and drug design, is essential to identify their modes of action, and ultimately to further advance towards their conscious design.

The hydrophobic interaction between chemicals and biological membranes results in non-specific toxicity, called baseline toxicity or narcosis, which is mainly governed by solubility and partitioning,⁹³ and, though argued, there are possibly both polar and non-polar narcosis mechanisms.⁹² However, chemicals can also interact with biological systems through hydrogen or covalent bonding and charge transfer, with higher (relative to baseline toxicity) effectiveness. Baseline toxicity has been mentioned often in this review, by suggestion if not implicitly, as the basis for the observed correlation between the ionic liquid lipophilicity (at the level of both the cation and the anion) and toxicity. It becomes important to select suitable descriptors for the lipophilicity of ionic liquids, such as the *K*_{ow} and the membrane/water partition coefficients.⁸⁹ Even though the significance of the *K*_{ow} coefficients is still under debate, they are often used, mostly because they can be retrieved by direct measurement, by reversed phase liquid chromatography, or predicted by computational methods.⁷⁰ Jastorff and coworkers observed a good correlation between the cations' lipophilicity (defined chromatographically) and the toxicity of the corresponding ionic liquid (focusing on hydrophilic and chemically stable anions, such as the halides).^{67,89} Likewise, the high correlation observed by us between the antimicrobial activity of several cholinium alkanoates and the calculated anion hydrophobicity log₁₀(*K*_{ow}),²² may be explained by the cation hydrophilicity and benign nature. Significantly, even when a good correlation between lipophilicity and toxicity was observed, this effect, probably due to limited solubility and/or steric limitations, was not universal. There are several examples where very lipophilic cations^{38–40,120,131} or anions,²² commonly carrying side chains longer than 10–12 carbon atoms, exhibited lower toxicity than that predicted. In the latest study, the cholinium dodecanoate exhibits lower toxicity than expected, probably due to its limited solubility. In addition, in a very recent study focusing on imidazolium ionic liquids, a strong relationship

between antimicrobial efficacy, structure of the cation and surface activity of the aqueous solutions containing [C_nmim]⁺ (*n* = 8 to 18) was reported.¹⁶⁵

Liposomes (phospholipid vesicles) constitute a simple and attractive methodology to analyse interactions of ionic liquids with cellular membrane.¹³⁶ Both [C₈mim]⁺ and [NTf₂][−] were demonstrated to cause severe disruptions in a supported phospholipid bilayer, with [NTf₂][−] leading to the most extensive loss of lipids.⁹⁷ The higher toxicity of the latter was tentatively explained using molecular simulations, predicting that [NTf₂][−] was readily incorporated into the cholesterol bilayer, strongly limiting the adsorption of the cation and, consequently, the insertion of the butyl chain of [C₄mim]⁺ into the bilayer.¹⁶⁶

As an attempt to rationalise the toxicity mode of ionic liquids, some processes have been proposed to play a key role. Nevertheless, one should bear in mind the high complexity of living organisms and the numerous physical and biochemical processes occurring simultaneously. The important observations on the critical alterations detected in fungal metabolism after exposure to sub-lethal concentrations of some hydrophilic ionic liquids⁵⁸ may suggest a specific mode of toxicity.

Quantitative structure–activity relationships (QSAR) for ionic liquids

Noteworthy efforts are increasingly being reported in order to define computational methods which can be used to reasonably predict ionic liquid toxicity. There is no doubt that computer modelling will, as more ecotoxicity data becomes available, develop further. Couling *et al.* applied QSAR modelling to their experimental data on the aquatic toxicity of twenty-five ionic liquids (containing imidazolium, pyridinium, tetraalkylammonium, and tetraalkylphosphonium cations) (correlation with *R*² = 0.78–0.88).⁸² Based on the derived descriptors, calculated at a low semi-empirical computational level, certain generalised predictions could be raised: toxicity is mainly governed by the cation, with the aromatics displaying higher toxicity than non-aromatic ones, and increasing with longer substituted alkyl chains. Additionally, in the aromatic ring, an increased number of nitrogen atoms leads to higher toxicity, while its methylation decreases it. A different approach was used by Luis *et al.*, since they designed an algorithm based on group contribution methods to estimate the aquatic toxicity of forty-three imidazolium, pyridinium and pyrrolidinium ionic liquids (correlation with *R*² = 0.92).¹⁶⁷ Based on this modelling, some new generalisations were suggested, such as a toxic ranking of the head groups (pyrrolidinium < imidazolium < pyridinium), while the anion effect remained less predictable, though some (bromide, dicyanamide and ethylsulfate) were considered highly toxic. Garcia-Lorenzo *et al.* built a QSAR model, according to the Topological Sub-Structural Molecular Design (TOSS-MODE) approach, which uses graph-based molecular descriptors (based on spectral moments) to predict the cytotoxicity of fifteen imidazolium-derived ionic liquids in CaCo-2 cells (correlation with *R*² = 0.98). Different mathematical models were proposed by Torrecilla *et al.*,¹⁶⁸ using empirical formulae (elemental composition) and

molecular weights of 153 ionic liquids (ammonium, imidazolium, morpholinium, phosphonium, piperidinium, pyridinium, pyrrolidinium and quinolinium salts) available in the open literature³³ to estimate their cytotoxicity (IPC-81 cells) and molecular toxicity (acetylcholinesterase) by neural network (NN) models (correlation with $R^2 = 0.98$ and 0.97 , respectively). More recently, COSMO-RS (Conductor like Screening Model for Realistic Solvents) molecular descriptors, which related cytotoxicity to the polar charge distribution of the cations and the anions, were used (non-linear neural network) (96 formulations in IPC-81 cells,³³ correlation with $R^2 = 0.98$): this leads to the highly significant conclusion that the cytotoxicity of ionic liquids cannot be systematically estimated by a summation of the independent contributions of the intrinsic toxicity of the cation and anion.^{168,169}

General considerations

In the light of the studies referred to above, it is clear that the numerous formulations of ionic liquids available provide a great pool of, and impetus for, many commercial applications, but not without significant toxicological and environmental concerns. The vast majority of toxicological studies on ionic liquids, available up to the present date, have focused on imidazolium ionic liquids. In addition, frequently, the ionic liquids “selected” for study tackled under a common assay were randomly chosen. Despite the scientific weight of these studies, the lack of systematisation (*e.g.* monitoring the effect of defined structural alterations in a specific head group) means that it is impossible at the moment to achieve a holistic analysis, which weakens conclusions and devalues the predictive algorithms under development.

Furthermore, the selected bioassay, despite its relevance, appears to be sometimes subjective. Ecotoxicity examination should be based on a realistic analysis of the risk of exposure, accounting predicatively for which environmental compartments are most likely to be involved, yet it is necessarily regionally-oriented. Legislation demands and standardised tests should be kept as a priority, engaging models of different complexity. However, one may doubt their suitability, since testing a vast number of organisms and the diversity of all available formulations is costly and irrational. Indeed, testing the most sensitive species and determining the chemical lethal concentration, will provide useful information, but this may lack a real ecological meaning, as sub-lethal concentrations significantly affect the ecology of the exposed niche (*e.g.* species interactions and food web balance). In addition, the environmental persistence of any chemical should be taken as one of the most critical ecotoxicological parameters (advancing their conscious design), and putatively their mitigation will involve the tolerant species able to metabolise it. The most frequently used toxicity tests establish median effective concentrations (inhibitory or lethal), which are useful parameters for comparison of different chemicals, but it is obvious that further standardisation should be pursued. The conditions selected, *e.g.* media composition, temperature, light/dark cycle, and more importantly the exposure time, have generated a considerable volume of data on ionic liquid toxicity that cannot be directly compared. Furthermore,

straightforward comparisons were not easy because of the use of diverse end-points and units.

The European Community regulation on chemicals and their safe use—REACH (Registration, Evaluation, Authorisation and Restriction of CHemical substances)¹⁷⁰—aims to increase the awareness of the industry on hazards and risk management. REACH registration, in force since 2007, is mandatory for any chemical produced in the quantity over one tonne per year. Although it is being criticised for its ever-increasing cost and the number of animals employed in testing,¹⁷¹ it undoubtedly provides a meaningful, and necessary, framework to raise human and environmental safety. Currently, only the ionic liquids which have already found application on industrial scale are undergoing REACH registration, such as $[C_2mim]X$ ($X = Cl^-$, $[C_2SO_4]^-$, $[C_1SO_3]^-$, $[O_2CMe]^-$ and $[NTf_2]^-$) and $[C_4mim]Cl$,¹⁷² but there are no doubts that this number will continuously increase. They have received the classification of substances, *i.e.* as salts, which assumes that in the water/soil matrix, the cation and the anion will behave as independent chemical entities, displaying distinct toxicity and biodegradability potential. But, as noted above, it appears (irrespective of speculation as to the cause) that the cytotoxicity of ionic liquids cannot be systematically estimated by a summation of the independent effects of the cation and anion.^{168,169} Moreover, it should also be noted that, to date, mixtures (binary or ternary) of ionic liquids have been rarely investigated.¹¹⁶

In conclusion, one should recognise that the ionic liquid scientific community needs to increase the public awareness of the immense diversity of possible formulations and properties included in such a classification. For example, despite the significance of the data, especially as the first study reporting aquatic toxicity,¹²⁵ the attention attracted by the headline “Warning shot for green chemistry” in *Nature*,¹⁷³ or “Ionic liquids toxic to fish” in *Dalton Transactions*,¹⁷⁴ was almost certainly counterproductive and misleading, distorting as it did the excellent data reported by the original authors, where thirteen out of fifteen tested ionic liquids were shown to have LC_{50} values above 100 mg l^{-1} after 96 h of exposure. In contrast, unwarranted claims that ionic liquids, as a class of materials, are “green” (and many papers erroneously claim this in their introductory sentences) are just as damaging to the field as claims that ionic liquids are “toxic”. With well over one million simple ionic liquids,¹⁷⁵ arguing from the specific to the generic is both misguided and intellectually dishonest. It should be recognised that sweeping generalisations do not favour our community, and it is the community itself that needs to protect ionic liquids indubitable broad utility, obviously by continuously following our duty to attain higher safety by their conscious design.

List of abbreviations

Cations

1-Alkyl-3-methylimidazolium	$[C_nmim]^+$
1-Alkylpyridinium	$[C_npy]^+$
1-Alkyl-3-methylpyridinium	$[C_nm\beta py]^+$
1-Alkyl-4-methylpyridinium	$[C_nm\gamma py]^+$

1-Alkoxymethylpyridinium cation	$[C_nOC_1py]^+$
1-Alkylquinolinium	$[C_nquin]^+$
1-Alkyl-1-methylpyrrolidinium	$[C_nmpyr]^+$
1-Alkyl-1-methylpiperidinium	$[C_nmpip]^+$
4-Alkyl-4-methylmorpholinium	$[C_nmmor]^+$
4-Alkyl-4-ethylmorpholinium	$[C_nemor]^+$
Generic tetraalkylammonium	$[N_{wxyz}]^+$
Generic tetraalkylphosphonium	$[P_{wxyz}]^+$

Anions

Tetrafluoroborate	$[BF_4]^-$
Hexafluorophosphate	$[PF_6]^-$
Hexafluoroantimonate	$[SbF_6]^-$
Dicyanamide	$[N(CN)_2]^-$
Methanesulfonate	$[C_1SO_3]^-$
Ethylsulfate	$[C_2SO_4]^-$
Octylsulfate	$[C_8SO_4]^-$
Ethanoate	$[O_2CMe]^-$ or $[O_2CC_1]^-$
Alkanoate	$[C_nH_{2n+1}CO_2]^-$
Bis{(trifluoromethyl)sulfonyl}amide	$[N(SO_2CF_3)_2]^-$ or $[NTf_2]^-$
Bis(trifluoromethyl)amide	$[N(CF_3)_2]^-$
Diethylphosphate	$[C_2PO_2]^-$

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