Effects of different head groups and functionalised side chains on the aquatic toxicity of ionic liquids

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In this study, the influence of different head groups, functionalised side chains and anions of ionic liquids on the marine bacteria *Vibrio fischeri*, the limnic green algae *Scenedesmus vacuolatus* and the fresh water plant *Lemna minor* was investigated. The aim of these experiments is to improve the knowledge base for the molecular design of ionic liquids leading to a reduced (eco)toxicological hazard potential. The analysed set of about 40 ionic liquids confirmed the interdependency between lipophilicity—as derived from gradient HPLC—and (eco)toxicity. The toxicity was clearly reduced for the test organisms (partially by six to seven orders of magnitude) when short functionalised side chains were used instead of non-polar alkyl chains. Furthermore, we could demonstrate strong interactions of hydrophobic ionic liquid cations with two different types of common biological lipid bilayers, indicating that the membrane system of organisms is probably a primary target site of toxic action. These systematic studies are addressed to producers, developers and downstream users of ionic liquids in different fields of application, to facilitate the selection of (eco)toxicologically favourable structural elements and thus to contribute to the design of inherently safer ionic liquids.

Introduction

The philosophy of green or sustainable chemistry is the development of highly efficient technical processes and applications using chemicals with a reduced or eliminated hazard potential for man and the environment.[1] The use of inherently safer substances is not only desirable from an ecological point of view but also with respect to economic aspects. For example, according to the new European chemical legislation REACH (registration, evaluation, authorisation and restriction of chemicals), the responsibility for the safety of chemicals and products is handed to the manufacturers.[2] The registration process demands *inter alia* (eco)toxicological data for all chemicals produced or imported into the European Union above one metric tonne per year. Especially for substances that cause (or to be under suspicion to cause) cancer, infertility, mutations or birth defects and to those which are persistent and accumulate in the environment, further investigations are required and they are subject to an authorisation procedure. According to the authorisation process, these substances have to be progressively replaced by safer alternatives if available. The possibility for a chemical product to fail the authorisation process would entail the loss of so far invested time and money. To avoid this financial risk, the development of chemicals with optimised technical properties should be accompanied with the minimisation of (eco)toxicological hazard potentials (in accordance to Paul Anasta's second principle of green chemistry).[1]

Following this strategy, we formed a university–industry partnership with the Merck KGaA to develop inherently safer ionic liquids, where (eco)toxicological studies are guided by the knowledge on current industrial processes. In the field of designing chemicals, ionic liquids represent an excellent model substance class because of their broad applicability, *e.g.* in synthesis,[3,4] electrochemistry[5,6] and (bio)catalysis[7,8] and their high structural diversity leading to an enormous number of possible compounds.

It is a goal of many researchers to be able to tune the physicochemical properties of ionic liquids *via* the choice of certain anionic and cationic components when designing a specific ionic liquid ideally suited for a specific process. Such task-specific[9] or functionalised[10] ionic liquids are created through incorporation of functional groups into the alkyl chains. For example, thiol and thioether functionality has been used to enhance the extraction of metals.[9] Additionally, ionic liquids with appended nitrile groups have been shown to stabilise catalysts.[10,11] Hydroxyl groups tend to increase hydrogen bonding strength,[12] while ethers can lead to decreases in viscosity.[13] Tertiary amine groups can increase nucleophilicity,[14] while primary amines can be used to capture protons or carbon dioxide.[15]

However, this general heterogeneity requires efficient testing strategies to generate data sets leading to profound insights in modes of toxic action and target sites of chemicals. Regarding this issue we follow a T-SAR (thinking in terms of structure activity relationships)[16–18] guided strategy to:

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i. Systematically select test compounds and structural elements according to the "test kit concept".[18,19]

ii. Test the selected substances in a flexible (eco)toxicological test battery at different levels of biological complexity (*e.g.* enzymes, cells, microorganisms and organisms).[18]

iii. Identify toxicophore substructures in chemicals and use this knowledge in the design of inherently safer chemical products.

iv. Improve the molecular understanding of (eco)toxicological results by relating them to physicochemical properties.

Following this approach we investigated the influence of the structural elements side chain,[18,20–23] head group[18,20,21] and the anionic moiety[18,23,24] on cytotoxicity and (eco)toxicity so far.

Different studies in the past few years have analysed the toxicity of certain ionic liquids using different organisms, and considering the aquatic and terrestrial compartment as well as different trophic levels, *e.g.* cells,[25] bacteria,[26–33] algae,[23,34,35] aquatic and terrestrial plants,[18,23,36] invertebrates[23,37] and vertebrates.[38–40] A complete overview on (eco)toxicological data of ionic liquids has recently been given elsewhere.[41]

The draining of contaminated process water represents the most likely release of ionic liquids into the environment. Thus, the aquatic compartment is of particular importance regarding the hazard assessment of ionic liquids.

Up to now, the influence of some ionic liquids on organisms of the aquatic compartment has been shown for the luminescent bacteria *Vibrio fischeri*,[22,23,42] various green algae species,[23,34,35] the crustacean *Daphnia magna*,[35,42,43,44] the aquatic plant *Lemna minor*,[18,23] the snail *Physa acuta*[45] and the zebrafish *Danio rerio*.[46]

In general, it was found that the cation species is the main effector for the observed toxicity, especially if substituted with a longer alkyl side chain. The anion can also contribute to toxicity, but in most cases anion effects are less drastic compared to the side chain effect.

Recently, we investigated the impact of 7 different head groups, 10 (functionalised) side chains and 4 anions on mammalian cell toxicity.[21] Briefly summarising the results obtained in this study, we found a correlation between a HPLC derived lipophilicity parameter and the observed cytotoxicity in a rat leukemia cell line. The side chain has been identified to be the main effector to alter both: cytotoxicity and lipophilicity. According to their relatively low lipophilicity, short functionalised side chains could clearly diminish the observed cytotoxcity compared to non-polar alkyl chains. Except for the 4-(dimethylamino)pyridinium head group, which strongly influenced cytotoxicity, the head group itself is of minor concern compared to the side chain. This study proved the possibility to design ionic liquids with a remarkably low (cyto)toxicity, compared to the alkyl ($C \geqslant 4$) substituted ionic liquids when appropriate structural elements were combined.

The aim of this study is to test the applicability of the above mentioned results obtained in our *in vitro* cytotoxicity assay with a test kit of 40 ionic liquids containing different head groups, (functionalised) side chains and 4 anions, to aquatic test organisms. As common representatives of the aquatic compartment, we have selected the test organisms *Vibrio fischeri* (marine bacteria), *Scenedesmus vacuolatus* (limnic green algae) and *Lemna minor* (a fresh water plant). Furthermore, we intended to confirm the correlation between toxicity and lipophilicity for bacteria and algae. In addition to HPLC derived lipophilicity parameters,[20] membrane water partitioning coefficients[47] were used as test systems to investigate the interactions between ionic liquids and biological membranes. In general, the lipid composition of membranes is highly variable in cell organelles and tissues of organisms. Therefore, we choose two different composed lipid bilayers for our investigations of membrane water partitioning.

These systematic studies are addressed to the users of ionic liquids in different fields of application to facilitate the selection of (eco)toxicologically favourable structural elements and contribute therefore to the design of inherently safer ionic liquids.

The test kit compounds

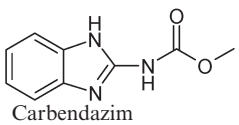
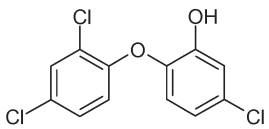
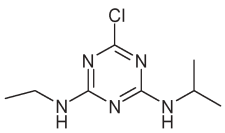
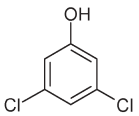
The test kit comprised of three aromatic head groups (4-(dimethylamino)pyridinium, pyridinium and imidazolium), three non-aromatic heterocycles (4-methylmorpholinium, 1-methylpiperidinium, 1-methylpyrrolidinium), and one non-cyclic quaternary ammonium head group (*N*,*N*-dimethylethylammonium), all substituted with a butyl side chain. The imidazolium head group is furthermore combined with seven different aliphatic side chains containing ether (in different positions), terminal hydroxyl and nitrile functions. For the correlation between lipophilicity and toxicity we added imidazolium ionic liquids with different alkyl side chains (-ethyl, -butyl, -hexyl, -octyl, -decyl, -tetradecyl, -hexadecyl, -octadecyl) to the test kit.

For all cationic head groups a halide (chloride, bromide or iodide) and the $[(CF_3SO_2)_2N]^-$ anion were tested in the different biological test systems.

Acronyms for ionic liquids

The cation is abbreviated according to the type of the head group as "Py-4NMe2" (dimethylamino)pyridinium, "Py" (pyridinium), "IM" (imidazolium), "Mor" (morpholinium), "Pip" (piperidinium), "Pyr" (pyrrolidinium) and as "N" (quaternary ammonium). The substituents at the nitrogen atom(s) of the head group are given as numbers corresponding to their alkyl chain length. For example the 1-butyl-3-methylimidazolium cation has the shorthand notation IM14. For two-digit side chains the numbers are separated by a hyphen (*e.g.* IM1-10 is 1-decyl-3-methylimidazolium). Ether containing side chains are indicated by splitting the chain in alkyl units with the symbol "O" for the oxygen in between (*e.g.* IM11O2 for 1-(ethoxymethyl)-3-methylimidazolium). Terminal hydroxyl or nitrile groups are shortened as OH (*e.g.* IM12OH is 1-(2-hydroxyethyl)-3-methyl-imidazolium) or CN (*e.g.* IM11CN is 1-cyanomethyl-3-methylimidazolium). The acronyms used for the halides are as in the periodic table. The bis(trifluoromethylsulfonyl)imide is written as $[(CF_3SO_2)_2N]^-$ according to its structural formula. The identifiers for the cation and for the anion—separated by a white space—represent the whole acronym for an ionic liquid.

Table 1 Toxic and relatively non-toxic compounds serving as references to demonstrate the range of toxicity of ionic liquids

	EC_{50}/μM		
	Vibrio fischeri (bacteria)	*Lemna minor* (duckweed)	*Scenedesmus vacuolatus* (algae)
 Carbendazim			2[a]
 Triclosan		0.2[b]	0.01[b]
 Atrazine		12[c]	0.05[d]
 3,5-Dichlorophenol	24[e]		
$H_3C–C\equiv N$ Acetonitrile	600 000[f]	5800[a]	180 000[a]
 Acetone	300 000[f]	90 000[a]	60 000[a]

[a] Data from ref. 48. [b] Data from ref. 52. [c] Data from ref. 50. [d] Data from ref. 49. [e] Data from ref. 51. [f] Data from ref. 22.

Results

In Table 1, the EC_{50} values from literature for toxic reference compounds and common solvents are shown for the three test organisms. All measured EC_{50} values and—if available—confidence intervals are presented in Table 2, Table 3 and Table 4. The results are described in the following sections.

Influence of the anion

The halides (chloride and bromide) do not exhibit an intrinsic anion effect in the tested concentration range of up to 20 mM for *Vibrio fischeri* and *Lemna minor* and 10 mM for *Scenedesmus vacuolatus*. Thus, it was concluded that all observed toxic effects for compounds with these anions (Table 2 and Table 3) can be attributed to the cation. The influence of the $[(CF_3SO_2)_2N]^-$ anion will be discussed in the following sections.

Influence of head group and anion

To investigate the influence of the head group on all three test organisms, we combined the seven different cationic cores with a butyl side chain and halides as counter ions (Table 2). For *Vibrio fischeri* the non-aromatic compounds exhibited a low toxicity ($EC_{50} > 10\ 000$ μM), whereas the aromatic substances showed an increased toxicity which is still in a moderate range ($EC_{50} > 1000$ μM), except for the 1-butyl-4-(dimethylamino)-pyridinium. For this compound the toxicity was clearly increased with an EC_{50} of approximately 300 μM.

For most of the compounds, a significant toxicity was observed ranging only one order of magnitude lower than the toxic reference compound atrazine (Table 1). For 1-butyl-4-(dimethylamino)pyridinium and the quaternary ammonium compound, drastic effects were found even below this toxic reference ($EC_{50} < 10$ μM). In contrast, the morpholinium compound exhibited only a moderate effect on *Lemna minor* ($EC_{50} > 1000$ μM).

For the algae *Scenedesmus vacuolatus* also a drastic toxicity was found for 1-butyl-4-(dimethylamino)pyridinium, whereas the quaternary ammonium and morpholinium compounds showed no toxicity.

Additionally, we investigated the influence of the head group being combined with the $[(CF_3SO_2)_2N]^-$, instead of the halides (Table 2). In a previous study, it was found that the $[(CF_3SO_2)_2N]^-$ showed no intrinsic toxicity to *Vibrio fischeri* ($EC_{50} > 20\ 000$ μM), a moderate toxicity for *Lemna minor* ($EC_{50} = 6300$ μM)[23] and a clear effect to *Scenedesmus vacuolatus* ($EC_{50} = 125$ μM).[23] In this study, an increased toxicity was found for all tested compounds combined with the $[(CF_3SO_2)_2N]^-$ for *Vibrio fischeri* and *Scenedesmus vacuolatus*. On the other hand, $[(CF_3SO_2)_2N]^-$ had no or even a positive influence on the observed effects on *Lemna minor*.

Influence of the side chain

To analyse the impact of functionalised side chains on toxicity we have chosen a test kit containing different imidazolium ionic liquids with halides as counter ions (Table 3). It was found that the toxicity towards all test organisms (except for IM11O2 $(CF_3SO_2)_2N$ and IM12O2 $(CF_3SO_2)_2N$ in the *Lemna minor* assay) decreased, compared to the butyl side chain when polar functional groups were introduced. For *Vibrio fischeri* and *Scenedesmus vacuolatus*, all compounds as halides (except for IM12O2 Br in the algae assay) were shifted to no or marginal toxicity ($EC_{50} \geqslant 10\ 000$ μM for *Vibrio fischeri* and $EC_{50} \geqslant 1000$ μM for *Scenedesmus vacuolatus*).

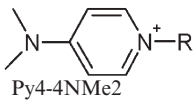
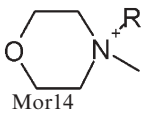
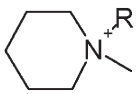
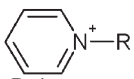
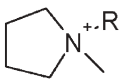
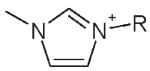
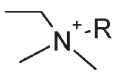
For *Lemna minor* this tendency is also observable but not as clearly.

Most of the ionic liquids with the $[(CF_3SO_2)_2N]^-$ (Table 3, column 4, 6 and 8) are more toxic for *Vibrio fischeri* and *Scendesmus vacuolatus* than the halides. For *Lemna minor*, again, no consistent trend for the influence of $[(CF_3SO_2)_2N]^-$ could be observed.

Correlation of toxicity with HPLC lipophilicity data and membrane water partitioning

To get more information on potential toxic modes of action of ionic liquids, the correlation between lipophilicity of the cation and the toxicity for *Vibrio fischeri* and *Scenedesmus vacuolatus* was investigated. For this analysis we used an HPLC derived lipophilicity parameter ($\log k_o$) and $\log EC_{50}$ values (Table 4). Most of the $\log k_o$ values have recently been published[20,21] and are supplemented with results generated for this study. Regarding toxicity, for some cations only data with BF_4^- instead of chloride were available, but this is of minor

Table 2 Structures and EC_{50} values of different head groups

R = butyl acronym	Type of halide	EC_{50}/μM (confidence interval)					
		Vibrio fischeri (bacteria)		*Lemna minor* (duckweed)		*Scenedesmus vacuolatus* (algae)	
		Halide	$[N(CF_3SO_2)_2]^-$	Halide	$[N(CF_3SO_2)_2]^-$	Halide	$[N(CF_3SO_2)_2]^-$
 Py4-4NMe2	Cl^-	330 (300–370)	70 (65–75)	3.4 (2.3–5.0)	5.8 (4.2–7.9)	0.6 (0.37–0.97)	0.4 (0.05–0.80)
 Mor14	Br^-	>20 000	310 (300–325)	1280 (930–1690)	1410 (1060–1910)	>10 000	100
 Pip14	Br^-	18 600 (15 100–23 400)	365 (340–390)	295	710 (610–830)	1850 (1410–2390)	120
 Py4	Cl^-	1500 (1300–1700)	n.d.	210 (135–315)	n.d.	390	n.d.
 Pyr14	Cl^-	>20 000	350 (330–370)	145 (80–255)	950 (490–2160)	2340 (1820–2950)	340
 IM14	Cl^-	2970[a] (2700–3300)	290[a] (250–320)	200[a]	280[a] (230–330)	180[a] (150–220)	65[a] (45–90)
 N1124	Cl^-	>20 000	1640 (1500–1800)	6.8 (1.6–17.9)	n.d.	>10 000	60 (40–90)

n.d. = not determined.[a] Data from ref. 23 (differences in EC_{50} values from recently published data result from additional follow-up measurements).

relevance because there are no significant differences in toxicity for ionic liquids containing chloride or BF_4^- in the assay with *Vibrio fischeri*.

As expected, a correlation between increasing chain length and increasing lipophilicity was found (ranges from log k_0 IM14 Cl = 0.63 up to log k_0 = 6.08 for IM1-16 Cl). Furthermore, a distinct decrease in lipophilicity could be observed when functionalised groups where introduced into the side chain (*e.g.* log k_0 = −0.28 for IM12OH I). For the different head groups substituted with a butyl side chain, the log k_0 values ranged from 0.18 for Mor14 Br to 1.08 for Py4-4NMe2 Cl (Table 4).

Plotting the logarithm of the log EC_{50} values (Table 4) derived from the luminescence inhibition assay *versus* the logarithm of the HPLC derived lipophilicity parameter k_0, reveals that ionic liquid cations in general span several orders of magnitude when considering their cation lipophilicity and toxicity (Fig. 1).

Concerning *Vibrio fischeri*, we found a strong correlation (r^2 = 0.942) for a set of imidazolium based ionic liquids with alkyl chain lengths of C4 and C6–C10 (■). Excluded from this correlation are data points for C14 and C16 (+)—for these compounds the elongation of the alkyl side chain does not lead to an increase in toxicity anymore, but the observed effects are still in a drastic range. This phenomenon is well known from literature for highly lipophilic substances (log $K_{ow} > 5$) as the "cutoff" effect. For this phenomenon, different explanations are discussed based either on insufficient solubility (nominal concentration deviating from real test concentration)[53] or on kinetic aspects (uptake is slowed down because of steric effects for compounds with a high molecular size).[54]

The results for the different head groups (▲) and the functionalised side chains (●) match with the above mentioned linear regression. Not all data points could be plotted, only when toxic effects were detected within the concentration range up to 20 mM.

Similar results for the limnic green algae *Scenedesmus vacuolatus* were achieved when correlating cation lipophilicity and toxicity for C4, C6, C8 and C10 alkyl chains (Fig. 2). Again, a cutoff from this correlation for C14 and C16 was observable (log EC_{50} values > −2; are not shown in Fig. 2).

The slope of the line resulting from linear regression (r^2 = 0.986) for *Scenedesmus vacuolatus* is clearly steeper than for *Vibrio fischeri*. This indicates a higher sensitivity for the algae to compounds with increasing cation lipophilicity.

The observed toxicities for *Scenedesmus vacuolatus* cover six to seven orders of magnitude, ranging from 0.3 nM for IM1-10 Cl (~150 times more toxic than the reference compound atrazine) to approximately 1 mM for IM12 Cl (Fig. 3).

Table 3 Structures and EC_{50} values of different functionalised side chains

R (acronym)	Type of halide	EC_{50}/μM (confidence interval)			
		Vibrio fischeri (bacteria)		*Lemna minor* (duckweed)	
		Halide	$[N(CF_3SO_2)_2]^-$	Halide	$[N(CF_3SO_2)_2]^-$
$-CH_2CH_2-OH$ (IM12OH)	I^-	7700 (7300–8200)	11 100 (9000–13 600)	1650 (1450–1860)	1650 (1450–1870)
$-CH_2CN$ (IM11CN)	Cl^-	>10 000	6500 (5400–7700)	n.d.	2800 (2400–3300)
$-CH_2CH_2CH_2CH_3$ (IM14)	Cl^-	2970[a] (2700–3300)	290[a] (250–320)	200[a]	280[a] (230–330)
$-CH_2CH_2CH_2-OH$ (IM13OH)	Cl^-	>20 000	6500 (5800–7500)	3350	3330 (2950–3760)
$-CH_2-O-CH_2CH_3$ (IM11O2)	Cl^-	12 000 (9000–16 000)	1000 (930–1050)	>5000	180
$-CH_2CH_2-O-CH_3$ (IM12O1)	Cl^-	15 000 (10 700–23 200)	670 (610–740)	420 (350–510)	580 (360–790)
$-CH_2CH_2-O-CH_2CH_3$ (IM12O2)	Br^-	19 200 (15 000–27 500)	890 (850–930)	300 (260–350)	140 (120–170)
$-CH_2-CH_2CH_2-O-CH_3$ (IM13O1)	Cl^-	>20 000	1600 (1500–1700)	980 (900–1070)	550 (350–820)

R (acronym)	*Scenedesmus vacuolatus* (algae)	
	Halide	$[N(CF_3SO_2)_2]^-$
$-CH_2CH_2-OH$ (IM12OH)	>1000	150 (130–170)
$-CH_2CN$ (IM11CN)	>1000	120
$-CH_2CH_2CH_2CH_3$ (IM14)	180[a] (150–220)	65[a] (45–90)
$-CH_2CH_2CH_2-OH$ (IM13OH)	>1000	95
$-CH_2-O-CH_2CH_3$ (IM11O2)	890 (620–1330)	90 (70–120)
$-CH_2CH_2-O-CH_3$ (IM12O1)	1820 (1510–2240)	95 (65–145)
$-CH_2CH_2-O-CH_2CH_3$ (IM12O2)	330	100
$-CH_2-CH_2CH_2-O-CH_3$ (IM13O1)	>1000	115 (100–130)

n.d. = not determined.[a] Data from[23] (differences in EC_{50} values from recently published data result from additional follow-up measurements)

Membrane water partitioning

In toxicology, the membrane water partitioning coefficient (*MA*) is used as a parameter to describe membrane interactions of highly polar, ionisable and permanently charged molecules.[47,55–57]

For the determination of these coefficients, we used solid-supported lipid membranes (TRANSIL™).[47,57] This technology is based on porous silica beads that are completely coated by a uni-lamellar lipid bilayer. For our investigations, two different types of coated lipids were used: 100% phosphatidyl choline (PCC; net charge of zero) and 80% phosphatidyl choline and 20% phosphatidyl serine (POPS; net charge of minus one). We analysed a set of imidazolium based ionic liquids with different alkyl side chain lengths (Table 5). For IM16 Cl no fixed coefficients could be measured because of its low affinity to the membrane (our technique described in "Experimental" is limited to substances with an $MA > 1.5$). An increased *MA* value is observable for compounds with elongated alkyl chains (Table 5). Furthermore, consistently higher *MA* values were measured for the negatively charged POPS lipids. Comparing the results for the ionic liquids with common organic compounds (Table 5), we found that the ionic liquid values range between 1-hexanol and 2,4,5-trichlorotoluene.

Discussion

The intention of the study presented here was to investigate the influence of (functionalised) side chains, head groups and anions of ionic liquids on different aquatic organisms (*Vibrio fischeri*, *Scenedesmus vacuolatus* and *Lemna minor*) with the aim to assert the trends observed in our *in vitro* cytotoxicity study.

We could confirm

i. the dependency between toxicity and lipophilicity of ionic liquid cations in the investigated systems with the marine bacterium *Vibrio fischeri* and the limnic green algae *Scenedesmus vacuolatus*.

ii. that the side chain is the main effector for changes in lipophilicity and concomitantly in toxicity.

iii. that the introduction of polar functionalised groups into a short side chain consistently diminishes the observed toxicities in all three test systems in comparison to the butyl side chain.

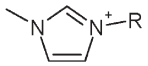
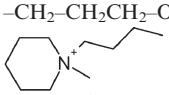
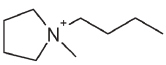
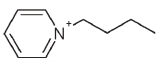
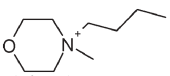
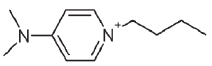
iv. that the influence of the head group is of minor relevance to alter toxicity/lipophilicity in most of the cases.

v. the well known side chain effect (increased toxicity with elongated alkyl side chain).

vi. that for $[(CF_3SO_2)_2N]^-$, a clear (eco)toxicological hazard potential is evident.

The general interdependency between ionic liquid cation lipophilicity and toxicity ascertained in *in vitro* and *in vivo* experiments suggests a mode of action that is called "baseline toxicity"[53,55,58] for aquatic system or "narcosis"[59–61] for mammals. This mode of action for hydrophobic compounds has been discussed several times for ionic liquids[20,21,23,44,45] and cationic surfactants.[62] The observed toxic effects can be explained by the assumption that these cationic hydrophobic compounds adsorb onto or intercalate into membranes

Table 4 List of log EC_{50} values and log k_o values of ionic liquids

	log EC_{50}/μM (confidence interval)			
	Vibrio fischeri		*Scenedesmus vacuolatus*	log k_o
R (acronym)	Halide	Tetrafluoroborate	Halide	Anion independent
–Ethyl (IM12)	4.33 (4.22–4.54)		2.78 (2.73–2.84)	n.d.
–Propyl (IM13)		3.94[a] (3.86–4.02)		0.42[b]
–Butyl (IM14)	3.47 (3.43–3.52)		2.25 (2.17–2.34)	0.63[b]
–Pentyl (IM15)		3.14[a] (3.11–3.17)		0.92[b]
–Hexyl (IM16)	2.91[a] (2.82–2.99)		0.08	1.24[b]
–Heptyl (IM17)		2.43[a] (3.43–3.52)		1.57[b]
–Octyl (IM18)	1.01 (0.95–1.08)		−2.67 (−3.06 to −2.32)	1.85[b]
–Nonyl (IM19)		0.72[a] (0.67–0.76)		2.1[b]
–Decyl (IM1-10)	−0.23 (−0.30 to −0.17)		−3.57 (−3.62 to −3.51)	2.37[b]
–Tetradecyl (IM1-14)	−0.15 (−0.22 to −0.07)		−2.48 (−2.68 to −2.27)	4.6
–Hexadecyl (IM1-16)	0.23 (0.15–0.31)		>−2	6.08
–Octadecyl (IM1-18)	1.45 (1.39–1.50)		>−2	>7
–CH_2CH_2–OH (IM12OH)	3.89 (3.86–3.91)		>3	−0.28[c]
–CH_2CN (IM11CN)	>4		>3	−0.29[c]
–$CH_2CH_2CH_2$–OH (IM13OH)	>4.3		>3	−0.23[c]
–CH_2–O–CH_2CH_3 (IM11O2)	4.01 (3.96–4.20)		2.95 (2.79–3.13)	0.21[c]
–CH_2CH_2–O–CH_3 (IM12O1)	4.18 (4.03–4.37)		3.26 (3.18–3.35)	−0.02[c]
–CH_2CH_2–O–CH_2CH_3 (IM12O2)	4.28 (4.18–4.44)		3.25	0.45[c]
–CH_2–CH_2CH_2–O–CH_3 (IM13O1)	>4		>3	0.4
 1(Mor14)	>4.3		>4	0.18[c]
 (Pyr14)	>4.3		3.37 (3.26–3.57)	0.51[c]
 (Py4)	3.18 (3.12–3.23)		2.59 [±0.11]	0.57[c]
 (Pip14)	4.27 (4.18–4.37)		3.27 (3.15–3.38)	0.68[c]
 (Py4-4NMe2)	2.52 (2.48–2.57)		−0.27 (−0.45 to −0.10)	1.08[c]

[a] Data from ref. 22. [b] Data from ref. 20. [c] Data from ref. 21.

resulting in membrane perturbation (expansion or swelling, increase in fluidity, lowering of the phase transition temperature, and alteration of the ion permeability of the membrane).[55,63] The high membrane water partitioning coefficients of long chain imidazolium ionic liquids support this hypothesis and indicate their strong potential to interact with biological membranes and to bioaccumulate, resulting in higher internal effect concentrations. Furthermore, a clear relationship between lipid composition and measured membrane water partitioning could be confirmed. An increased membrane affinity of the analysed ionic liquid cations was found for the membrane containing 20% negatively charged phosphatidyl serine. This circumstance is easily explained with the increased ionic interactions between ionic liquid cations and negatively charged lipids.

For very lipophilic compounds with an alkyl side chain >C10, a cutoff effect (divergence from correlation between toxicity and lipophilicity) was found for algae and luminescent bacteria. This phenomenon is probably based on insufficient solubility (nominal concentration deviating from real test concentration).

In contrast to our previous cytotoxicity study, the 1-butyl-4-dimethylaminopyridinium compound does not deviate in our *in vivo* test from the toxicity/lipophilicity correlation. However, we found consistently drastic toxicities towards all three test organisms for this head group. In this study, the toxic effects of the dimethylaminopyridinium head group can be explained by its higher lipophilicity (log k_0 = 1.08)—due to the broadly delocalised positive charge—compared to the other butyl substituted head groups (log k_0 ranges between 0.18–0.68).

The $[(CF_3SO_2)_2N]^-$ anion increases the toxicity—compared to the halides—for *Vibrio fischeri* and *Scenedesmus vacuolatus*, like in our cytotoxicity assay. Regarding *Scenedesmus vacuolatus*, this fact is probably based on the intrinsic toxicity of the $[(CF_3SO_2)_2N]^-$ anion (EC_{50} of Li $(CF_3SO_2)_2N$ = 125 μM), whereas for *Vibrio fischeri*, no intrinsic toxicity for this anion was detectable (EC_{50} of Li $(CF_3SO_2)_2N$ > 20.000 μM). Nevertheless, an increased toxicity towards *Vibrio fischeri* for $[(CF_3SO_2)_2N]^-$ containing ionic liquids is obvious. This mixture effect of cation and anion can possibly be explained by temporary formed ion pairs in aqueous solutions, leading to a higher bioavailability and a higher membrane disturbance,

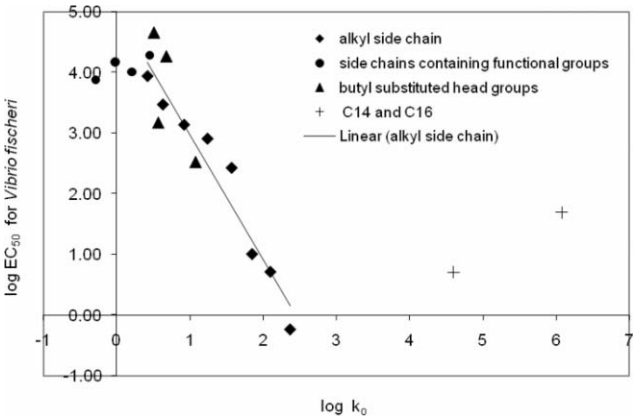


Fig. 1 Correlation for *Vibrio fischeri*. The correlation between lipophilicity (log k_o) and toxicity (log EC_{50}) for the bacteria *Vibrio fischeri* is demonstrated for the cations as halides. The linear regression relates to imidazolium cations with different alkyl side chains (■, r^2 = 0.942). The alkyl side chains C14 and C14 (+) deviate from this correlation ("cutoff" effect). Additional data points for ionic liquids with functionalised side chains (●) and butyl substituted head groups (▲) are additionally shown if a defined log EC_{50} was detectable within the tested concentration range.

resulting therefore in stronger toxic effects. For *Lemna minor*, the negative influence of the $[(CF_3SO_2)_2N]^-$ is not observed. Here, the toxicity is mainly driven by the cation.

In consideration of the structural design of ionic liquids, the vulnerable algae are an optimal test system to identify (eco)toxicologically favourable structural elements. We could demonstrate the possibility to alter ionic liquid cation toxicity by 6 to 7 orders of magnitude, ranging from extreme toxicity for IM1-10 Cl (EC_{50} = 0.3 nM) to low/no toxicity for *e.g.* IM13OH Cl (EC_{50} > 1 mM), just by varying the lipophilicity of the side chain.

For the head groups we found very low toxicities for the morpholinium and ammonium compounds in the algae assay,

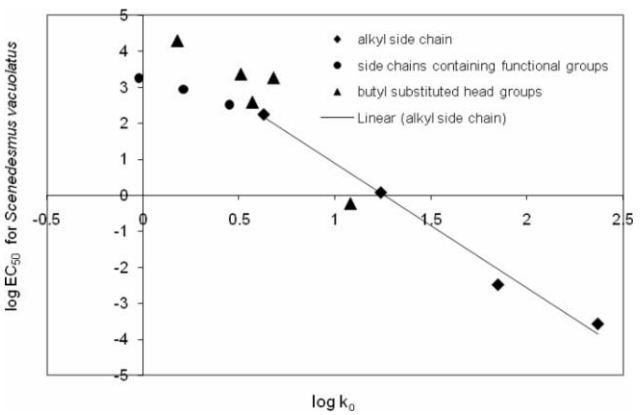


Fig. 2 Correlation for *Scenedesmus vacuolatus*. The correlation between lipophilicity (log k_o) and toxicity (log EC_{50}) for the algae *Scenedesmus vacuolatus* is demonstrated for the cations as halides. The linear regression relates to imidazolium cations with different alkyl side chains (■, r^2 = 0.986). Additional data points for ILs with functionalised side chains (●) and butyl substituted head groups (▲) are presented within this chart if a defined log EC_{50} was detectable within the tested concentration range.

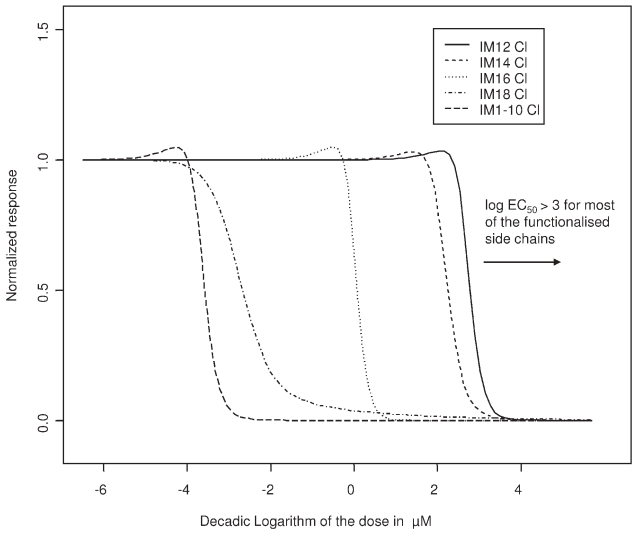


Fig. 3 Ranges of toxicity for *Scenedesmus vacuolatus*. Dose response curves of imidazolium ionic liquids substituted with different alkyl side chains to confirm the drastic shift in toxicities by changing this structural element for this organism.

but the N1124 cation can not be recommended due to the drastic toxicities in the test with *Lemna minor* (EC_{50} = 6.8 µM).

The very low toxicity found for the morpholinium ionic liquid can be related to the low lipophilicity of its cation (k_0 = 0.18). Regarding the molecular design of ionic liquids, this head group seems to be promising from the viewpoint of aquatic toxicity, especially when it would be combined with functionalised side chains.

Conclusion

We used the test organisms *Vibrio fischeri*, *Lemna minor* and *Scenedesmus vacuolatus* to investigate the influence of different head groups and functionalised side chains of about 40 ionic liquids on aquatic toxicity, with the aim to confirm the results obtained in our *in vitro* cytotoxicity assay. In general, a good accordance between *in vivo* and *in vitro* experiments could be found, indicating the relevance of the cytotoxicity assay for aquatic organisms. We could demonstrate that the lipophilicity—determined as HPLC parameter—is the key factor to

Table 5 Log membrane water partitioning coefficients (*MA*)

	Log *MA*	
	PCC[a]	POPS[b]
IM16 Cl	⩽1.5	
IM18 Cl	2.06 ± 0.15	2.59 ± 0.14
IM1-10 Cl	3.15 ± 0.19	3.52 ± 0.12
IM1-14 Cl	4.09 ± 0.17	4.47 ± 0.19
IM1-16 Cl	4.48 ± 0.03	
1-Hexanol	1.91 ± 0.05[c]	
Chlorobenzene	2.81 ± 0.03[c]	
1,3,5-Trichlorobenzene	3.95 ± 0.14[c]	
2,4,5-Trichlorotoluene	4.77 ± 0.07[c]	
Benzocaine (literature)	2.55[d]	
Benzocaine (measured)	2.39 ± 0.17	

[a] 100% phosphatidyl choline. [b] 80% phosphatidyl choline and 20% phosphatidyl serine. [c] Data from ref. 56. [d] Data from ref. 47.

alter toxicities in the investigated test systems. Due to their low lipophilicity, short functionalised side chains can clearly reduce the toxicity (partially by six to seven orders of magnitude) compared to the non-polar alkyl chains in all three test organisms. Furthermore, based on its low lipophilicity/toxicity the morpholinium head group seems to be recommendable, whereas the dimethylaminopyridinium core should be avoided because of its drastic effects on aquatic organisms. Considering these facts, our data open up the possibility to design task specific ionic liquid cations with a reduced (eco)toxicological hazard potential, just by adjusting their lipophilicity.

For many applications, hydrophobic water non-soluble ionic liquids are necessary and therefore the anion moiety is an important element to alter this physicochemical property. Thus, investigations regarding (eco)toxicity, and in particular (bio)degradability of the anion species forming hydrophobic ionic liquids (especially when combined with non-toxic polar cations), are of major concern for the structural design and a sound hazard assessment of ionic liquids.

Experimental

Chemicals

All tested ionic liquids were received by the Merck KGaA (Darmstadt, Germany). Carbendazim, acetic acid, acetonitrile, methanol, ethanol, propanol and dimethylsulfoxide were purchased from Sigma–Aldrich Cooperation (Deisenhofen, Germany).

Determination of membrane–water partition coefficients

The lipid–water partitioning has been ascertained using solid-supported lipid membranes (TRANSIL™) purchased from Nimbus Biotechnologie GmbH (Leipzig, Germany). This technology is based on porous silica beads that are completely coated by a uni-lamellar lipid bilayer (100% phosphatidyl choline or 80% phosphatidyl choline and 20% phosphatidyl serine) from egg yolk. 500 μM stock solutions of ionic liquids (containing 10% dimethyl sulfoxide (DMSO)) were prepared in PBS puffer (adjusted at pH 7.4). The experiment was carried out in Eppendorf test tubes in a total volume of 300 μL containing 50 μM ionic liquid, 1% DMSO and PBS buffer. DMSO was used to ensure the solubility of the ionic liquids. In this percentage, it has been proven not to influence the membrane coating of the silica beads and the *MA* value in general according to the manufacturer. The volume of added TRANSIL beads depended on the lipophilicity of the ionic liquid cation. For IM17 Cl, IM18 Cl and IM19 Cl, 50 μL beads were used. 10 μL and 5 μL TRANSIL suspensions were necessary for IM1-10 Cl and IM1-16 Cl, respectively. After addition of the beads, the mixtures were thoroughly shaken and incubated for 30 min at 37 °C (Eppendorf thermomixer 5436, shaker level 10). Afterwards, the samples were centrifuged (Eppendorf bench centrifuge) at 10 000 rpm for 5 min. The ionic liquid concentration in the supernatant ($n_{\text{supernatant}}$) was determined *via* HPLC. For all substances, controls without beads were measured as a reference ($n_{\text{reference}}$). The calculation of lipid–water partition coefficients (*MA*) is derived in detail in ref. 57 and can be simplified to:

$$\log MA = \log \frac{V_{\text{total}}}{V_{\text{lipid}}} \frac{n_{\text{supernatant}} - n_{\text{reference}}}{n_{\text{supernatant}}}$$

V_{total} represents the total volume (300 μL) and V_{lipid} is known from the certificate of analysis. The validity of the obtained log *MA* has been confirmed by determination of a reference compound (benzocaine) for this assay. Our results for benzocaine agree very well with literature data.

Luminescence inhibition assay with marine bacteria

The bioluminescence inhibition assay is a highly standardised and widely used bioassay.[64] This test with the marine bacterium *Vibrio fischeri* was performed according to DIN EN ISO 11348-2.[65] The freeze-dried bacteria were purchased from Dr Lange GmbH (Düsseldorf, Germany). The tests were carried out at least twice for each substance. First a range-finding was undertaken with two replicates per dilution series. Those results were validated within a second test using three replicates for each concentration and substance. Within each test, at least 4 controls (2% NaCl solution, phosphate buffered) were used. To exclude pH-effects all selected substances were prepared as phosphate-buffered solutions (0.02 M, pH 7.0, including 2% sodium chloride) and the test solutions were prepared at least 12 h before testing, to ensure complete dissolution of the substances. The tests were performed at 15 °C using thermostats (LUMIStherm, Dr Lange GmbH, Düsseldorf, Germany). The luminescence was measured with a luminometer (LUMIStox 300, Dr Lange GmbH, Düsseldorf, Germany).

The freeze-dried bacteria were rehydrated according to the test protocol, then 500 μL aliquots of the bacteria solution were pre-incubated for 15 min at 15 °C. After measuring the initial luminescence, 500 μL of the diluted samples were added to the pre-aliquoted bacteria. The bioluminescence was measured again after an incubation time of 30 min. The relative toxicity of the samples was expressed as a percentage inhibition compared to the controls. Luminescent bacteria assays were done without any co-solvents.

Reproduction inhibition assay with limnic green algae

For this assay, the unicellular limnic green algae *Scenedesmus vacuolatus* (strain 211-15, SAG (Culture Collection of Algae), Universität Göttingen, Göttingen) was used and toxicity tests were done using a synchronised culture.[66] The stock culture was grown under photoautotrophical conditions at 28 °C (±0.5 °C) in an inorganic, sterilized medium (pH 6.4), with saturating white light (intensity of 22 to 33 kilolux) (Lumilux Daylight L 36 W-11 and Lumilux Interna L 36 W-41, Osram, Berlin, Germany). Cells were aerated with 1.5 vol% CO_2 and synchronised by using a 14 h light and 10 h darkness cycle. The stock culture was diluted every day to a cell density of 5×10^5 cells mL^{-1}.

This test is a modified version of the assay described in ref. 67 and its sensitivity is comparable to the standardised 72 h test (ISO, 1989).[68]

The toxicity tests started with autospores (young algal cells at the beginning of the growth cycle). Algae were exposed to the test substances for one growth cycle (24 h). The endpoint of this assay is the inhibition of algal reproduction, measured as inhibition of population growth. All cell numbers (stock culture and test) were determined with the Coulter Counter Z2 (Beckmann, Nürnberg, Germany). The tests were performed in sterilized glass tubes (20 mL Pyrex tubes sealed with caps containing a gas tight Teflon membrane), algae were stirred over the whole test period of 24 h and the test conditions were the same as for the stock culture, except for the CO_2 source. Here. 150 μL of $NaHCO_3$ solution was added to each test tube. The methods for stock culturing and testing are described in detail in ref. 69,70. Laboratory facilities allowed parallel testing of up to 60 tubes. All substances were tested at least twice: first a range finding was undertaken (4 concentrations, two replicates) and in a second test the results were verified with 8 concentrations per substance in two replicates. The growth inhibition was calculated using the cell counts of the treated samples in relation to the untreated controls (pure medium). For each assay, at least 6 controls were used.

Growth inhibition assay with *Lemna minor*

The growth inhibition assay with *Lemna minor* was performed as described in detail in ref. 50. The plants were grown in open Erlenmeyer flasks in sterilised Steinberg medium (pH 5.5 ± 0.2), in a climate chamber with a constant temperature of 25 ± 2 °C. To exclude pH effects on plant growth, the pH values were checked at the test beginning and the end. The chamber was illuminated continuously with a maximum of 125 μE m^{-2} s^{-1}. The assays were also conducted in Erlenmeyer flasks. All substances were tested at least twice, with a minimum of 6 controls (pure Steinberg medium) for each test. The test started with 12 fronds, endpoints were inhibition of growth rate and frond area calculated in relation to the controls. The growth was determined on the basis of the counted fronds; for the detection of the frond area (mm^2) a Scanalyzer from Lemnatec GmbH (Würselen, Germany) was used.

Effect data modelling

Dose-response curve parameters and plots were obtained using the drfit[71] package (version 0.05-86) for the R language and environment for statistical computing (www.r-project.org).

The HPLC systems

The HPLC system used for deriving the lipophilicity parameters was a Hewlett Packard system Series 1100, with gradient pump, online degasser, autosampler and a Bruker esquire ESI-MS ion trap detector. The column was a MetaChem Polaris Ether bridged RP-18 column with 150 mm length, 3 mm inner diameter and 3 μm particle size. A guard column with octadecylsilica material was also used (both Varian, Inc.). The eluent was composed of 0.25% acetic acid (*p.a.*) in Milipore (TM) water (pH = 3.2), mixed with gradient grade acetonitrile. The column dead time t_o was calculated from the retention time difference of thiourea with and without column. The equipment dwell volume t_D was quantified by switching from water to 0.1 mM $NaNO_3$ in 10 min. Cation retention times from a single gradient run with a gradient time t_G of 10 min were obtained for all substances listed in Table 4. The theoretical background and the calculation of the log k_o values were recently described in detail in ref. 20.

The HPLC system used for determination of lipid–water partitioning was a VWR Hitachi system containing the L-2130 HTA-pump, L-2130 degasser, L-2200 autosampler, L-2300 column oven, L-2450 diodearray-detector and EZChrom Elite software. The used cation exchange column (250/3 NUCLEOSIL 100-5 SA) with guard column was purchased from Macherey–Nagel (Dürren, Germany). The mobile phase was composed of 55% acetonitrile (HPLC grade) and 45% aqueous 20 mM HK_2PO_4–3.9 mM H_3PO_4 buffer. A flow rate of 0.5 mL min^{-1} and a detection wavelength of 212 nm were employed.

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