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Zwitterionic compounds are less ecotoxic than their analogous ionic liquids

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Zwitterionic compounds (ZIs) have been attracting much attention due to their properties. Unlike ionic liquids – constituted by separated ions – the ZIs structure – with the cation and anion covalently bonded – results on an added complexity and diversity, suggesting that their ecotoxicological behavior should probably not be extrapolated from those of ionic liquids. This study addresses the aquatic toxicity of ZIs towards the bacterium *Allivibrio fischeri* and the microalga *Raphidocelis subcapitata*. Sixteen ZIs, comprising five different cationic groups – ammonium, imidazolium, pyridinium, pyrrolidinium and piperidinium – and two anionic groups – sulfonate and carboxylate – were studied, and the relationships between their structure and toxicity are reported. All studied ZIs are harmless or practically harmless for both microalga and bacterium (median effective concentration, EC₅₀ >100 mg·L⁻¹), presenting a significantly lower hazardous potential to aquatic species than their ionic liquids counterparts. The results also show that the increased hydrophobicity of ZIs, promoted by the increase of cation alkyl chain or spacer size, has a significant influence on EC₅₀ values for microalga, reflected on a higher toxicity. However, no significant differences were observed when considering the various cationic groups of the ZIs studied, unlike what is known for the ionic liquids ecotoxicity. Also, no relationships were found between the chemical structure of ZIs and EC₅₀ values estimated for the bacterium *A. fischeri*. The structural differences between ZIs and ionic liquids results into different mechanisms of interaction with microalgae and bacteria membranes, which may explain why the ecotoxicity heuristic rules previously reported for ionic liquids do not seem to apply to ZIs.

Introduction

Solvents are essential in many chemical processes, influencing the environmental performance of chemical industries. Despite their wide application, conventional organic solvents employed in chemical industries present several drawbacks, such as high volatility, flammability, and toxicity, which represents a clear handicap for the development of sustainable processes in line with the Green Chemistry principles. In this context, the search for novel and alternative environmentally benign solvents has gained increased attention.¹ During the past years, ionic liquids gained high relevance as alternative solvents to hazardous volatile organic compounds in the most diverse chemical and engineering applications, such as catalysis, organic synthesis, polymerization, biomaterials dissolution, extraction and separation processes.^{2,3} The studies of ionic liquids lead to the synthesis of novel families of compounds based on them.^{4–6} One such effort was successfully achieved in 2001 when Ohno and co-workers⁷ reported the synthesis and application of zwitterionic compounds (ZIs) derived from ionic liquids as

alternative electrolyte materials. Zwitterions are compounds characterized by the simultaneous presence of both positively and negatively charged groups in the same chemical structure, bearing a total zero net charge. The covalently bonded anionic and cationic groups, which are linked by an alkyl or other type of spacer, confer to the ZIs both a global neutral charge and an extremely high polarity. Zwitterions are widely spread in nature, amino acids being probably the better known example of this type of molecules. The large number of anionic and cationic groups available in the chemical inventory allows the synthesis of a wide range of zwitterionic structures. Most systems studied use, as cationic groups, quaternary ammonium or imidazolium, whereas the most commonly used anionic groups are sulfonate and carboxylate.^{8,9} However, the number of possible structural variations by the modification of headgroups, spacers and hydrophobic tails is theoretically endless, which supports their tunable character. The ZIs may be considered task-specific compounds, *i.e.*, their properties can be tuned by structural modifications, to fit a specific requirement.^{9–13}

After the initial report,⁷ research on the design and development of novel ZIs with high structural similarity to ionic liquids followed, accompanied by their application in novel fields, including separation techniques, material chemistry and biotechnology.^{9,11,13–15} Recently, the use of aqueous solutions of ZIs derived from ionic liquids as alternative solvents in extraction and separation systems was demonstrated. Ferreira

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et al.^{13,14} studied the ability of ammonium-based ZIs (also known as sulfobetaines) to induce the formation of aqueous biphasic systems when mixed with polymers or salts. These systems presented a high performance as selective separation platforms¹³ and allowed the development of integrated bioreaction–separation processes.¹⁴ Kuroda et al.¹¹ also demonstrated how the tunable character of ZIs can be implemented, by designing a specific solvent with a high capacity to dissolve cellulose, while keeping its biocompatible character towards the cell walls.

The increased interest in the application of alternative solvents, like ionic liquids and ZIs, and their consequent industrialization, will result in the release of their residues to aquatic systems, potentially affecting the aquatic biota. As commonly referred, legislation concerning these scenarios is nowadays stricter in Europe as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals)¹⁶ requires ecotoxicological data for all chemicals produced or imported into the European Union above 1 ton/year. Therefore, the optimization of the technical performance must run in parallel with the minimization of hazardous potential, to simultaneously improve the chemical's economic viability and reduce their environmental impact. Nevertheless, and although we recognize the importance of creating ecotoxicological databases to characterize the various classes of compounds allowing their licensing, in this work, we intend to go further and provide experimental evidence on the toxicology of ZIs derived from ionic liquids. In literature, only a few studies dedicated to the environmental behavior of ZIs^{17–23} can be found, and without consistency to be used for the understanding of their ecotoxicity and allowing a comparison with ionic liquids congeners. This work addresses thus the study of the aquatic ecotoxicity of a set of sixteen ZIs, towards the marine bacterium *Allivibrio fischeri* and the freshwater

microalga *Raphidocelis subcapitata*. Included in the set of ZIs under study are some of the most common charged groups (cationic groups: ammonium, imidazolium, pyridinium, pyrrolidinium and piperidinium; anionic groups: sulfonate and carboxylate). The toxicity of these ZIs and its relation to their chemical structure are discussed, and further compared with the ecotoxicity data available in literature or determined in this work for their ionic liquid congeners.

Experimental

Materials

All ZIs presented in Table 1 and Figure 1 were synthesized in our laboratory, following procedures previously reported.^{11–13,24} Triamylamine (TCI, >98.0 wt% pure), tributylamine (Acros-Organics, 99 wt% pure), tri-*N*-propylamine (Alfa Aesar, 98 wt% pure), triethylamine (Fisher Chemical, HPLC grade), trimethylamine (TCI, 13 wt% in ACN *ca* 2M), 1-methylimidazole (Iolitec, 99 wt% pure), methylpyrrolidine (Alfa Aesar, 98 wt% pure), 1-vinylimidazole (TCI, >98.0 wt% pure), pyridine (Sigma-Aldrich, 99.8 wt% pure), 1-ethylpiperidine (Aldrich, 99 wt% pure), 1-butylimidazole (Aldrich, 98 wt% pure), 1,3-propanesultone (TCI, >99 wt% pure), 1,4-butanedisultone (TCI, >99 wt% pure), ethyl 4-bromobutyrate (Sigma-Aldrich, 95 wt% pure) and ethyl 6-bromohexanoate (TCI, > 98 wt% pure) were used as reagents in the synthesis of ZIs. The following solvents and ion-exchange resin were also necessary: acetonitrile (Fisher Chemical, 99.99 wt% pure), diethyl ether (Panreac, 99.8 wt% pure), ethyl acetate (Fisher Scientific, analytical grade), methanol (Fisher Scientific, HPLC grade) and Amberlite® IRN78 hydroxide form (Sigma-Aldrich). After synthesis, the structure and purity of the ZIs was evaluated by ¹H NMR spectroscopy.

Table 1. Name, acronym, and properties – molecular weight (M_w) and logarithmic function of the octanol-water partition coefficient (K_{ow}) – of the studied ZIs.

ID	Name	Acronym	M_w (g·mol ⁻¹)	$\log(K_{ow})^{25}$	Ref.
i	3-(tripentylammonio)propane-1-sulfonate	N ₅₅₅ C3S	349.57	2.44	¹²
ii	3-(tributylammonio)propane-1-sulfonate	N ₄₄₄ C3S	307.49	1.10	This work
iii	3-(tripropylammonio)propane-1-sulfonate	N ₃₃₃ C3S	265.41	-0.23	¹²
iv	4-(triethylammonio)butane-1-sulfonate	N ₂₂₂ C4S	237.36	-1.28	²²
v	3-(triethylammonio)propane-1-sulfonate	N ₂₂₂ C3S	223.33	-1.80	¹²
vi	3-(triethylammonio)propane-1-carboxylate	N ₂₂₂ C3C	187.28	-2.13	This work
vii	4-(trimethylammonio)butane-1-sulfonate	N ₁₁₁ C4S	195.28	-2.35	¹²
viii	3-(trimethylammonio)propane-1-sulfonate	N ₁₁₁ C3S	181.25	-2.87	¹²
ix	3-(1-butylimidazolium-3-yl)propane-1-carboxylate	C ₄ ImC3C	210.28	-2.37	This work
x	5-(1-methylimidazolium-3-yl)pentane-1-carboxylate	C ₁ ImC5C	197.26	-2.58	This work
xi	4-(1-methylimidazolium-3-yl)butane-1-sulfonate	C ₁ ImC4S	218.27	-5.05	²²
xii	3-(1-methylimidazolium-3-yl)propane-1-carboxylate	C ₁ ImC3C	168.20	-3.70	¹⁰
xiii	4-(1-vinylimidazolium-3-yl)butane-1-sulfonate	VImC4S	230.28	-4.54	²²
xiv	4-(1-methylpyrrolidinium-1-yl)butane-1-sulfonate	C ₁ PyrC4S	221.32	-1.95	²²
xv	4-(pyridinium-1-yl)butane-1-sulfonate	PyC4S	215.27	-4.77	²²
xvi	4-(1-ethylpiperidinium-1-yl)butane-1-sulfonate	C ₂ PipC4S	249.37	-1.14	²²

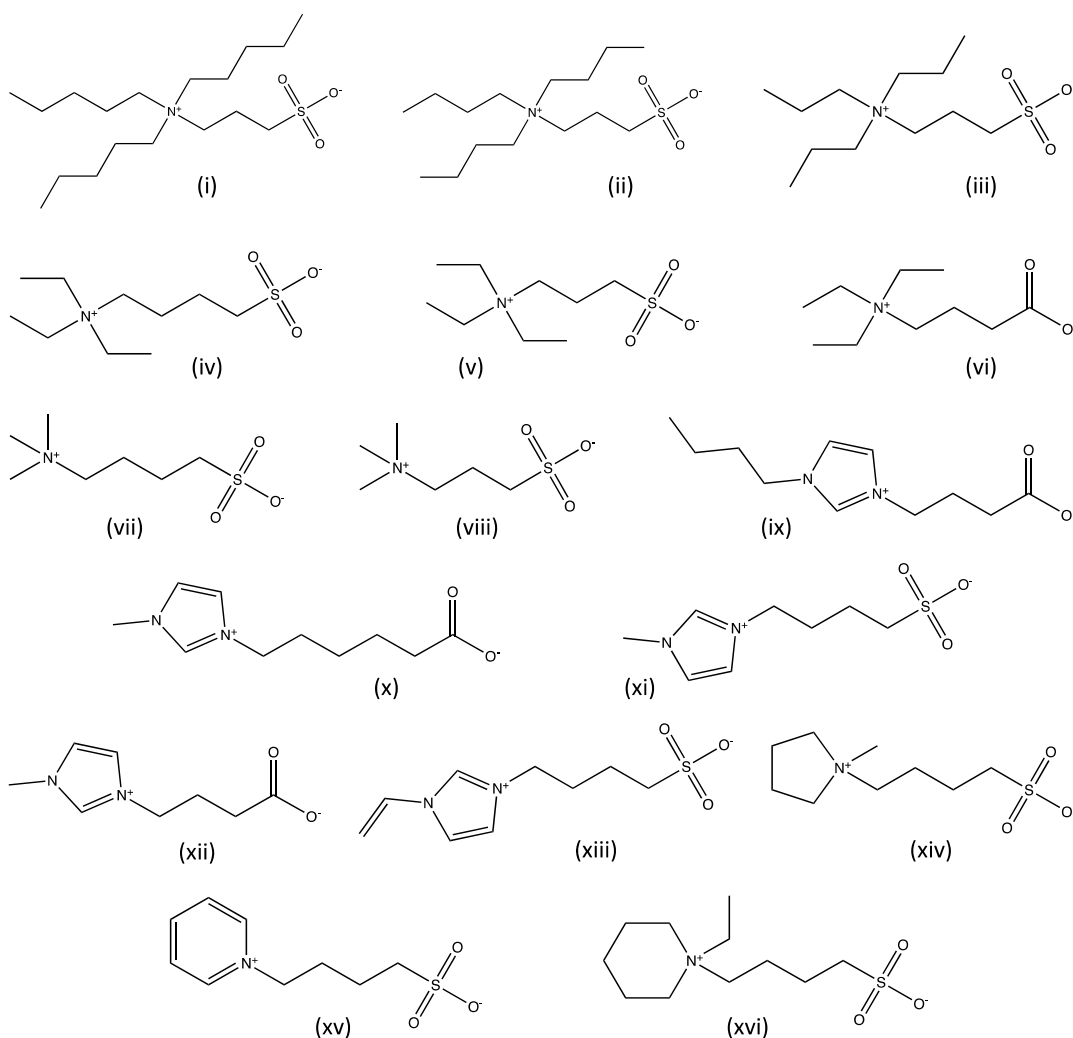


Fig. 1. ZIs chemical structures and respective IDs. Names and acronyms are presented in Table 1.

The chemical structures of the ZIs, $N_{444}C3S$, $N_{222}C3C$, C_4ImC3C , and C_1ImC5C were confirmed by 1H NMR spectroscopy and elemental analysis, and their thermal properties were determined by differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA) (*cf.* the Electronic Supplementary Information, ESI), since these are reported for the first time in this work. The water content of each ZI was confirmed by Karl Fischer titration and verified to be always below 1 wt%. Trimethylglycine ($N_{111}C1C$), commonly designated as betaine, was included in this ecotoxicological assessment for comparison purposes, as some of the studied ZIs share the same cationic and anionic groups as betaine. Betaine, 98 wt% pure, supplied by Acros Organics was used without further purification. The ecotoxicity of the ionic liquids, namely tetrabutylammonium chloride ($[N_{4444}]Cl$, Sigma-Aldrich, 97 wt% pure), tetrabutylammonium methanesulfonate ($[N_{4444}][C_1SO_3]$, Sigma-Aldrich, ≥ 97 wt% pure), 1-ethyl-3-methylimidazolium methanesulfonate ($[C_2C_1Im][C_1SO_3]$, lolitec, 99 wt% pure), 1-

ethyl-3-methylimidazolium acetate ($[C_2C_1im][C_1CO_2]$, lolitec, 98 wt% pure) and 1-ethyl-3-methylpyrrolidinium methanesulfonate ($[C_2C_1Pyr][C_1SO_3]$, lolitec, >97 wt% pure) were also evaluated for comparison purposes.

Methods

Toxicity to *Raphidocelis subcapitata*

Bulk cultures of the freshwater microalga *R. subcapitata* were maintained in batch cultures of Woods Hole MBL medium at $(20 \pm 2)^\circ C$, with continuous aeration, under a 16h:8h (light:dark) photoperiod cycle. Four days before starting the tests, an inoculum was harvested from the bulk microalgae culture and incubated at $(23 \pm 1)^\circ C$ under continuous illumination (cool-white fluorescent light, intensity about 7000 lux). This inoculum was used to start the test, which initiated with 1.0×10^4 cells mL^{-1} of *R. subcapitata* in the log exponential growth phase, following the OECD guideline N201²⁶ adapted to the use of 24-well

microplates.²⁷ Microalgae were exposed to a geometric range of concentrations of each chemical, which was obtained by dilution of a stock solution. Both the stock solution and the dilutions were prepared using Woods Hole MBL medium. Each treatment consisted of a blank control (test solution with no algae), an algae control (no chemical) and three replicates of the tested concentration. The test conditions were the same as mentioned above for the inoculum. After 96 h of exposure, the absorbance at 440 nm was measured (Shimadzu UV-1800) and used to estimate the microalgae densities using a previously established calibration curve.²⁸ To exclude any interference in the absorbance, each respective blank control was used as the blank in the absorbance measurement of each replicate. Since the ZI C₂PipC4S, betaine and the ionic liquid [C₂C₁Im][C₁CO₂] interfere with the absorbance measurements at 440 nm, microalgae densities for these chemicals were determined by counting under a microscope in a Neubauer chamber. Cell densities were used for yield calculations.

Toxicity to *Allivibrio fischeri*

Acute toxicity to the Gram-negative bacteria *A. fischeri* was assessed using the Microtox® Toxicity Test (Microtox® M500 analyzer, Modern Water, UK), by determining the luminescence inhibition after 30 min of exposure to each compound. Bacteria, purchased freeze-dried, were activated by rehydration, and exposed to a range of diluted aqueous solutions (from 0 to 81.9 % in volume) of each compound. Dilutions were made following the manufacturer protocol with the supplied diluent reagent (2 wt% NaCl solution in water), thus ensuring optimal osmotic conditions for the bacteria. The light emission of the bacteria was measured and compared to that of the blank control (bacteria in blank diluent), to determine the light emission relative to control.

Statistical analysis

The obtained data (light emission relative to control for *A. fischeri*, and yield for *R. subcapitata*) were used to estimate the effective concentrations (EC₅₀ and EC₂₀ values, *i.e.* concentrations eliciting 50% and 20% of effect, respectively) and the corresponding 95% confidence intervals. This estimative was achieved by fitting the data to the logistic equation, using the least-squares method (non-linear regression) in the STATISTICA software package (version 8, StatSoft Inc., USA). Correlations were assessed by Pearson's correlation (Statistica, StatSoft). An alpha of 0.05 was used in all analyses.

Results and discussion

A total of sixteen ZIs constituted by five different cationic (ammonium, imidazolium, pyridinium, pyrrolidinium and piperidinium) and two anionic (sulfonate and carboxylate) groups were studied on this work (see Table 1 and Figure 1). The EC₅₀ values of ZIs for *R. subcapitata* (96 h of exposure) and *A. fischeri* (30 min of exposure) are given in Table 2. For a more detailed view on the ecotoxicological profile of these ZIs, EC₂₀ values are additionally provided in Table S1 of the ESI. All experimental data are in mass units (g·L⁻¹) to be in line with the toxicological categories adopted by the European Commission²⁹ and to facilitate the comparison with data previously reported in literature, which is commonly presented in weight *per* volume units.

A global evaluation of the EC₅₀ values (Table 2) suggests that none of these chemicals could be considered hazardous to the aquatic environment according to the United Nations Globally Harmonized System of Classification and Labelling Chemicals (GHS),³⁰ as their EC₅₀ values are above 100 mg·L⁻¹. Such a low toxicity of the tested compounds agrees with the low toxicity commonly attributed to ZIs.^{9,10,31} This is particularly valid for ZIs

Table 2. Median effective concentration (EC₅₀) values and the respective 95% confidence intervals (within brackets), obtained for *R. subcapitata* after 96 h of exposure (EC₅₀-96 h) and *A. fischeri* after 30 min of exposure (EC₅₀-30 min) to different ZIs, including betaine.

ZI	<i>R. subcapitata</i> EC ₅₀ -96 h (g·L ⁻¹)	<i>A. fischeri</i> EC ₅₀ -30 min (g·L ⁻¹)
N ₅₅₅ C3S	0.1354 (0.1051 – 0.1657)	1.962 (1.500 – 2.425)
N ₄₄₄ C3S	0.9867 (0.8512 – 1.122)	2.534 (2.370 – 2.698)
N ₃₃₃ C3S	2.936 (2.728 – 3.144)	0.6714 (0.6314 – 0.7113)
N ₂₂₂ C4S	21.13 (18.38 – 23.87)	55.79 (37.55 – 74.03)
N ₂₂₂ C3S	41.26 (37.15 – 45.37)	1.527 (1.178 – 1.877)
N ₂₂₂ C3C	22.39 (18.93 – 25.84)	106.3 (91.08 – 121.5)
N ₁₁₁ C4S	48.85 (43.34 – 54.37)	1.327 (1.233 – 1.422)
N ₁₁₁ C3S	46.83 (41.84 – 51.81)	1.756 (1.581 – 1.930)
C ₄ ImC3C	16.44 (14.61 – 18.28)	22.43 (19.14 – 25.72)
C ₁ ImC5C	2.744 (2.265 – 3.223)	13.40 (11.98 – 14.83)
C ₁ ImC4S	28.34 (26.22 – 30.45)	3.417 (2.420 – 4.414)
C ₁ ImC3C	13.07 (12.39 – 13.75)	13.42 (11.01 – 15.83)
ViImC4S	29.54 (27.35 – 31.73)	10.69 (8.068 – 13.32)
C ₁ PyrC4S	27.21 (23.66 – 30.76)	231.1 (123.7 – 338.5)
PyC4S	19.88 (18.24 – 21.52)	75.91 (61.85 – 89.97)
C ₂ PipC4S	13.70 (12.71 – 14.70)	6.981 (3.777 – 10.18)
Betaine (N ₁₁₁ C1C)	19.19 (16.52 – 21.86)	141.6 (129.7 – 153.5)

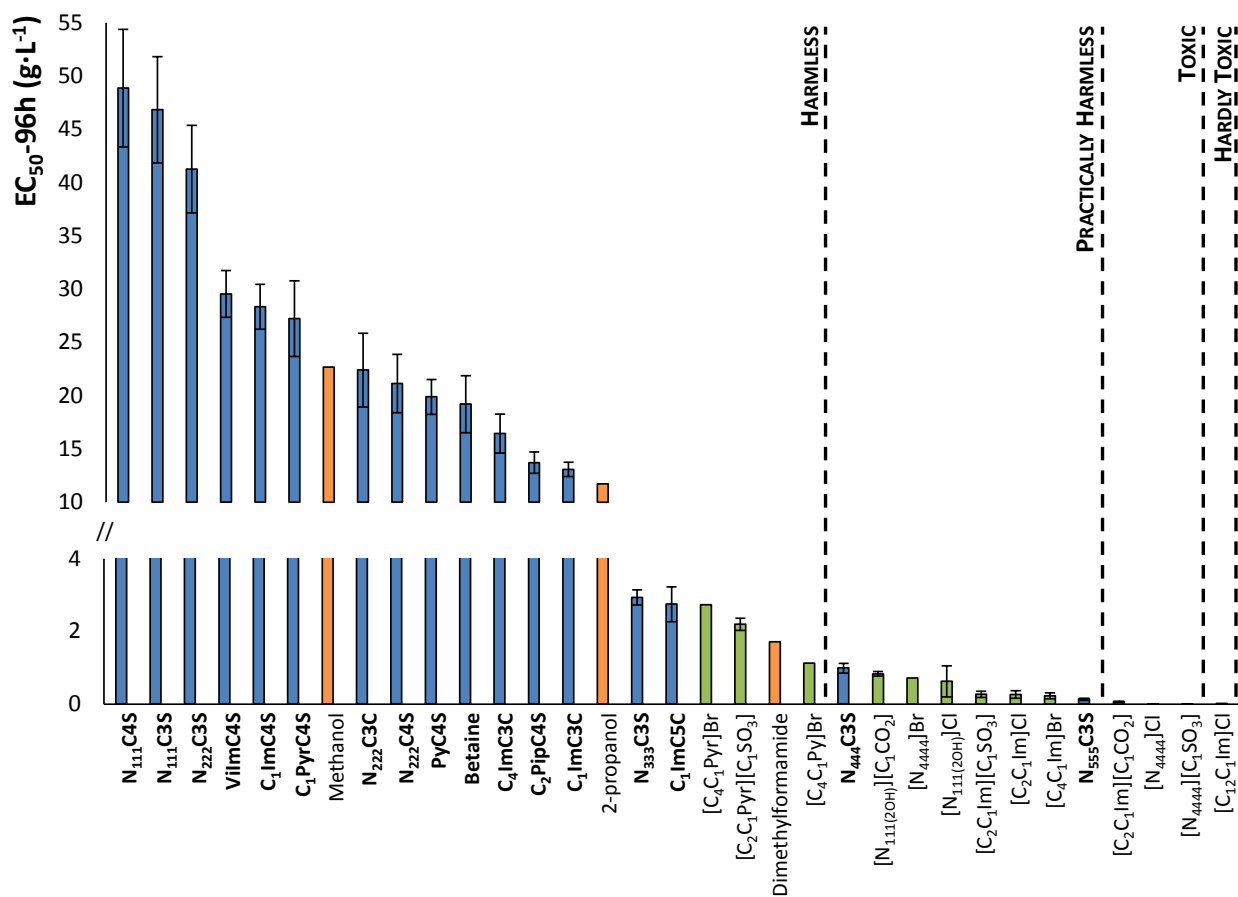


Fig. 2. Median effective concentration (EC₅₀), in g·L⁻¹, obtained for the microalga *R. subcapitata* after 96 h of exposure to several zwitterionic compounds (blue bars), ordered by increasing toxicity. The results for betaine (determined in this work), and some ionic liquids of similar structure^{27,32–34} (green bars) (cf. ESI) and organic solvents³² (orange bars) are also represented. The error bars represent standard error. The hazard classification was adapted from Passino and Smith, 1987.³⁵

with short alkyl chains, as the elongation of alkyl chains seems to promote an increase in toxicity, as previously observed for sulfobetaines¹⁸ – ZIs composed of ammonium-based cationic groups and sulfonate anionic groups – and alkyl dimethyl amine oxides.¹⁷ The increased toxicity with the elongation of the alkyl chains reveals an effect of the ZIs chemical structure on their toxicity. To better understand these effects, a detailed analysis of the relationship between the structural features of the ZIs chemical (cationic and anionic groups, and spacer length) and EC₅₀ values determined for both *R. subcapitata* and *A. fischeri* will be carried out in the following sections.

Toxicity to *R. subcapitata*

The median effective concentrations of the tested ZIs to the microalga *R. subcapitata* are presented in Figure 2. For comparison, in this figure are also presented the results obtained for betaine – a well-known naturally occurring, non-toxic and biodegradable zwitterionic compound – and ionic liquids of similar chemical structure^{27,32–34} and some organic solvents.³² More details about name, acronym and chemical

structure of ionic liquids used in this work for comparison purposes can be found in Table S2 and Figure S1.

According to the Global Harmonized System (GHS) of classification and labelling of chemicals by the United Nations UN³⁰ all studied ZIs are considered not hazardous to the aquatic environment, concerning short-term (acute) aquatic hazard, as the EC₅₀-96h values are above 100 mg·L⁻¹. Furthermore, the results presented in Figure 2 show that ZIs exhibit higher EC₅₀ values than the correspondent ionic liquids, which are considered practically harmless or hardly toxic in accordance with the Passino & Smith³⁵ classification: C₁PyrC₄S (27.21 g·L⁻¹) vs [C₂C₁Pyr][C₁SO₃] (2.198 g·L⁻¹); C₁ImC₄S (28.34 g·L⁻¹) vs [C₂C₁Im][C₁SO₃] (0.2732 g·L⁻¹); C₁ImC₃C (13.07 g·L⁻¹) vs [C₂C₁Im][C₁CO₂] (0.074 g·L⁻¹); N₄₄₄C₃S (0.9867 g·L⁻¹) vs [N₄₄₄₄4][C₁SO₃] (1×10⁻³ g·L⁻¹). On the other hand, organic solvents such as methanol, 2-propanol and dimethylformamide, exhibited intermediate EC₅₀ values between the ZIs and the ionic liquids.

The EC₅₀ of ZIs varied 361-fold, with N₅₅₅C₃S presenting the lowest value (0.1354 g·L⁻¹) and N₁₁₁C₄S the highest one (48.85 g·L⁻¹). Such a wide range suggests a role of the chemical

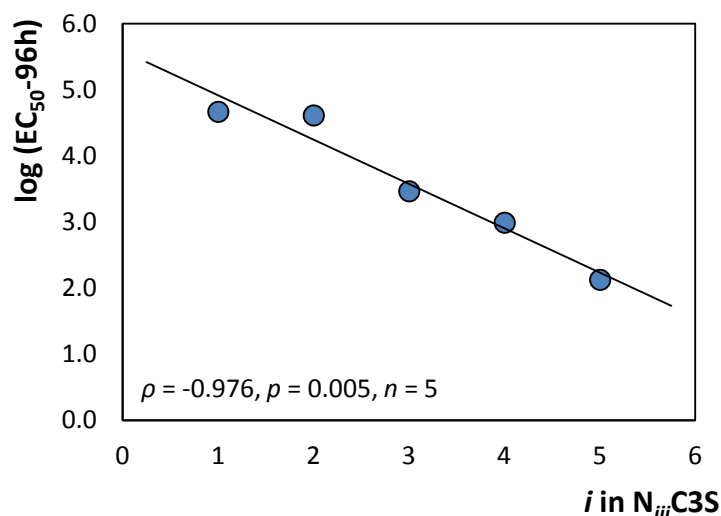


Fig. 3. Linear relationship between the logarithmic function (\log_{10}) of EC_{50-96h} ($g \cdot L^{-1}$) for *R. subcapitata* and the cationic group alkyl chain length (i) for $N_{iii}C3S$ ZIs.

structure and thus chemical properties, on the environmental profile of the tested ZIs. Indeed, despite the harmless character of ZIs for the microalga, it is possible to identify some dependency of the EC_{50} values on their chemical structures – cf. Figure 2. Considering the sulfobetaines ($N_{iii}C3S$ and $N_{iii}C4S$), it is clear that the length of the alkyl chains in the cationic group has a pronounced effect on the EC_{50} values. The increase of alkyl chain from 1 ($N_{111}C3S$) to 5 carbons ($N_{555}C3S$) lead to a decrease of the EC_{50} by 2 orders of magnitude (346-fold). This trend was already reported for microalgae, invertebrates and/or fishes exposed to sulfobetaines,^{19,22,36,37} amine oxides,^{17,20,21} quaternary ammonium surfactants,³⁸ and ionic liquids.^{39–41} It is true that the increase of the alkyl chain length in ZIs leads to an increased hydrophobicity, as given by the logarithmic function of the octanol-water partition coefficient ($\log(K_{OW})$) values presented in Table 1 (for a hydrophobic compound $\log(K_{OW}) > 0$). Indeed, a similar trend was previously observed for the ecotoxicity of ionic liquids for the same aquatic species.⁴² This relationship is supported by the linear correlation found between the logarithm function of EC_{50} and the number of carbons in alkyl chains of the sulfobetaines family ($N_{iii}C3S$) depicted in Figure 3 (Pearson correlation, $\rho = -0.976$, $p = 0.005$, $n = 5$). However, for ZIs based in the imidazolium cations and carboxylate anions, the elongation of alkyl chain from 1 (C_1ImC3C) to 4 carbons (C_4ImC3C) induces only a slight decrease in the EC_{50} values (1.2-fold), despite the pronounced hydrophobicity augment (cf. Table 1), suggesting that hydrophobicity changes do not always affect in the same extent the (eco)toxicity of a compound.

Concerning the size of the spacer between the cationic and anionic groups (cf. Figure 2) – $N_{111}C3S$ vs $N_{111}C4S$, $N_{222}C3S$ vs $N_{222}C4S$ and C_1ImC3C vs C_1ImC5C – our results suggest that the increase in the spacer length translates into a decreased on the EC_{50} values, except for the pair $N_{111}C3S$ vs $N_{111}C4S$. Probably,

this trend is also related with the hydrophobicity of ZIs, in accordance with data previously reported by Davies et al.¹⁸ concerning the toxicity of sulfobetaines to *Daphnia magna*. Indeed, the small difference in hydrophobicity between $N_{111}C3S$ and $N_{111}C4S$ (cf. Table 1) should explain the negligible effect of the spacer length in the (eco)toxicity of these ZIs to microalga. Different cationic groups were studied; among them ammonium, imidazolium, pyrrolidinium, pyridinium and piperidinium. However, it was not possible to identify any specific trend regarding the impact of this structural feature on the EC_{50} values (cf. Figure 2). The cation confers to the ZIs an aromatic or non-aromatic character. ZIs based on imidazolium and pyridinium families are aromatic, whereas ZIs derived from ammonium, pyrrolidinium and piperidinium families are non-aromatic (cf. Figure 1). The role of ZIs hydrophobicity on the EC_{50} values for the whole EC_{50-96h} dataset is depicted in Figure 4, distinguishing both aromatic and non-aromatic ZIs. For non-aromatic ZIs, the correlation between hydrophobicity (expressed as $\log(K_{OW})$), and toxicity (as $\log(EC_{50})$), is strong and significant (Pearson correlation: $\rho = -0.9814$, $p < 0.001$, $n = 10$) but weak and non-significant for the aromatic ones ($\rho = 0.121$, $n = 6$). For the latter, a pronounced decrease of $\log(K_{OW})$ does not translate into an increase of EC_{50} values.

A linear relationship between hydrophobicity and toxicity of non-aromatic ZIs was reported in a previous study, which proposed to use $\log(K_{OW})$ as a single physicochemical property for defining sulfobetaines toxicity to the freshwater planktonic crustacean *Daphnia magna*.³⁶ However, this study was carried out for quaternary alkylammonium sulfobetaines with longer radicals than those used here. In the same study, it was proposed that ZIs with lower molecular weight can cross the cellular membrane directly or be carried by an ion channel, which may outweigh the dependence of the (eco)toxicity on hydrophobicity.³⁶ This might explain why ZIs with similar

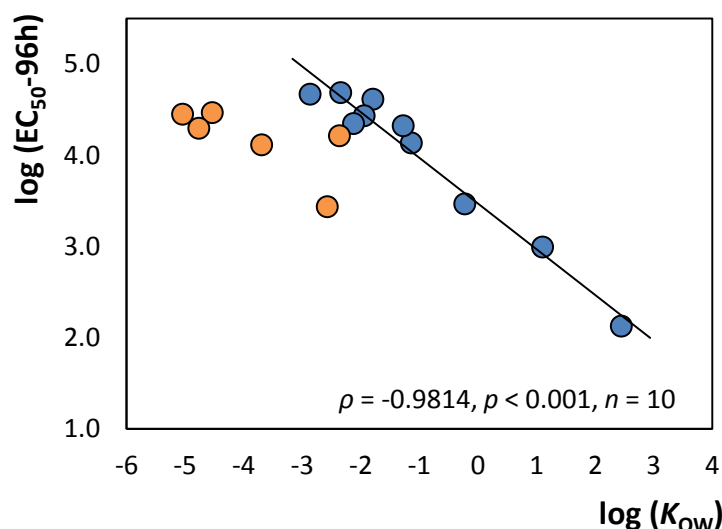


Fig. 4. Relationship between the logarithmic function of the octanol-water partition coefficient of ZIs (expressed as $\log(K_{OW})$) and the corresponding toxicity (expressed as $\log(EC_{50-96h})$, $g \cdot L^{-1}$) for *R. subcapitata*. Aromatic ZIs correspond to the orange symbols and non-aromatic ZIs correspond to the blue symbols. The correlation represented by the black straight line (statistical summary presented below) refers only to the latter.

$\log(K_{OW})$, but structurally different, can exhibit significantly different toxicities, as is the case of $N_{111}C4S$ and C_4ImC3C ($\log(K_{OW}) = -2.35$ and -2.37 , and $EC_{50} = 48.85$ and $16.44 g \cdot L^{-1}$, respectively). It is interesting to note that the studied aromatic mechanism driving the organisms' response may be taking place here. Nevertheless, in order to clarify whether the relationship between hydrophobicity and (eco)toxicity is significant for aromatic ZIs, further ecotoxicological tests should be performed. Interestingly, the opposite trend was observed for ionic liquids, with aromatic compounds (e.g. $[C_4C_1Im]Br$) exhibiting higher toxicity than the non-aromatic ones (e.g. $[C_4C_1Pyr]Br$). This was observed for the *R. subcapitata* microalga^{43,44} and other freshwater species,^{43–46} and it was explained by the increased water solubility of ionic liquid-based aromatic cations.⁴⁴

The effect of ZIs anionic group (sulfonate vs carboxylate) can be addressed by comparing the toxicity of $N_{222}C3C$ and $N_{222}C3S$ – cf. Figure 2. Following their differences in hydrophobicity ($\log(K_{OW}) = -2.13$ and -1.80 , respectively), a higher EC_{50} value was expected for $N_{222}C3C$ than for $N_{222}C3S$, which was not observed ($EC_{50} = 22.39$ and $41.26 g \cdot L^{-1}$, respectively). Nevertheless, due to the higher hydrophilic character of the carboxylate group, carboxybetaines tend to present larger solubility in water and lower self-association effects,⁹ which may result in a higher interaction of ZIs with the microalga membrane.

Toxicity to *A. fischeri*

The median effective concentrations estimated for the ZIs following exposure of the bacterium *A. fischeri* are presented in Figure 5. The results obtained for betaine and previously reported for structurally similar ionic liquids^{33,45,47–50} and organic solvents^{51,52} are also presented for comparison. The

EC_{50} values of studied ZIs varied by 344-fold, with $N_{333}C3S$ presenting the lowest ($EC_{50} = 0.6714 g \cdot L^{-1}$) and $C_1PyrC4S$ the highest value ($EC_{50} = 231.1 g \cdot L^{-1}$), thus a range similar to that observed for the microalga. Following the hazard ranking from Passino & Smith,³⁵ all ZIs are harmless or practically harmless also for the bacterium. Furthermore, the equivalent ionic liquids used for comparison (cf. Table S2 of ESI) exhibit significantly higher toxicity than the studied ZIs: for example, $C_1PyrC4S$ ($231.1 g \cdot L^{-1}$) vs $[C_2C_1Pyr][C_1SO_3]$ ($1.798 g \cdot L^{-1}$); C_1ImC4S ($3.417 g \cdot L^{-1}$) vs $[C_2C_1Im][C_1SO_3]$ ($0.5355 g \cdot L^{-1}$); C_1ImC3C ($13.42 g \cdot L^{-1}$) vs $[C_2C_1Im][C_1CO_2]$ ($0.9133 g \cdot L^{-1}$); $N_{444}C3S$ ($2.534 g \cdot L^{-1}$) vs $[N_{444}][C_1SO_3]$ ($0.2377 g \cdot L^{-1}$), again showing the hazardous behavior of ionic liquids towards the ZIs. The determined EC_{50-30} min of betaine to *A. fischeri* ($141.6 mg \cdot L^{-1}$) is lower than the value reported in a previous study ($242 mg \cdot L^{-1}$);⁵³ but the fact that the exposure period in that work was not clarified prevents further comparison. The reduced antimicrobial activity of ZIs, in particular of sulfobetaines, is in agreement with the results of a previous study, in which sulfobetaines were shown to exhibit a lower toxicity to bacterium than quaternary ammonium salts.⁵⁴ Unlike to what was observed for the median effective concentration of ZIs to the freshwater microalga, it is difficult to identify any trend regarding the toxicity dependency for the bacterium on the ZI chemical structures. The ZIs harmless character may explain the absence of clear trends in the data obtained, although the quantification of the EC_{50} values was well established. Moreover, previous works concerning the ecotoxicity of other type of solvents, which also record very high EC_{50} values for bacterium, were able to observe a relationship between the toxicity and the chemical structure of the compounds under study. However, the trends reported are

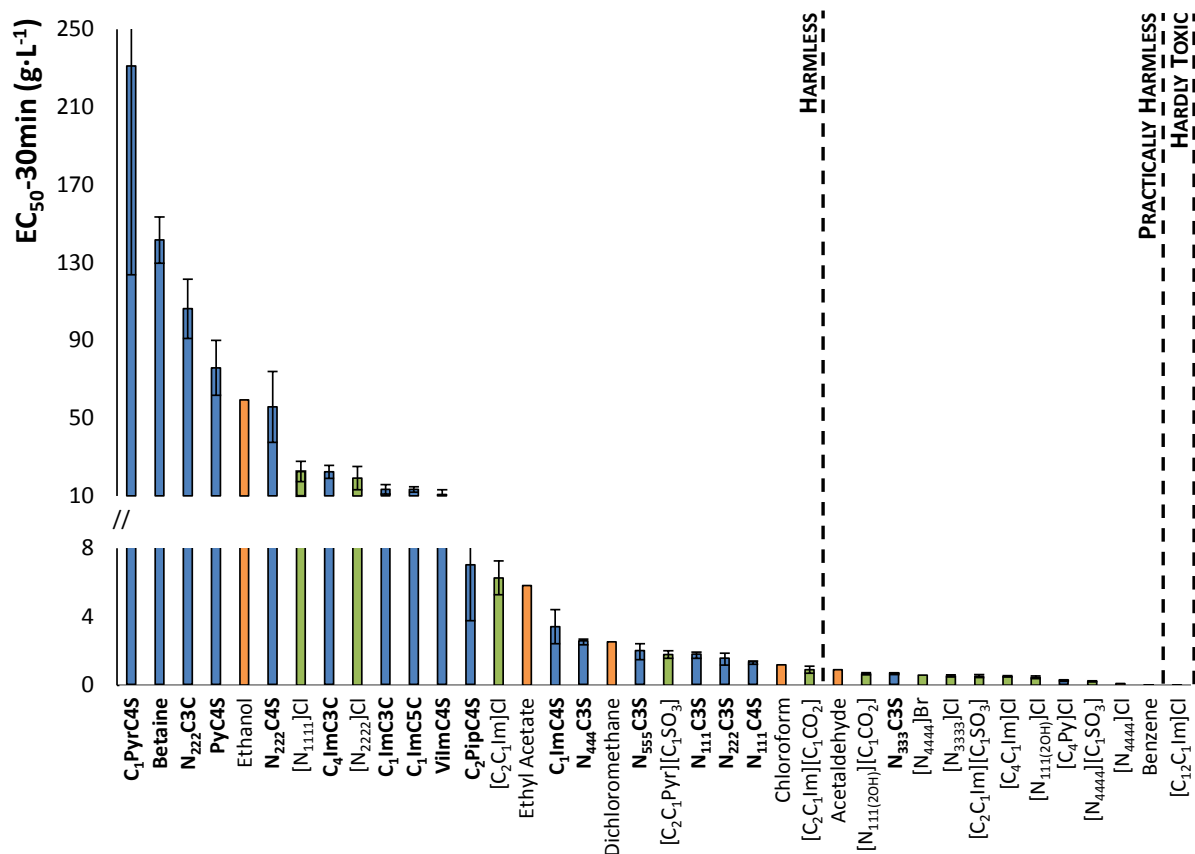


Fig. 5. Median effective concentration (EC₅₀), in g·L⁻¹, obtained after 30 min of exposure of the bacteria *A. fischeri* to each ZI tested, ordered by the level of toxicity. The results for betaine (determined in this work), some ionic liquids of similar structure^{33,45,47–50} (cf. ESI) and organic solvents^{51,52} – some considering 15 min of exposure – are also presented. The error bars represent standard error.

variable according to the compounds and biological species used. For instance, *A. fischeri* exposed to amine oxides showed increased EC₅₀ values with increased alkyl chain length from 12 to 14 carbons, which is the opposite trend from that reported for freshwater organisms, such as *Daphnia magna* and *R. subcapitata*.^{20,21} On the other hand, for several Gram-positive and Gram-negative bacteria, increased toxicity of sulfobetaines was caused by increasing chain length from 10 to 16 carbons.⁵⁵ In particular, sulfobetaines with 10 carbon atoms did not exhibit antimicrobial activity to the tested bacteria.⁵⁵ The same trend was observed for bacteria exposed to ZIs composed of both a pyridinium and a sulfonate charged groups.⁵⁶ Here, the following trends were observed: EC₅₀ of N₄₄₄C3S > N₅₅₅C3S ≥ N₁₁₁C3S ≥ N₂₂₂C3S > N₃₃₃C3S and C₄ImC3C > C₁ImC3C. Applying the rationale used before there is an apparent inconsistency on the trend observed. For the sulfobetaines, there is a decrease of EC₅₀ values from N₁₁₁C3S to N₃₃₃C3S but, comparing the EC₅₀ values of N₃₃₃C3S and N₄₄₄C3S, there is a significant increase of EC₅₀ with the alkyl chain length. Some authors suggested that this behavior may be related with self-aggregation effects that may become more pronounced as the size of cationic group

alkyl chain increases.⁹ Considering the reported data for the ecotoxicity of tetraalkylammonium chloride ionic liquids ([N_{iiii}]Cl) to the bacteria *A. fischeri*,⁴⁷ where alkyl chains varying from 1 to 3 carbons were tested, and the experimental value determined in this work for [N₄₄₄₄]Cl (cf. Table S1), it is possible to conclude that the EC₅₀ of the ionic liquids decreases continually with the increase of cation alkyl chain length from 1 to 4 carbons, presenting a distinct behavior to the one here presented for ZIs. Nevertheless, similar trend shifts in ZIs properties/behavior were previously reported.¹³ Concerning imidazolium-based ZIs, a trend for decreased toxicity with increasing hydrophobicity can be identified through a strong correlation between the two variables, but this correlation was not found to be significant, possibly due to the low sample size ($\rho = 0.844$, $p = 0.072$, $n = 5$). When comparing the anionic groups, carboxylate and sulfonate, is the former that results in higher EC₅₀ values, probably due to the higher hydrophilicity of carboxylated ZIs. Interestingly, both trends are the opposite to that observed for the microalga. While further studies are needed to better understand how these compounds interact with this type of microorganism, the role of the salinity

of the test medium in modulating the observed trends should not be ruled out. The presence of electrolytes/salts may interfere with the complex balance of forces governing the structure and the solution properties of ZIs,^{9,10} and consequently the resultant toxicity. However, the exposure to the ZIs was carried out under the same conditions of salinity, which allows comparing the ZIs toxicity data.

Despite the harmless character of studied ZIs for *A. fischeri*, most ZIs are commonly reported as antibacterial agents.^{55–58} Their mechanism of toxic action against Gram-negative bacteria is assumed to be bacteriostasis, indicating that ZIs may interfere with the bacterial metabolism or ability to reproduce, but do not destroy their cell membrane.⁵⁷ Concerning their mechanism of toxic action to aquatic species in general, it is believed to occur through narcosis, a nonspecific disturbance of the membrane integrity and functioning, resulting from the partition of pollutants into biological membranes.⁵⁹ ZIs are suggested to act as polar narcotics,³⁶ also known as “baseline toxicants”. When considering sulfobetaines, the toxicity mechanism suggested by Davies et al.³⁶ is supported by other studies concerning pharmaceutical zwitterionic compounds.^{60,61}

The differences in the toxicity of different chemicals to Gram-negative bacteria like *A. fischeri* can be attributed to differences in the molecular interactions between the chemical and key cellular events, namely (i) the mechanisms involved in the uptake, *i.e.*, concerning composition of the cell wall, transport and diffusion across the membrane, (ii) changes induced by the toxic chemicals in metabolic pathways which supply cell energy, and (iii) interactions with the luciferase complex which is responsible for the luminescence.⁶² The cell wall limits the uptake and further toxic effects of chemicals. In the Gram-negative bacteria, the cell wall is characterized by the presence of an external membrane, exhibiting negatively charge lipopolysaccharides in the surface. This external membrane, which is not present in Gram-positive bacteria, has been assumed as the main responsible for the decreased sensitivity of Gram-negative bacteria to chemicals, compared to Gram-positive bacteria.^{55,63}

The complex interactions of ZIs with the components of the cell wall allied to chemical interactions with the medium¹⁰ might explain the lack of obvious trends in the toxicity of ZIs to *A. fischeri*. The non-hazardous character of the tested ZIs to the bacterium and the macroalga are quite promising in supporting of their environmentally friendliness, which allied to their interesting properties, contributes to the attractiveness of these compounds. However, to address the limited knowledge on their ecotoxicological assessment, future studies should address their chronic toxicity, biodegradability and potential for bioaccumulation.

Conclusions

The ecotoxicity of sixteen hydrophilic ZIs composed of cationic and anionic groups commonly found in ionic liquids, was here determined considering the microalga *R. subcapitata* and the bacterium *A. fischeri*. The studied ZIs were shown to present a

harmless character to both tested aquatic species, with EC₅₀ values above 100 mg·L⁻¹. Their EC₅₀ values to the freshwater microalgae *R. subcapitata* could be related to the chemical structure through the featured hydrophobicity (most pronounced for non-aromatic ZIs), following the general heuristic rule of increased toxicity with increased hydrophobicity. However, no general consistent relationships were found for the marine bacteria *A. fischeri*, suggesting that the chemical structure may have no relevant toxicity repercussions depending on the test model. Furthermore, despite the structural similarity between the studied ZIs and some ionic liquids, the heuristic rules commonly used to explain the ecotoxicological behavior of most ionic liquids, are not able to explain the behavior of all ZIs.

When compared with their ionic liquid counterparts, the ZIs studied present a more benign character, supporting the idea that these compounds may become attractive in several chemical and engineering applications. Nevertheless, a deeper understanding of the ZIs toxic behavior and underlying mechanisms is required for their more comprehensive ecotoxicological profiling. This effort will certainly allow the establishment of a more robust basis for the rational design of sustainable ZIs.

Conflicts of interest

There are no conflicts of interest to declare.

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