Ecotoxicology and Environmental Safety 108 (2014) 302–310

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Sustainable design for environment-friendly mono and dicationic cholinium-based ionic liquids

Francisca A. e Silva [a,1], Filipa Siopa [b,1], Bruna F.H.T. Figueiredo [a], Ana M.M. Gonçalves [c,d], Joana L. Pereira [c], Fernando Gonçalves [c], João A.P. Coutinho [a], Carlos A.M. Afonso [b], Sónia P.M. Ventura [a,*]

[a] CICECO, Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[b] Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal
[c] Departamento de Biologia, CESAM (Centro de Estudos do Ambiente e do Mar), Universidade de Aveiro, 3810-193 Aveiro, Portugal
[d] IMAR-Centro do Mar e Ambiente (IMAR CMA), Departamento das Ciências da Vida, Universidade de Coimbra, 3004-517 Coimbra, Portugal

ARTICLE INFO

Article history:
Received 31 March 2014
Received in revised form
27 June 2014
Accepted 3 July 2014
Available online 7 August 2014

Keywords:
Monocationic
Dicationic
Ionic liquids
Cholinium
Microtox® test
Median effect concentration (EC_{50})

ABSTRACT

Cholinium-based ionic liquids are receiving crescent interest in diverse areas of application given their biological compatibility and potential for industrial application. In this work, mono and dicationic cholinium ionic liquids as well as cholinium derivatives were synthesized and their toxicity assessed using the luminescent bacteria *Vibrio fischeri*. A range of cholinium derivatives was synthesized, using different amines and the correspondent brominated derivatives, through the alkylation of the amine with the halide in MeCN. The results indicate that their toxicity is highly dependent on the structural modifications of the cholinium cation, mainly related to the alkyl side or linkage chain length, number of hydroxyethyl groups and insertion of carbon–carbon multiple bonds. The data indicated that it is possible to perform environmentally advantageous structural alterations, namely the addition of double bonds, which would not negatively affect *V. fischeri*. Moreover, the dicationic compounds revealed a significantly lower toxicity than the monocationic counterparts. The picture emerging from the results supports the idea that cholinium derivatives are promising ionic liquids with a low environmental impact, emphasizing the importance of a careful and directed design of ionic liquid structures.

1. Introduction

"Green Chemistry" is commonly defined as the "design of chemical products and processes which reduce or eliminate the use and generation of hazardous substances" (Anastas and Warner, 1998). Notable efforts have been made focusing on the design of safer and more environmentally benign solvents, having in mind their increasing importance in the development of clean manufacturing processes. Because conventional organic solvents are often toxic, flammable and volatile, there is a crescent need for the search of new solvents and reaction media. In the last 10 years, ionic liquids (ILs) were the focus of several studies contemplating diverse areas of expertise, namely organic transformations (Haumann and Riisager, 2008), electrochemistry (Hapiot and Lagrost, 2008), nanotechnology (Ichikawa et al., 2007), biotechnology (van Rantwijk and Sheldon, 2007), process engineering (Greaves and Drummond, 2008), organic synthesis (Olivier-Bourbigou et al., 2010), and separation technologies (Han and Armstrong, 2007). Particular applications in the extraction and recovery of different (bio)molecules from fermentation broths (Ventura et al., 2013a, 2012a) or pharmaceutical wastes (e Silva et al., 2014) have been investigated. Most of the ILs used so far are characterized by a bulky organic cation (imidazolium, pyridinium, pyrrolidinium, piperidinium, ammonium, phosphonium and more recently cholinium also known as choline), substituted with alkyl chains of different lengths, types and functionalizations, and conjugated with distinct anions. Although a "more benign" nature of ILs relatively to traditional solvents is normally claimed by the authors in the field, supported on their negligible vapor pressure (reduced potential as atmospheric pollutants), ILs are wrongly labeled as "greener solvents". In fact, ILs are thermally and chemically stable, are non-flammable and have a tunable solubility in several organic compounds. However, they have a notably variable solubility in water (Freire et al., 2008, 2009, 2010) which is the most probable route favoring ILs inflow to aquatic ecosystems. Their physicochemical

* Corresponding author at: Campus Universitário de Santiago, University of Aveiro, Aveiro, Portugal. Fax: +351 234 370084.
E-mail address: spventura@ua.pt (S.P.M. Ventura).
[1] Both authors, Francisca A. e Silva and Filipa Siopa contributed equally to this work.

http://dx.doi.org/10.1016/j.ecoenv.2014.07.003
0147-6513/

properties depend upon inter and intramolecular interactions and are, subsequently, strictly related with the countless cation/anion combinations. Nevertheless, such high flexibility can be problematic when the aim is to establish which ILs may cause serious human and environmental damage. Despite several efforts trying to make predictions regarding untested ILs through mathematical models based on quantitative structure–activity relationships, QSAR, the limitations as to the prediction of IL ecotoxicity are considerable (Matzke et al., 2010). Although model predictions should gain favor in the future as a relevant tool within the sustainable development of new ILs, these limitations make it still necessary to carry out systematic experimental studies considering the full characterization of ILs, in particular for the new ionic species synthesized. An ultimate use for comprehensive databases enclosing the (eco)toxicological profile of ILs is the support of compliance with regulatory demands when large-scale applications are intended, *e.g.* sense the REACH directive (EC, 2007) in the European Union and the United Nations Agenda 21 (Agenda 21, 2004) worldwide. According to the REACH rules, extensive IL toxicity studies with different test systems are required (Kulacki et al., 2011; Matzke et al., 2010; Petkovic et al., 2011; Pham et al., 2010; Ventura et al., 2012b, 2010, 2013b, 2013c). This knowledge is needed to provide a relevant set of information on the properties of these new chemicals, allowing their prospective risk assessment that supports the final decision on marketing authorization. In this context, the body of literature dealing with ILs, and specifically contributing to the related toxicity database, has been increasing. However, it should be noticed that most of the studies lie on the toxicity analysis of conventional ILs, namely those containing the imidazolium cation, conjugated with several anions (Frade and Afonso, 2010; Kulacki and Lamberti, 2008; Matzke et al., 2010; Petkovic et al., 2011; Pham et al., 2010; Ventura et al., 2012b; 2010, 2013b; 2013c). A considerable amount of data already related the toxicity with the introduction of oxygenated groups, mainly in the imidazolium family, the results indicating that such modification decreases the toxicity (Frade et al., 2007; Pretti et al., 2009; Samori et al., 2007; Stolte et al., 2007a; 2007b).

In fact, the number of new IL families is continuously increasing and, with it, the need for their full toxicological evaluation. Two of the most recent IL cation structures are the quaternary ammonium and its derivative, the cholinium, commonly known as choline. Albeit the increasing interest in the cholinium cation as a promising candidate to design "greener" ILs, provided that choline is a naturally occurring nutrient (Zeisel and Da Costa, 2009), the toxicity of this family is still poorly studied and the interpretation of its toxicological behavior is consequently very deficient and overgeneralized. Therefore, researchers are paying increasing attention on the toxicities of ILs, particularly on those containing the cholinium cation or cholinium derivative structures (Frade et al., 2013; Hou et al., 2013; Nockemann et al., 2007; Pernak and Chwala, 2003; Pernak et al., 2007; Ventura et al., 2014; Wang et al., 2007). Pernak et al. (2007) for example, synthesized a wide range of cholinium derivatives sharing distinct anions. With the *N* in alkoxy group varying between 6 and 12 carbons, the derivatives were found to be increasingly active against the microorganisms investigated.

Dicationic ILs are new structures characterized by the presence of two cations conjugated with two anions and connected by a principal alkyl chain where each cation has also substituted alkyl chains (Messali et al., 2013). The interest in dicationic ILs is increasing due to their higher thermal stability when compared with their monocationic counterparts. The suitability of dicationic compounds as reaction media was now studied when high temperatures are required (Han and Armstrong, 2005), to be used for capillary gas chromatography columns coating as stationary phases (Liu et al., 2012). The use of dicationic ILs enables a higher flexibility in the manipulation/tunability of their physicochemical properties; yet the knowledge available on their (eco)toxicological profile is limited (Steudte et al., 2014). Steudte et al.(2014), have performed a systematic toxicological evaluation where imidazolium-based dicationic ILs (central chain varying from 1 to 6 carbons) and their toxic effects towards several model systems, namely the acetylcholinesterase (AChE) enzyme, the promyelocytic leukemia rat cell line (IPC-81 cells), the green algae *Scenedesmus vacuolatus* and the cladoceran *Daphnia magna*, were included (Steudte et al., 2014).

In a former study (Ventura et al., 2014) the ecotoxicological effects of structural changes in the anion moiety of cholinium-based ILs, measured on the basis of the luminescence inhibition induced in *Vibrio fischeri,* were focused. As a follow-up, variations in the cation core are covered in the present work, which aims specifically at describing the synthesis of mono and dicationic cholinium derivatives, having in mind the characterization of each one of these structures in terms of their toxicity towards the same test system *V. fischeri*. In fact, the works are connected, since they both contribute for the development of knowledge of the ILs' structures/toxicity relationship, with the ultimate final goal of creating heuristic rules for the ILs design, which is often mentioned, but rarely studied. The synthesis of 17 monocationic and 8 dicationic cholinium (or cholinium-derivative) ILs conjugated with the bromide anion (Br^-) is reported and their toxicity against the luminescent marine bacteria *V. fischeri* is evaluated and reported. The monocationic structures were selected to evaluate the influence of alkyl chains lengths and their functionalization with a double or triple bond, the number of hydroxyethyl groups at the cation and the substitution of an alkyl chain by a hydrogen atom. The dicationic cholinium derivatives allow evaluating the effects of the length of the linkage alkyl chain (used to connect the two cations) and the number of hydroxyethyl groups substituted in the alkyl chains.

2. Material and methods

2.1. General procedure for the synthesis of choline derivatives

A range of choline derivatives was synthesized, using different amines, *e.g.* 2-(dimethylamino)ethanol, *N*-methyldiethanolamine, diethanolamine and triethanolamine, and the correspondent brominated derivatives. Most of the quaternary ammonium salts (Series **A** and **B**; Table 1) were prepared by alkylation of a tertiary amine with the correspondent brominated derivative. Likewise, the bisquaternary ammonium salts (Series **C**; Table 2) were synthesized by alkylation of a tertiary amine with an alkyl dihalide (Scheme 1). The synthesized ILs are stable at room temperature, and thus they can be stored at this temperature once carefully closed (to avoid the accumulation of water, since some of them are hygroscopic).

The synthesis was run in general as follows (details for each salt are provided as Supplementary information; S4–S14).

Series **A** (Table 1)—In an Aldrich ace pressure tube (Z181064) at room temperature, a solution of the correspondent brominated derivative (45 mmol) dissolved in MeCN (5 mL) was added to a solution of 2-(dimethylamino)ethanol (4.1 g, 45 mmol) in MeCN (5 mL). The reaction mixture was heated overnight at 60 °C, unless stated. For compounds **A1** and **A3**, the salt precipitated during the reaction, while for compound **A7**, the salt precipitated when the Aldrich ace pressure tube was cooled until room temperature. Diethyl ether was added and the salt was filtered and dried under vacuum. For compounds **A2**, **A4**, **A5**, **A6** and **A8**, in which the reaction mixture was liquid-, the solvent was evaporated and diethyl ether was added to precipitate the final product. The salt (IL) was then filtered and dried under vacuum.

Series **B** (Table 1)—In an Aldrich ace pressure tube (Z181064) at room temperature, the brominated derivative (45 mmol), the tertiary amine or diethanolamine (for **B1**) (45 mmol) and MeCN (5 mL) were added. If indicated, a catalytic amount of sodium iodide was added to the reaction mixture. The reaction mixture was heated at 60 °C overnight, unless stated. For compounds **B1**, **B2**, **B3**, **B5**, **B6**, **B7** and **B8**, the solvent was removed by evaporation under vacuum to obtain the correspondent ammonium salt. For compound **B4** after the reaction reach the room temperature, diethyl ether was added to precipitate the salt. This final product was then filtered and dried under vacuum.

Series **C** (Table 1)—In an Aldrich ace pressure tube (Z181064) at room temperature the brominated derivative (42 mmol), the tertiary amine (84 mmol) and MeCN (5 mL) were added. If indicated, a catalytic amount of sodium iodide was added to the reaction

Table 1
EC_{50} values (mg L^{-1}) estimated for Series **A** and **B** of monocationic cholinium-derivative-compounds at 30 min of exposure of the luminescent marine bacteria *Vibrio fischeri*, with the respective 95 percent confidence limits (within brackets). The structural features varying along the ecotoxicological evaluation are highlighted in different colors: alkyl side chain length in salmon; insertion of multiple bonds in blue; addition of hydroxyethyl groups in green; and the substitution of an alkyl chain by a hydrogen atom (protic IL) in red.

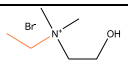
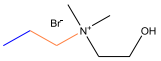
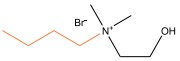
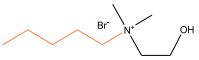
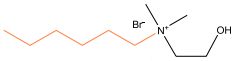
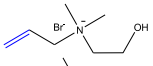
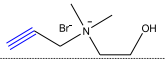
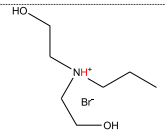
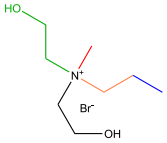
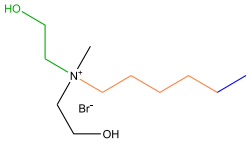
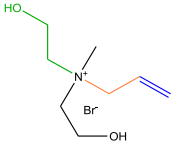
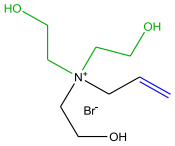
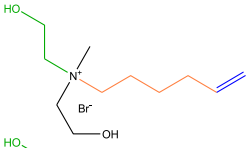
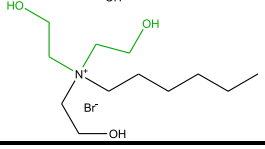
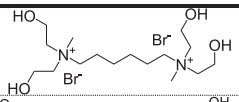
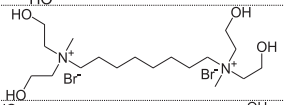
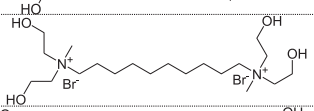
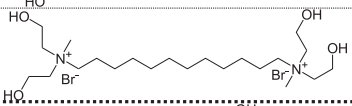
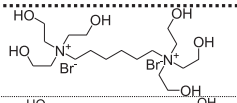
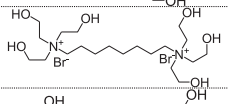
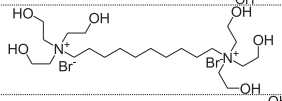
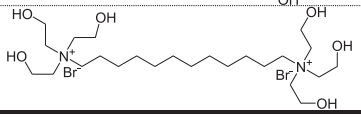
Compound	Chemical structure	EC_{50} (mg.L^{-1}) 30 min (lower limit; upper limit)
A1		**25619.07** (17592.81; 33645.32)
A2		**33972.39** (27766.06; 40178.71)
A3		**13442.88** (8796.98; 18088.78)
A4		**3016.96** (2340.91; 3693.01)
A5		**746.30** (699.73; 792.86)
A6		**162.96** (158.59; 167.32)
A7		**0.81** (0.79; 0.82)
A8		**20798.81** (15494.22; 26103.40)
A9		**235.92** (126.13; 345.71)
B1		**370.07** (318.50; 421.65)
B2		**1601.29** (872.32; 2330.27)
B3		**276.96** (220.22; 333.70)
B4		**8.45** (6.70; 10.21)
B5		**2745.90** (2187.13; 3304.68)
B6		**2522.02** (2301.58; 2742.46)
B7		**1311.66** (1002.73; 1620.60)
B8		**19.74** (18.20; 21.27)

Table 2
EC_{50} values (mg L^{-1}) estimated for Series **C** composed of dicationic cholinium-derivative compounds at 30 min of exposure of the luminescent marine bacteria *Vibrio fischeri*, with the respective 95 percent confidence limits (in brackets).

Compound	Chemical structure	nº CH_2CH_2OH	*N*	EC_{50} (mg.L^{-1}) 30 min (lower limit; upper limit)
C1		2	6	**6117.15** (4657.16; 7577.14)
C2			8	**5579.82** (4962.60; 6197.04)
C3			10	**388.95** (335.27; 442.63)
C4			12	**97.89** (81.31; 114.48)
C5		3	6	**417.50** (357.28; 477.73)
C6			8	**6.97** (5.97; 7.97)
C7			10	**11.19** (9.53; 12.86)
C8			12	**6.15** (6.06; 6.24)

mixture. The reaction mixture was heated overnight at 90 °C, unless stated otherwise. For compounds **C1** and **C2**, the salt precipitated during the reaction. Diethyl ether was added and the salt was filtered and dried under vacuum. For compounds **C3**, **C4**, **C5**, **C6**, **C7** and **C8** where the reaction mixture was liquid, the solvent was removed by evaporation under vacuum to obtain the correspondent ammonium salt.

Additional experimental details and selected ^1H and ^{13}C NMR spectra are presented in Supporting information (S4–S38).

2.2. Standard Microtox® liquid-phase assays

Standard Microtox® liquid-phase assays (Microbics Corporation, 1992) were used to evaluate the luminescence inhibition of the bacteria *V. fischeri* (strain NRRL B-11177) following exposure to each compound at 15 °C. The bacteria was exposed to a range of geometrically diluted aqueous solutions (typically from 0 to 81.9 percent; geometric factor=2) of each compound, where 100 percent of IL corresponds to a known concentration of a previously prepared stock solution. Prior Microtox® testing, the water content of each IL sample was always measured for an adequate preparation of each stock solution. The amount of water was determined by Karl Fischer (KF) titration using a Metrohom 831 KF coulometric titrator. Then, the mass concentration of each IL was corrected for the water content of the bulk IL used to prepare the stock solution, thus guaranteeing the accuracy of the estimated EC_{50} values.

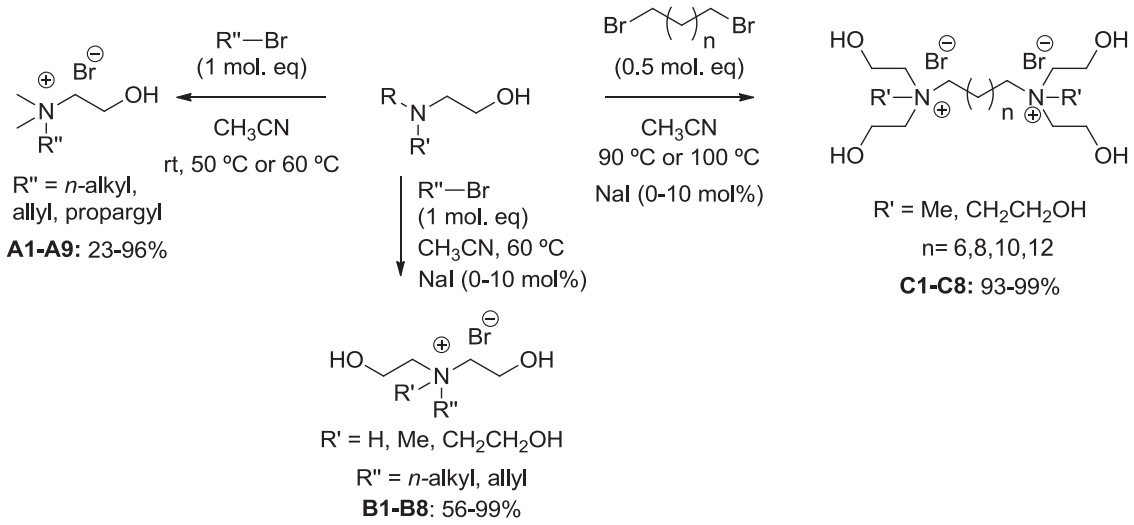
The light output of *V. fischeri* was measured after 5, 15 and 30 min of exposure to each IL, and compared with the light output of a blank control sample, allowing the calculation of the luminescence inhibition *per* treatment. The higher the inhibition record, the higher the IL toxicity effect provided that luminescence changes as a direct function of the bacteria overall viability. On the basis of this parameter, 5 min-, 15 min- and 30 min-EC_{50} values (EC_{50} being the estimated concentration yielding 50 percent effect), plus the corresponding 95 percent confidence intervals were estimated for each IL through non-linear regression, using the least-squares method to fit the data to the logistic equation.

3. Results and discussion

A variety of cholinium-derivative compounds sharing the Br^- anion, divided into three distinct series (Tables 1 and 2 and Table S1 in the Supporting information), were synthesized and their ecotoxicity towards the luminescent marine bacteria *V. fischeri* evaluated.

The synthetic methodology involves in general the alkylation of the amine with the corresponding halide in MeCN, overnight at 60 °C (Scheme 1). In some cases, the starting material was not consumed during this time (reaction followed by ^1H NMR experiments) requiring some modification to the general experimental procedure such as, higher temperatures, and/or longer reaction times, and/or the addition of sodium iodide. The synthesized quaternary (series **A** and **B**) and bisquaternary ammonium salts (series **C**) are present in Table 1. Some products were obtained as white solids and others as liquids. The yield of the products synthesis varied between 23 percent and quantitative yields being the majority above 80 percent (see Table S1 and the scheme associated in the page S40 of Supporting information).

As an attempt to design more sustainable ionic structures, the role of several structural modifications on the variation in the ecotoxicity of both mono and dicationic compounds was analyzed. The analysis was centered namely in structural changes regarding the alkyl side chain (described by the number of carbons and represented by *N*) or central chain length (described by the number of carbons of the central alkyl chain and represented by $N_{linkage}$), the insertion of multiple covalent bonds, the addition of CH_2CH_2OH groups and the replacement of an alkyl chain by an H atom (protic IL). In order to facilitate the readability of the main conclusions, results and discussion are henceforth presented by grouping mono and dicationic compounds and their different structural features. All EC_{50} values following 5-,15- and 30-min exposure were provided as a general contribution to the ecotoxicological database on ILs (Tables 1 and 2 complemented by



Scheme 1. General synthetic route for the preparation of mono and dicationic cholinium salts A, B and C..

Tables S2 and S3 in the Supporting information), as well as a way to provide the necessary grounds for any intended more refined analysis on the toxicokinetics within each tested IL by the reader. Although the decrease of the EC_{50} estimated, compared to the corresponding for shorter exposure periods, was the most common trend observed in the three IL series, absolute consistency of the time-dependent effects in the toxicity cannot be recognized. In fact, a slight increase in the luminescence yield was often observed at the lowest IL concentrations as the exposure period enlarges, which may reflect a slight recovery of the bacteria following the initial stress challenge pulse. This promotes differential adjustment of the predictive model to the datasets, translating into an increase of the estimated EC_{50} values at longer exposure periods. Because these were only slight fluctuations, pictured more or less randomly, in the sense that they do not reflect any particular structural trend in the toxicant, only the EC_{50} values obtained after 30 min of exposure were considered for further discussion, hence facilitating the readability of a systematic comparison of the chemicals toxicity. Also, conservativeness as to full capturing of effects is ensured by assuming the largest exposure period for discussion.

3.1. Monocationic cholinium-based ILs

The Series **A** and **B** of monocationic cholinium derivatives sharing the Br^- anion allow the assessment of the impact of several structural features on the bacterial toxicity of such compounds: (a) the increasing in the alkyl chain length in the *R* group of the cation (structures from **A1** to **A7** and from **B2** to **B4** depicted in Table 1); (b) the insertion of double and triple bonds between carbons (structures **A2**, **A8** and **A9**; **B2** and **B5**; **B3** and **B7**, shown in Table 1); (c) the addition of CH_2CH_2OH groups (structures **A2** and **B2**; **A7** and **B4**; **A5**, **B3** and **B8**; **A8**, **B5** and **B6**); and (d) the replacement of an alkyl chain by an H atom—protic IL (**B1**).

3.1.1. Alkyl side chain length

By the analysis of the EC_{50} values represented in Table 1, for the cholinium derivatives from Series **A** as the increase in the alkyl chain length is considered, it is possible to rank their ecotoxicity according to the following tendency [IL (*N*=number of carbon atoms)]: **A2** (*N*=3) < **A1** (*N*=2) < **A3** (*N*=4) ≪ **A4** (*N*=5) ≪ **A5** (*N*=6) ≪ **A6** (*N*=8) ≪ **A7** (*N*=12). Fig. 1 depicts the EC_{50} values

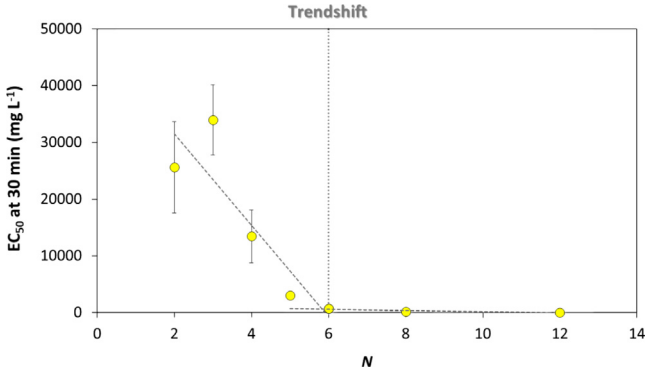


Fig. 1. Trendshift evidences in the relationship between the EC_{50} values at 30 min in mg L^{-1} and the alkyl chain length (*N*) for the **A1** to **A7** cholinium-derivative compounds. The error bars represent the 95 percent confidence limits.

after 30 min of exposure of the cholinium derivative compounds against the number of carbons (*N*) composing the *R* group. For Series **A** of cholinium derivatives with *R* composed of alkyl chains with lengths up to 6 carbon atoms (from **A1** to **A5**), the increase in the toxicity against *V. fischeri* is more significant than for the compounds with longer alkyl chains (**A5** to **A7**). In fact, these results show changes of magnitude between them of 5 orders, being however, in general agreement with the main conclusions found for the imidazolium cation (Stolte et al., 2007b, Ventura et al., 2012b). Nevertheless, it should be stressed out that the apparent contradiction between the EC_{50} values obtained for the **A1** and **A2** compounds relates to the very low toxicity typical of short alkyl chains. Trend shifts in ILs' behavior such as that described in Fig. 1 have also been noticed and received considerable attention in other domains of ILs research, namely in terms of thermophysical properties (Rocha et al., 2012, 2011) and in the extraction of biomolecules using IL-based aqueous biphasic systems (Passos et al., 2013).

The data hence confirm the two general tendencies described for the alkyl chain influence, referred to as the "*side chain effect*", *i.e.* the alkyl chain elongation increases the hydrophobicity/lipophilicity of the IL, and the "*cut-off*" effect – Fig. 1 – (Stolte et al., 2007b). The picture emerging from the measured data indicates that the alkyl side chain length is a key structural feature when attempting the sustainable design of cholinium derivative compounds. This has been intensively discussed in the domain of ILs'

toxicity (Petkovic et al., 2009; Stolte et al., 2007a, 2007b; Ventura et al., 2012c). These former studies ground the evidence that in the new cholinium family also the toxicity mechanism behind the increase of the alkyl chain comprises the disruption of cell membranes. In addition, some compounds comprised in Series **B** – **B2**, **B3** and **B4**; **B5** and **B7** – further support the presence of the "side chain effect", independently of the cholinium derivative cation structure, being different in the number of CH_2CH_2OH groups or double bond from that in Series **A**: **B2** ($N=3$) < **B3** ($N=6$) ≪ **B4** ($N=12$); **B5** ($N=3$) < **B7** ($N=6$).

3.1.2. Insertion of multiple covalent bonds

The impact of inserting multiple covalent bonds into the *R* group of the cholinium cation in the ecotoxicity towards the *V. fischeri* was assessed through testing the luminescence inhibition effect of the addition of carbon–carbon double (**A8**) and triple (**A9**) covalent bonds in the alkyl chain of the **A2** compound. The EC_{50} values are shown in Table 2 and their ecotoxicity increases according to the following trend: **A2** (single bond) < **A8** (double bond) ≪ **A9** (triple bond). It should be highlighted in particular the boost in ecotoxicity delivered by the insertion of the triple bond translated by a decrease of more than two orders of magnitude in the EC_{50} value retrieved for **A9** relatively to that found for **A8** and to the effect found for **A2** (20,798.81 mg L^{-1} for **A8** vs. 235.92 mg L^{-1} for **A9**). Aiming at explaining the phenomena behind the impact of the insertion of carbon–carbon double and triple bonds here observed for **A2**, **A8** and **A9** compounds, the results by Schultz and Yarbrough (2004) can be used as a reference. The authors investigated the toxicity of aliphatic polarized α,β-unsaturated derivatives of esters, aldehydes and ketones and observed that the compounds containing carbon–carbon triple bonds presented an enhanced toxicity when compared to the compounds with double bonds. This fact was justified based on the greater reactivity toward nucleophiles of the carbon–carbon triple bond (Schultz and Yarbrough, 2004). This mechanism may also be assumed for the understanding of the impact of the triple bond on the toxicity of the cholinium derivatives observed here. Moreover, using the data found for some compounds belonging to series **B**, it is possible to infer the impact of the insertion of a carbon–carbon double bond, namely comparing **B2** with **B5** and **B3** with **B7**. In these cases, the insertion of double bonds considerably decreased the ecotoxicity of the cholinium derivatives as shown by the EC_{50} values (**B2** vs. **B5**: 1601.29 mg L^{-1} vs. 2745.90 mg L^{-1}; **B3** vs. **B7**: 276.96 mg L^{-1} vs. 1311.66 mg L^{-1}). The results here described show a distinct behavior when the same effect is studied considering the cholinium compounds with just one hydroxyethyl group. It is reported that double bond-containing side chains reduce the ILs viscosity, which can be considered a technological advantage of such structural alterations for some specific applications (Pinkert et al., 2009). Since there are no appreciable changes in the Microtox®-based ecotoxicity of the compounds by the introduction of a double bond, such technological advantage can be recognized also as environment friendly.

3.1.3. Addition of hydroxyethyl groups

The Series **B** of cholinium derivatives is composed of a set of compounds possessing, at least, one additional hydroxyethyl (CH_2CH_2OH) group compared to the ionic compounds enclosed in Series **A**. The EC_{50} dataset is reported in Table 1 and the toxicity of the compounds allowing further analysis can be ranked as follows: **A2** (one CH_2CH_2OH group) < **B2** (two CH_2CH_2OH groups), **A5** (one CH_2CH_2OH group) < **B3** (two CH_2CH_2OH groups) ≪ **B8** (three CH_2CH_2OH groups) and **A8** (one CH_2CH_2OH group) ≪ **B5** (two CH_2CH_2OH groups) < **B6** (three CH_2CH_2OH groups). Summing up, it is possible to conclude that the insertion of CH_2CH_2OH groups considerably increases the toxicity towards the bacteria [for example, EC_{50} drastically decreasing from 746.30 mg L^{-1} (**A5**) to 19.74 mg L^{-1} (**B8**)]. The incorporation of oxygenated alkyl chains in the ILs' structure has also been subject of considerable interest in the literature due to its promising applications (Galletti et al., 2007; Liu et al., 2005) and higher biodegradable character of engineered ILs (Coleman and Gathergood, 2010). Herein, the results suggest that the addition of CH_2CH_2OH groups increases the toxicity of these cholinium-based ILs, contrarily to the literature results for the imidazolium family functionalized with polar groups (Frade et al., 2007; Pretti et al., 2009; Stolte et al., 2007b). This is likely to indicate that the cholinium derivatives possess a different mode of toxic action, as compared to the imidazolium-based ILs. The same evidences were recently reported by our research group for a plethora of cholinium-based compounds (Ventura et al., 2014), where the cholinium chloride, [Ch]Cl, revealed higher ecotoxicity when compared to the *N*-ethyl-*N,N*-dimethyl-1-butanaminium chloride, $[N_{1,1,2,4}]Cl$ (EC_{50} value retrieved from Stolte et al., 2007b), suggesting that the oxygenation of the alkyl chain present in the choline did not contribute for a decrease in toxicity. However, it should be stressed out that also a superposition of effects, between the ethyl (increasing hydrophobicity) and hydroxyl (increasing hydrophilicity) units may also influence the mechanism of toxic action. In fact, Samori et al. (2010) brought some evidences of higher sensitivity of *V. fischeri* to the elongation of the alkyl side chain rather than to the presence of oxygen units. A reversed trend from that established above can be retrieved when comparing the EC_{50} values for the compounds **A7** and **B4**. In this specific case, the addition of an extra CH_2CH_2OH group is capable of augmenting the EC_{50} value from 0.81 mg L^{-1} to 8.45 mg L^{-1}, *i.e.* reducing toxicity. Taking into account that these compounds have a 12 carbon atoms side chain, this reversed phenomenon can be justified on the basis of their possibly higher volume, which makes it reasonable to consider that the capacity of the IL to enter in the organism can be reduced.

3.1.4. Substitution of an alkyl chain by a hydrogen atom

A single protic IL derived from the cholinium family (**B1**) was also investigated and its ecotoxicity compared with its aprotic counterpart (**B2**), making possible the evaluation of the impact of the substitution of an alkyl chain by a hydrogen atom. The EC_{50} data depicted in Table 1 indicates that **B1** (EC_{50} = 370.07 mg L^{-1}) is significantly more toxic than **B2** (EC_{50} = 1601.29 mg L^{-1}). Pretti et al. (2009) analyzed the toxicity of some imidazolium-based protic ILs towards cladocerans and algae, concluding that they were less toxic than their aprotic counterparts (even with $N=1$ or $N=4$ side chains). Again, signs of a different mode of toxic action of the cholinium derivatives as compared to conventional ILs appear, as already noticed for the insertion of additional CH_2CH_2OH groups and in agreement with the evidences brought by our previous work (Ventura et al., 2014).

3.2. Dicationic cholinium derivatives

Series **C** (Table S1 in Supporting information) is exclusively composed of dicationic cholinium derivatives. The set of compounds here investigated allows studying the impact of two different structural features on the toxicity towards the marine bacteria: (a) the increase of the central chain length (from structure **C1** to **C4** and from **C5** to **C8**) and b) the addition of CH_2CH_2OH groups (structures from **C1** to **C4** compared to **C5** to **C8**). The estimated 30-min EC_{50} values are presented in Table 2.

3.2.1. Alkyl linkage chain length

In order to assess the role of the linkage chain between both cations on the toxicity towards *V. fischeri*, 8 dicationic compounds were synthesized and their toxicity evaluated. Two distinct groups of cholinium derivatives containing either 2 (**C1** to **C4** in Table 1) or 3 (**C5** to **C8** in Table 1) CH_2CH_2OH groups were analyzed and their linkage chain length was changed from $N_{linkage}=6$ to $N_{linkage}=12$. The ecotoxicity of the first group of compounds can be ranked as follows: **C1** ($N_{linkage}=6$) < **C2** ($N_{linkage}=8$) ≪ **C3** ($N_{linkage}=10$) < **C4** ($N_{linkage}=12$). It is clear that the increasing length of the central chain is turning the dicationic compounds more toxic, following the behavior observed for the effect of the alkyl side chain length on the ecotoxicity of monocationic compounds. In fact, by the simple increase of the linkage chain length from $N_{linkage}=6$ to 12 carbon atoms, the toxicity parameter (EC_{50}) was drastically decreased by about 2 orders of magnitude, from 6117.15 mg L^{-1} down to 97.89 mg L^{-1}. Again, the hydrophobicity/lipophilicity seems to be controlling the ecotoxicity results. The results found here for the cholinium dicationic-based ILs are in general agreement with the results described elsewhere (Steudte et al., 2014). Regarding the second group of compounds, their toxicity follows the tendency **C5** ($N_{linkage}=6$) ≪ **C7** ($N_{linkage}=10$) ≈ **C6** ($N_{linkage}=8$) ≈ **C8** ($N_{linkage}=12$). The EC_{50} value significantly decreases from 417.50 to 11.19 mg L^{-1} stabilizing then at, approximately, 6 mg L^{-1}. Herein, the elongation of the alkyl linkage chain is inducing the occurrence of the well-known *"cut-off"* effect *i.e.*, beyond the $N_{linkage}=8$ chains the toxicity seems to stabilize (Stolte et al., 2007b; Ventura et al., 2012b). Although many explanations for such phenomenon have been previously suggested (Könemann, 1981; Mayer and Reichenberg, 2006; Stolte et al., 2007b; Opperhulzen et al., 1985) in this specific case, it seems that steric effects are limiting the uptake due to the volume of the dicationic compounds.

3.2.2. Addition of hydroxyethyl groups to the cations

The effect of the number of $-CH_2CH_2OH$ groups in the cations composing the dicationic compounds on the toxicity towards the luminescent bacteria was investigated through paired comparison between the compounds from **C1** to **C4** and **C5** to **C8** (Table S1 in Supporting information), respectively. The results depicted in Fig. 2 indicate that the increase in the number of CH_2CH_2OH groups from 2 to 3 (in both cations) induces a higher toxicity on the dicationic compounds. In fact, a maximum reduction in three orders of magnitude of the EC_{50} value was achieved by the addition of one CH_2CH_2OH group (5579.82 mg L^{-1} for **C2** vs. 6.97 mg L^{-1} for **C6**). A similar phenomenon was observed when addressing the toxicity of monocationic cholinium derivatives. Again, evidences of a distinct mode of toxic action of the cholinium derivatives as compared to the conventional ILs appear

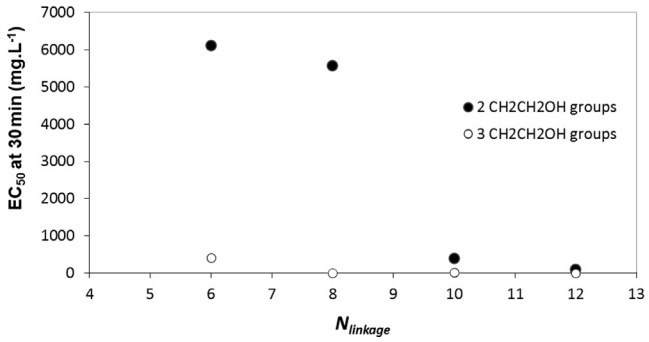


Fig. 2. EC_{50} values at 30 min vs the number of carbons in the linkage chain ($N_{linkage}$) considering the dicationic ILs series: ●, **C1**–**C4** (2 CH_2CH_2OH groups) and ○, **C5**–**C8** (3 CH_2CH_2OH groups).

(Ventura et al., 2014). However, the higher susceptibility of the bacteria to the alkyl chain elongation rather than to the presence of oxygen units should also be noticed (Samori et al., 2010), indicating the need for further testing such compounds using a more complex test battery.

3.3. Mono vs. dicationic cholinium-based ILs

As an overarching discussion item adding to the fine-tuned structural analysis above, direct comparison of the toxicity of mono and dicationic cholinium and cholinium derivatives towards *V. fischeri* deserves attention. In fact, up to date, most of the works related to ILs' toxicity are dealing with monocationic ILs (Matzke et al., 2010; Petkovic et al., 2011; Pham et al., 2010), being scarce the available knowledge on the toxicity of dicationic compounds (Steudte et al., 2014). This fact is slowing down the development of effectively "greener" dicationic ILs, which appear as promising alternatives to be applied in several areas of expertise. The EC_{50} values represented in both Tables 1 and 2 suggest that it is possible to synthesize dicationic compounds revealing putatively lower ecotoxicity than their monocationic counterparts, with this conclusion being limited by the single biological model used in this study. As the direct comparison is made between mono- and dicationic equivalents, *i.e.* **B3** vs. **C1**; **B8** vs. **C5** and **B4** vs. **C4**, a noticeable decrease by more than one order of magnitude in the ecotoxicity of the dicationic cholinium derivatives is consistently recorded. This may be related with the size of the compounds, which constrains their interactions with the membranes. Accordingly, Steudte et al. (2014) demonstrated that the toxicity of dicationic ILs is, in general, lower than that revealed by the monocationic ILs under investigation. The authors also draw the attention for their deficient capability to undergo biodegraded, indicating the need for further research in the design of dicationic ILs (Steudte et al., 2014). Herein, the cholinium cation, which was already proven to be more easily biodegraded than the heterocyclic ones (Boethling et al., 2007), seems to be a promising route for the sustainable design of ILs.

4. Conclusions

Regarding the 25 cholinium-based ionic liquids investigated in this work, it is possible to pinpoint some trends relating their structure with their ecotoxicity. The results suggest that the toxicity of these cholinium compounds towards the bacteria is highly dependent on their structural features. This is an evidence that should definitively be considered in the sustainable design of new compounds, mostly when the attempt of specific applications and/or improving physicochemical or, even, toxicological properties, is investigated. First of all, and in contrast to what has been intensively claimed on the literature, the family of cholinium compounds is not devoid of toxicity. Long alkyl side or linkage chains, the increase in the number of CH_2CH_2OH groups and multiple covalent bonds are structural features capable of significantly increase the toxicity of such compounds. Moreover, the present results reinforce that the mechanisms of toxicity that have been proposed for ILs on the basis of studies made with the conventional families may not be fully adequate to explain the cholinium-based ILs toxicity.

The development of dicationic compounds, with a brand new range of applications, but concomitantly possessing low toxicity, was here achieved. Supplying new knowledge about the ecotoxicity of cholinium derivatives, hence straightening the gap in literature data, is an offer of the present study that is worth noticing in this field where new developments will be scrutinized and limited by tight regulation highly considering environmental

safety. Unquestionably, the synthesis and toxicological evaluation of new cholinium derivatives should not be unvalued, especially when attempting the design of truly "greener" ILs.

Acknowledgments

This work is financed by FEDER through Programa Operacional Fatores de Competitividade – COMPETE and national funds through FCT – Fundação para a Ciência e Tecnologia within CICECO project - FCOMP-01-0124-FEDER-037271 (Ref[a]. FCT PEst-C/CTM/LA0011/2013), PEst-C/MAR/ LA0017/2013, PEst-OE/SAU/UI4013/2011, PTDC/QEQ-PRS/2824/ 2012 and PTDC/AAC-AMB/119172/2010. The authors also thank financial support through the post-doctoral and doctoral grants SFRH/BPD/79263/2011, SFRH/BPD/79307/2011, SFRH/BPD/88666/2012, SFRH/BD/94901/2013 and SFRH/BPD/44733/2008 of S.P.M. Ventura, A.M.M. Gonçalves, F. Siopa, F.A. e Silva and J.L. Pereira, respectively.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2014.07.003.

References

Agenda 21, 2004. Environmentally Sound Management of Toxic Chemicals, Including Prevention of Illegal International Traffic in Toxic and Dangerous Products. In: United Nations Department of Economic and Social Affairs, United Nations, ed. 2004; vol. Chapter 19.

Anastas, P.T., Warner, J.C., 1998. Green Chemistry: Theory and Practice. 1998. Oxford University Press, New York p. 30.

Boethling, R.S., Sommer, E., DiFiore, D., 2007. Designing small molecules for biodegradability. Chem. Rev. 107, 2207–2227.

Coleman, D., Gathergood, N., 2010. Biodegradation studies of ionic liquids. Chem. Soc. Rev. 39, 600–637.

e Silva, F.A., Sintra, T., Ventura, S.P.M., Coutinho, J.A.P., 2014. Recovery of paracetamol from pharmaceutical wastes. Sep. Purif. Technol. 122, 315–322.

EC, 2007. ⟨http://ec.europa.eu/environment/chemicals/reach⟩.

Frade, R.F.M., Afonso, C.A.M., 2010. Impact of ionic liquids in environment and humans: an overview. Hum. Exp. Toxicol. 29, 1038–1054.

Frade, R.F.M., Matias, A., Branco, L.C., Afonso, C.A., Duarte, C.M., 2007. Effect of ionic liquids on human colon carcinoma HT-29 and CaCo-2 cell lines. Green Chem. 9, 873–877.

Frade, R.F.M., Simeonov, S., Rosatella, A.A., Siopa, F., Afonso, C.A.M., 2013. Toxicological evaluation of magnetic ionic liquids in human cell lines. Chemosphere 92, 100–105.

Freire, M.G., Carvalho, P.J., Gardas, R.L., Marrucho, I.M., Santos, L.M.N.B.F., Coutinho, J.A.P., 2008. Mutual solubilities of water and the [Cnmim][Tf_2N] hydrophobic ionic liquids. J. Phys. Chem. B 112, 1604–1610.

Freire, M.G., Carvalho, P.J., Silva, A.M.S., Santos, L.M. N.B. F., Rebelo, L.P.N., Marrucho, I.M., Coutinho, J.A.P., 2009. Ion specific effects on the mutual solubilities of water and hydrophobic ionic liquids. J. Phys. Chem. B 113, 202–211.

Freire, M.G., Neves, C.M.S.S., Ventura, S.P.M., Pratas, M.J., Marrucho, I.M., Oliveira, J., Coutinho, J.A.P., Fernandes, A.M., 2010. Solubility of non-aromatic ionic liquids in water and correlation using a QSPR approach. Fluid Phase Equilib 294, 234–240.

Galletti, P., Moretti, F., Samori, C., Tagliavini, E., 2007. Enzymatic acylation of levoglucosan in acetonitrile and ionic liquids. Green Chem. 9, 987–991.

Greaves, T.L., Drummond, C.J., 2008. Protic ionic liquids: properties and applications. Chem. Rev. 108, 206–237.

Han, X., Armstrong, D.W., 2007. Ionic liquids in separations. Acc. Chem. Res. 40, 1079–1086.

Han, X., Armstrong, D.W., 2005. Using geminal dicationic ionic liquids as solvents for high-temperature organic reactions. Org. Lett. 7, 4205–4208.

Hapiot, P., Lagrost, C., 2008. Electrochemical reactivity in room-temperature ionic liquids. Chem. Rev. 108, 2238–2264.

Haumann, M., Riisager, A., 2008. Hydroformylation in room temperature ionic liquids (RTILs): catalyst and process developments. Chem. Rev. 108, 1474–1497.

Hou, X.-D., Liu, Q.-P., Smith, T.J., Li, N., Zong, M.-H., 2013. Evaluation of toxicity and biodegradability of cholinium amino acids ionic liquids. PLoS One 8, e59145.

Ichikawa, T., Yoshio, M., Hamasaki, A., Mukai, T., Ohno, H., Kato, T., 2007. Self-organization of room-temperature ionic liquids exhibiting liquid-crystalline bicontinuous cubic phases: formation of nano-ion channel networks. J. Am. Chem. Soc. 129, 10662–10663.

Könemann, H., 1981. Quantitative structure–activity relationships in fish toxicity studies Part 1: Relationship for 50 industrial pollutants. Toxicology 19, 209–221.

Kulacki, K.J., Chaloner, D.T., Larson, J.H., Costello, D.M., Evans-White, M.A., Docherty, K.M., Bernot, R.J., Brueseke, M.A., Kulpa Jr., C.F., Lamberti, G.A., 2011. Proactive aquatic ecotoxicological assessment of room-temperature ionic liquids. Curr. Org. Chem. 15, 1918–1927.

Kulacki, K.J., Lamberti, G.A., 2008. Toxicity of imidazolium ionic liquids to freshwater algae. Green Chem. 10, 104–110.

Liu, Q., Janssen, M.H.A., van Rantwijk, F., Sheldon, R.A., 2005. Room-temperature ionic liquids that dissolve carbohydrates in high concentrations. Green Chem. 7, 39–42.

Liu, T., Zhang, L., Sun, L., Luo, A., Novel geminal dicationic ionic liquid as stationary phase for capillary gas chromatography. In: Zhang H. (Ed.), Advanced Research on Advanced Structure, Materials and Engineering, 2012, pp. 477–480.

Matzke, M., Arning, J., Ranke, J., Jastorff, B., Stolte, S., 2010. Design of inherently safer ionic liquids. In: Handbook of Green Chemistry, Toxicology and Biodegradation. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 225–290.

Mayer, P., Reichenberg, F., 2006. Can highly hydrophobic organic substances cause aquatic baseline toxicity and can they contribute to mixture toxicity? Environ. Toxicol. Chem. 25, 2639–2644.

Messali, M., Moussa, Z., Alzahrani, A.Y., El-Naggar, M.Y., ElDouhaibi, A.S., Judeh, Z.M.A., Hammouti, B., 2013. Synthesis, characterization and the antimicrobial activity of new eco-friendly ionic liquids. Chemosphere 91, 1627–1634.

Microbics Corporation, 1992. Microtox® manual—A Toxicity Testing Handbook, pp. 1–5.

Nockemann, P., Thijs, B., Driesen, K., Janssen, C.R., Van Hecke, K., Van Meervelt, L., Kossmann, S., Kirchner, B., Binnemans, K., 2007. Choline saccharinate and choline acesulfamate: ionic liquids with low toxicities. J. Phys. Chem. B 111, 5254–5263.

Olivier-Bourbigou, H., Magna, L., Morvan, D., 2010. Ionic liquids and catalysis: recent progress from knowledge to applications. Appl. Catal., A 373, 1–56.

Opperhulzen, A., Volde, E.W.v.d., Gobas, F.A.P.C., Liem, D.A.K., Steen, J.M.D.v.d., Hutzinger, O., 1985. Relationship between bioconcentration in fish and steric factors of hydrophobic chemicals. Chemosphere 14, 1871–1896.

Passos, H., Trindade, M.P., Vaz, T.S.M., da Costa, L.P., Freire, M.G., Coutinho, J.A.P., 2013. The impact of self-aggregation on the extraction of biomolecules in ionic-liquid-based aqueous two-phase systems. Sep. Purif. Technol. 108, 174–180.

Pernak, J., Chwala, P., 2003. Synthesis and anti-microbial activities of choline-like quaternary ammonium chlorides. Eur. J. Med. Chem. 38, 1035–1042.

Pernak, J., Syguda, A., Mirska, I., Pernak, A., Nawrot, J., Prądzyńska, A., Griffin, S.T., Rogers, R.D., 2007. Choline-derivative-based ionic liquids. Chem. Eur. J. 13, 6817–6827.

Petkovic, M., Ferguson, J., Bohn, A., Trindade, J., Martins, I., Carvalho, M.B., Leitao, M.C., Rodrigues, C., Garcia, H., Ferreira, R., Seddon, K.R., Rebelo, L.P.N., Silva Pereira, C., 2009. Exploring fungal activity in the presence of ionic liquids. Green Chem. 11, 889–894.

Petkovic, M., Seddon, K.R., Rebelo, L.P.N., Silva Pereira, C., 2011. Ionic liquids: a pathway to environmental acceptability. Chem. Soc. Rev. 40, 1383–1403.

Pham, T.P.T., Cho, C.-W., Yun, Y.-S., 2010. Environmental fate and toxicity of ionic liquids: a review. Water Res. 44, 352–372.

Pinkert, A., Marsh, K.N., Pang, S., Staiger, M.P., 2009. Ionic liquids and their interaction with cellulose. Chem. Rev. 109, 6712–6728.

Pretti, C., Chiappe, C., Baldetti, I., Brunini, S., Monni, G., Intorre, L., 2009. Acute toxicity of ionic liquids for three freshwater organisms: Pseudokirchneriella subcapitata, *Daphnia magna* and *Danio rerio*. Ecotoxicol. Environ. Saf. 72, 1170–1176.

Rocha, M.A.A., Coutinho, J.A.P., Santos, L.M. N.B. F., 2012. Cation symmetry effect on the volatility of ionic liquids. J. Phys. Chem. B 116, 10922–10927.

Rocha, M.A.A., Lima, C.F. R.A. C., Gomes, L.R., Schröder, B., Coutinho, J.A.P., Marrucho, I.M., Esperança, J.M. S.S., Rebelo, L.P.N., Shimizu, K., Lopes, J.N.C., Santos, L.M. N. B. F., 2011. High-accuracy vapor pressure data of the extended [CnC1im][NTf_2] ionic liquid series: trend changes and structural shifts. J. Phys. Chem. B 115, 10919–10926.

Samori, C., Pasteris, A., Galletti, P., Tagliavini, E., 2007. Acute toxicity of oxygenated and nonoxygenated imidazolium- based ionic liquids to *Daphnia magna* and *Vibrio fischeri*. Environ. Toxicol. Chem. 26, 2379–2382.

Samori, C., Malferrari, D., Valbonesi, P., Montecavalli, A., Moretti, F., Galletti, P., Sartor, G., Tagliavini, E., Fabbri, E., Pasteris, A., 2010. Introduction of oxygenated side chain into imidazolium ionic liquids: evaluation of the effects at different biological organization levels. Ecotoxicol. Environ. Saf. 73, 1456–1464.

Schultz, T.W., Yarbrough, J.W., 2004. Trends in structure–toxicity relationships for carbonyl-containing α,β-unsaturated compounds. SAR QSAR Environ. Res. 15, 139–146.

Steudte, S., Bemowsky, S., Mahrova, M., Bottin-Weber, U., Tojo-Suarez, E., Stepnowski, P., Stolte, S., 2014. Toxicity and biodegradability of dicationic ionic liquids. RSC Adv 4, 5198–5205.

Stolte, S., Arning, J., Bottin-Weber, U., Muller, A., Pitner, W.-R., Welz-Biermann, U., Jastorff, B., Ranke, J., 2007a. Effects of different head groups and functionalised side chains on the cytotoxicity of ionic liquids. Green Chem. 9, 760–767.

Stolte, S., Matzke, M., Arning, J., Boschen, A., Pitner, W.-R., Welz-Biermann, U., Jastorff, B., Ranke, J., 2007b. Effects of different head groups and functionalised side chains on the aquatic toxicity of ionic liquids. Green Chem. 9, 1170–1179.

van Rantwijk, F., Sheldon, R.A., 2007. Biocatalysis in ionic liquids. Chem. Rev. 107, 2757–2785.

Ventura, S.P.M., Santos-Ebinuma, V.C., Pereira, J.F.B., Teixeira, M.F.S., Pessoa, A., Coutinho, J.A.P., 2013a. Isolation of natural red colorants from fermented broth using ionic liquid-based aqueous two-phase systems. J. Ind. Microbiol. Biotechnol. 40, 507–516.

Ventura, S.P.M., de Barros, R.L.F., de Pinho Barbosa, J.M., Soares, C.M.F., Lima, A.S., Coutinho, J.A.P., 2012a. Production and purification of an extracellular lipolytic enzyme using ionic liquid-based aqueous two-phase systems. Green Chem. 14, 734–740.

Ventura, S.P.M., de Barros, R.L.F., Sintra, T., Soares, C.M.F., Lima, Á.S., Coutinho, J.A.P., 2012b. Simple screening method to identify toxic/non-toxic ionic liquids: agar diffusion test adaptation. Ecotoxicol. Environ. Saf. 83, 55–62.

Ventura, S.P.M., Gonçalves, A.M.M., Gonçalves, F., Coutinho, J.A.P., 2010. Assessing the toxicity on [C3mim][Tf_2N] to aquatic organisms of different trophic levels. Aquat. Toxicol. 96, 290–297.

Ventura, S.P.M., Gonçalves, A.M.M., Sintra, T., Pereira, J.L., Gonçalves, F., Coutinho, J. A.P., 2013b. Designing ionic liquids: the chemical structure role in the toxicity. Ecotoxicology 22, 1–12.

Ventura, S.P.M., Gurbisz, M., Ghavre, M., Ferreira, F.M.M., Gonçalves, F., Beadham, I., Quilty, B., Coutinho, J.A.P., Gathergood, N., 2013c. Imidazolium and pyridinium ionic liquids from mandelic acid derivatives: synthesis and bacteria and algae toxicity evaluation. ACS Sustainable Chem. Eng. 1, 393–402.

Ventura, S.P.M., Marques, C.S., Rosatella, A.A., Afonso, C.A.M., Gonçalves, F., Coutinho, J.A.P., 2012c. Toxicity assessment of various ionic liquid families towards *Vibrio fischeri* marine bacteria. Ecotoxicol. Environ. Saf. 76, 162–168.

Ventura, S.P.M., e Silva, F.A., Gonçalves, A.M.M., Pereira, J.L., Gonçalves, F., Coutinho, J.A.P., 2014. Ecotoxicity Analysis of cholinium-based ionic liquids to *Vibrio fischeri* marine bacteria. Ecotoxicol. Environ. Saf. 102, 48–54.

Wang, X., Ohlin, C.A., Lu, Q., Fei, Z., Hu, J., Dyson, P.J., 2007. Cytotoxicity of ionic liquids and precursor compounds towards human cell line HeLa. Green Chem. 9, 1191–1197.

Zeisel, S.H., Da Costa, K.-A., 2009. Choline: an essential nutrient for public health. Nutr. Rev. 67, 615–623.