



## Glycine-betaine-derived ionic liquids: Synthesis, characterization and ecotoxicological evaluation

Juan J. Parajó<sup>a</sup>, Inês P.E. Macário<sup>b</sup>, Yannick De Gaetano<sup>c</sup>, Laurent Dupont<sup>c</sup>, Josefa Salgado<sup>a</sup>, Joana L. Pereira<sup>b</sup>, Fernando J.M. Gonçalves<sup>b</sup>, Aminou Mohamadou<sup>c</sup>, Sónia P.M. Ventura<sup>d,\*</sup>

<sup>a</sup> NaFoMat Group, Applied Physic Department, University of Santiago de Compostela. Campus Vida, 15782, Santiago de Compostela, Spain

<sup>b</sup> Department of Biology, CESAM (Centro de Estudos Do Ambiente e Do Mar), University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

<sup>c</sup> ICMR, Institute of Molecular Chemistry of Reims, CNRS UMR 7312, University of Reims Champagne-Ardenne, BP 1039, F-51687, Reims Cedex 2, France

<sup>d</sup> CICECO, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

### ARTICLE INFO

#### Keywords:

Glycine-betaine ionic liquids

Ecotoxicity profile

*Allivibrio fischeri*

*Raphidocelis subcapitata*

EC<sub>50</sub>

### ABSTRACT

Ionic Liquids (ILs) are generically regarded as environmentally “harmless” and thus, assumed as “non-toxic”. However, due to the endless design possibilities, their ecotoxicological profile is still poorly known. An accurate knowledge on the toxicity of a substance is required, under the scope of environmental regulation worldwide, before their application and commercialization. Knowledge on the relationship between the chemical structure and toxic effects is essential for the future design of more biocompatible solvents. Focusing on the use of ILs as base lubricants, lubricant additives, or even as potential working fluids for absorption heat pumps, the knowledge on its environmental impact is of great importance, due to the possibility of spills. In this specific context, four analogues of glycine-betaine-based ILs (AGB-ILs) and four glycine-betaine based ILs (GB-ILs) were synthesized and characterized. Their ecotoxicity was assessed using representatives of two trophic levels in aquatic ecosystems, the bacteria *Allivibrio fischeri* (commonly used as a screening test organism) and the microalgae *Raphidocelis subcapitata* (as an alternative test organism that has been proven very sensitive to several IL families). The microalgae were more sensitive than the bacteria, hence, following a precautionary principle, we recommend considering the toxicity towards microalgae as an indicator in future studies regarding the focused ILs. Although four of the studied ILs were derived from a natural amino acid, all were considered hazardous for the aquatic environment, disproving the primary theory that all ILs derived from natural compounds are benign. Furthermore, the modification in the structure of anion and the cation can lead to the increase of toxicity.

### 1. Introduction

Ionic liquids (ILs) have been investigated for more than 20 years, and new structures have been synthesized aiming the design of task-specific compounds (chemicals, additives, solvents, etc) to be applied in the most varied fields. These compounds present interesting properties, namely their low flammability, high stability in water, high solvation potential, and improved chemical and thermal stability (Ruß and König, 2012).

In the last years, the task-specificity of these ionic solvents is being allied with the awareness of the need for more sustainable and benign chemical structures. Indeed, this is the demand nowadays. However, overgeneralizations about their “non-toxic” and “greener” nature have been questioned, and need clarification. A classic example is the use of the attribute “green” to ILs only because most of them have a negligible

vapour pressure (e.g. Lovelock et al., 2010). However, even the most hydrophobic ILs have enough solubility in water (Freire et al., 2007) to configure a putative exposure scenario for aquatic organisms. Indeed, several studies showed that ILs present appreciable toxicity (Amdé et al., 2015; Bado-Nilles et al., 2015; Biczak et al., 2014; Bubalo et al., 2014; Gouveia et al., 2014; Pawłowska and Biczak, 2016; Salgado et al., 2017), being their environmental impact strongly dependent on the cation and anion combinations. The toxicity of ILs towards different model organisms has been investigated, namely considering bacteria (Ventura et al., 2013), green microalgae, crustaceans (Santos et al., 2015), fish (Pretti et al., 2006), amphibians (Li et al., 2009), plants and soil microorganisms (Salgado et al., 2017), or model cell cultures (Kumar and Malhotra, 2009). The environmental impact of ILs has been given reviewing attention even by researchers that primarily focus on the development and synthesis of these compounds for diverse

\* Corresponding author.

E-mail address: [spventura@ua.pt](mailto:spventura@ua.pt) (S.P.M. Ventura).

industrial applications (Jordan and Gathergood, 2015; Petkovic et al., 2011; Ranke et al., 2007; Silva et al., 2019), denoting the relevance of this aspect. Such relevance certainly relates to the regulatory requirements regarding the environmental risk assessment of new chemicals or processes intended to be marketed, which includes (eco)toxicity profiling (e.g. EU, 2007). It is known that the toxicity of ILs depends not only on their structure (Matzke et al., 2010; Oliveira et al., 2016), i.e. alkyl chain size (Matzke et al., 2007) and molecular architecture (Alvarez-Guerra and Irabien, 2011; Petkovic et al., 2011), cation and anion cores (Luis et al., 2007; Petkovic et al., 2011; Romero et al., 2008; Stolte et al., 2007), but also on some properties, e.g. their hydrophobic/hydrophilic nature (Stolte and Stepnowski, 2011). However, despite the increased number of studies attempting the toxicity evaluation of ILs, the knowledge of their ecotoxicity and biodegradability is still an open question, while the literature often presents contradictory conclusions.

ILs based on amino acids have been given recent research attention since these ligands have a natural-based origin [e.g. they can be present in biological fluids (Santi et al., 2011)], thus they are expected to be harmless to the environment. Betaine is a methyl derivative of the amino acid glycine that can be found in microorganisms, plants, animals and foods, such as wheat, shellfish, spinach and sugar beets (Zeisel et al., 2003). This quaternary ammonium works as an organic osmolyte to protect cells under stress, and as a catabolic source of methyl groups via trans-methylation for use in many biochemical pathways (Craig, 2004). This work aims to analyse the toxic effects of eight ILs, four analogues of glycine-betaine-based ILs (AGB-ILs) and four glycine-betaine-based ILs (GB-ILs) (Table 1). Once proven environmentally safe, these ILs can apply as suitable alternatives as zwitterionic solvents in the purification of different biomolecules (Ferreira et al., 2017; Pereira

et al., 2018). The ecotoxicological behaviour of the studied betaine-derived ILs was addressed in this study using standard ecotoxicological models, namely the marine bacteria *Aliivibrio fischeri* and the freshwater microalgae *Raphidocelis subcapitata*. These two test organisms are amongst the most sensitive to ILs. The selection of two test models reflects the secondary aim of the study, on gaining an insight regarding the consistency of toxic responses between representatives of two trophic levels of the aquatic food web, as well as of prokaryotic and eukaryotic cells, following the rationale and outcomes of previous studies (Santos et al., 2015; Ventura et al., 2013). A relevant role of the cation, the alkyl chain of the cation and the anion in modulating toxic responses is hypothesised.

## 2. Materials and methods

### 2.1. Materials

Glycine-betaine, methanesulfonic acid, sodium docusate (bis(2-ethylhexyl)sulfosuccinate), potassium thiocyanate, *n*-dodecanol, *n*-tetradecanol, ethyl bromoacetate, ethyl 4-bromobutyrate, tri(*n*-butyl)amine, tri(*n*-butyl)phosphine and sodium saccharinate were analytical-grade materials purchased from Sigma-Aldrich, used in this work without further purification. All aqueous solutions were prepared with distilled water.

### 2.2. Synthesis of ILs

Both alkyl ester betainium methanesulfonate (dodecylbetainium methanesulfonate and tetradecylbetainium methanesulfonate) were

**Table 1**  
Name, abbreviation, chemical structure and molecular weight of the ILs studied.

Name	Abbreviation	Chemical structure	Molecular weight (g·mol <sup>-1</sup> )
N,N,N-tri(methyl)(2-dodecyloxy-2-oxomethyl)-1-ammonium docusate	[Me <sub>3</sub> NC <sub>2</sub> OC <sub>12</sub> ][Doc]		735.1
N,N,N-tri(methyl)(2-tetradecyloxy-2-oxomethyl)-1-ammonium docusate	[Me <sub>3</sub> NC <sub>2</sub> OC <sub>14</sub> ][Doc]		754.1
N,N,N-tri(methyl)(2-dodecyloxy-2-oxomethyl)-1-ammonium thiocyanate	[Me <sub>3</sub> NC <sub>2</sub> OC <sub>12</sub> ][SCN]		344.3
N,N,N-tri(methyl)(2-tetradecyloxy-2-oxomethyl)-1-ammonium thiocyanate	[Me <sub>3</sub> NC <sub>2</sub> OC <sub>14</sub> ][SCN]		376.2
N,N,N-tri( <i>n</i> -butyl)(2-ethoxy-2-oxoethyl)-1-ammonium saccharinate	[Bu <sub>3</sub> NC <sub>2</sub> ][Sac]		482.7
N,N,N-tri( <i>n</i> -butyl)(4-ethoxy-4-oxobutyl)-1-ammonium saccharinate	[Bu <sub>3</sub> NC <sub>4</sub> ][Sac]		467.2
Tri( <i>n</i> -butyl)(2-ethoxy-2-oxoethyl)-1-phosphoniumsaccharinate	[Bu <sub>3</sub> PC <sub>2</sub> ][Sac]		514.1
Tri( <i>n</i> -butyl)(4-ethoxy-4-oxobutyl)-1-phosphoniumsaccharinate	[Bu <sub>3</sub> PC <sub>4</sub> ][Sac]		487.8

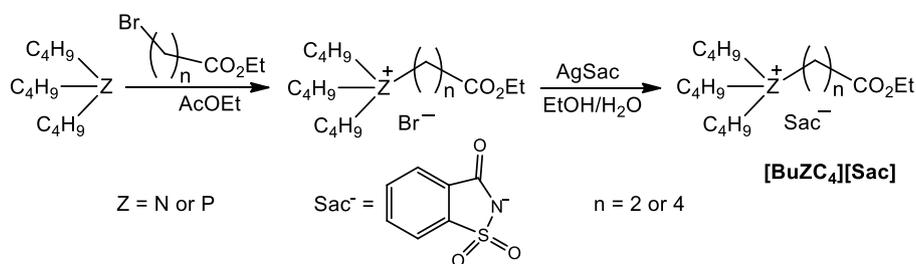


Fig. 1. Synthetic route of the hydrophobic saccharinate-based AGB-ILs.

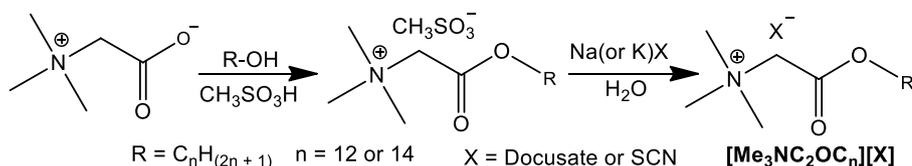


Fig. 2. Synthetic route of  $[\text{Me}_3\text{NC}_2\text{OC}_n][\text{X}]$ -based ILs.

synthesized by the standard protocol described elsewhere (De Gaetano et al., 2015). The four bromide salts, namely bromide  $[\text{Bu}_3\text{NC}_2]\text{Br}$ ,  $\text{N,N,N}$ -tri(*n*-butyl)(4-ethoxy-4-oxobutyl)-1-ammonium bromide  $[\text{Bu}_3\text{NC}_4]\text{Br}$ , tri(*n*-butyl)(2-ethoxy-2-oxoethyl)-1-phosphonium bromide  $[\text{Bu}_3\text{PC}_2]\text{Br}$  and tri(*n*-butyl)(4-ethoxy-4-oxobutyl)-1-phosphonium bromide  $[\text{Bu}_3\text{PC}_4]\text{Br}$ , were obtained following the experimental methodology previously presented (Messadi et al., 2013). Here, two main steps were performed as described in Figs. 1 and 2. The first step comprised the synthesis of (i) bromide salt by the reaction between the corresponding tri(*n*-butyl)amine or tri(*n*-butyl)phosphine and 2-bromoacetic acid ethyl ester or 4-bromobutyric acid ethyl ester, respectively; or (ii) the methanesulfonic salt of the cationic ester of glycine-betaine by esterification of glycine-betaine with primary alcohols using methanesulfonic acid (MSA) as catalyst following Goursaud et al. (2008).

### 2.2.1. Saccharinate-based ILs

Metathesis for bromide salt to saccharinate (Sac) salt was done as follows. Briefly, a slight excess of freshly prepared solid silver saccharinate (9.1 g, 31.5 mmol) was added to a solution of tri(*n*-butyl) [2(or 4)-ethoxy-2-oxoethyl(or 4-oxobutyl)]-1-ammonium (or 1-phosphonium) bromide (30 mmol) in 100 mL ethanol:water (50:50, v:v) and gently heated (30–40 °C) for 1 h and stirred overnight at room temperature. The solid precipitate (composed of AgBr and excess of AgSac) was filtered and the solvent removed by rotary evaporation. The resultant sample was dissolved in dichloromethane to precipitate a small quantity of silver saccharinate, which was filtered. Then, the filtrate was washed several times with water until neither bromide ions nor silver cations were detected in the washings by the silver nitrate and sodium chloride tests (Messadi et al., 2013). The organic layer was dried over  $\text{MgSO}_4$ , filtered and the solvent is removed by a rotary evaporator. In the end, the saccharinate-based ILs obtained were dried under vacuum overnight. The elemental analysis performed to each saccharinate-based ILs synthesized is presented in supplementary section S1.

### 2.2.2. Docusate-based ILs

All docusate alkyl ester betanium-based ILs were synthesized as follows. Briefly, in a 500 mL round-bottomed flask charged with a magnetic stir bar, 30 mmol of sodium docusate was dissolved in hot water (200 mL). To this stirred solution, an equimolar portion of the alkyl ester betanium methanesulfonate (30 mmol dissolved in 60 mL of water) was added dropwise. Then, the suspension was heated to reflux for 1 h, and stirred at room temperature during 6 h. Afterwards, the product was extracted three times with ethyl acetate (70 mL  $\times$  3), and

the organic layer obtained was evaporated to dryness. The residue was recovered by applying dichloromethane, being the solution washed several times with water to remove any inorganic salt. The organic layer was dried over  $\text{MgSO}_4$ , filtered and the solvent removed by the rotary evaporator. In the end, a viscous liquid was obtained. The elemental analysis performed to each docusate-based IL synthesized is presented in supplementary section S1.

### 2.2.3. Thiocyanate based-ILs

The thiocyanate-based ILs were synthesized according the methodology described for docusate based ILs. The thiocyanate anion/betanium cation ratio was equal to 2. The product was obtained as a white powder. The elemental analysis performed to each thiocyanate-based IL synthesized is presented in supplementary section S1.

## 2.3. Physicochemical analysis

A complete elemental analysis (C, H, N and S) was carried on a PerkinElmer 2400 C, H, N and S element analyser. IR spectra were recorded at room temperature with a PerkinElmer UATR Two spectrometer.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectrum were recorded at room temperature with a Bruker AC 30 spectrometer (250 MHz for  $^1\text{H}$ , 62.5 MHz for  $^{13}\text{C}$ ) using  $\text{CDCl}_3$  as solvent. Chemical shifts (in ppm) for  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were referenced to residual protic solvent peaks.

Differential Scanning Calorimetry (DSC) experiments were performed on a TA Instruments Q100 under a nitrogen atmosphere, following the same procedure described in literature (De Gaetano et al., 2015). The decomposition temperatures of the ILs were measured using a Netzsch TG 209 F3 Tarsus thermogravimetric analyser under nitrogen atmosphere, with the mass of TGA samples varying between 10 mg and 20 mg. The samples were heated from 30 °C to 500 °C with a heating rate of 10 °C.min $^{-1}$ .

The water contents in the ILs were determined by Karl Fischer titration as detailed in literature (Messadi et al., 2013). To ensure the highest purity of the glycine-betaine-derived ILs under study, all samples for the measurements of physicochemical properties and ecotoxicity were further purified. A standard protocol was applied, i.e. the samples were dried under vacuum (10 Pa), at room temperature for a minimum of 48 h. After this second purification step, the water content was measured again through a Metrohm 831 Karl Fisher coulometer. The water content determined after this second purification step was considered for the preparation of the stock solutions used in the ecotoxicity study.

## 2.4. *Microtox*<sup>®</sup> acute Toxicity Test

The toxicity of all ILs was assessed using the *Microtox*<sup>®</sup> Toxicity Test kit (Microbics Corporation, 1992) following the same procedure detailed elsewhere (Macário et al., 2018). Briefly, the luminescence production of the bacteria is understood as a metabolic effect to test treatments, which comprise a range of aqueous solutions (0–81.9% of a stock with a known concentration) of each IL. The light output of the luminescent bacteria following 5, 15 and 30 min of exposure was read and compared to that of a blank control. Concentrations promoting 50%, 20% and 10% of luminescence inhibition (EC<sub>50</sub>, EC<sub>20</sub> and EC<sub>10</sub>, respectively) and the corresponding 95% confidence intervals were estimated through a non-linear regression, using the least-squares method to fit the data to the logistic equation.

## 2.5. *Microalgae* bioassay

This assay was conducted as described in literature (Santos et al., 2015) with the microalgae *Raphidocelis subcapitata*, following the guidelines of OECD (OECD, 2011) adapted to the use of 24-well microplates (Geis et al., 2000). The test begun with an initial test cell density of 10<sup>4</sup> cells.mL<sup>-1</sup>, adjusted on the basis of microscopic cell counting (Neubauer haemocytometer) of a microalgae inoculum culture grown in MLB medium (Stein, 1973) under controlled incubation conditions (23 ± 1 °C and permanent illumination). The microalgae were exposed to a range of concentrations of each IL. All treatments, including the MBL blank control were run with three replicates. The microplates were incubated for 72 h under controlled incubation conditions as used for the inoculum. At the end of the test, the microalgae yield in each individual treatment was calculated as the difference between the cell densities at the end and beginning of the test. EC<sub>50</sub>, EC<sub>20</sub> and EC<sub>10</sub> and the corresponding 95% confidence intervals were calculated through a non-linear regression, using the least-squares method to fit the data to the logistic equation.

## 3. Results and discussion

### 3.1. (A)GB-ILs physico-chemical characterization

The methane-sulfonic acid route was selected due to its higher compatibility with what is called a “greener” synthetic route, known as less aggressive when compared with other inorganic acids, taking part of the natural sulphur cycle and being readily biodegradable (Higgins et al., 1996). The second step comprehended the synthesis of AGB-ILs by anion metathesis from the bromide salt using silver saccharinate in water-ethanol. The control of the absence of Br<sup>-</sup> or Ag<sup>+</sup> ions was done by using AgNO<sub>3</sub> and NaCl tests, respectively. The anionic metathesis from the methanesulfonate salt was performed using the salt of the anion constitutive of GB-ILs (Fig. 2). In the end, the AGB-ILs and GB-ILs were obtained with more than 80% of yield, being isolated as colorless viscous liquids ([Bu<sub>3</sub>NC<sub>4</sub>][Sac] and [Bu<sub>3</sub>PC<sub>4</sub>][Sac] crystallised after standing at room temperature for three days), and [Me<sub>3</sub>NC<sub>2</sub>OC<sub>12</sub>][SCN] and [Me<sub>3</sub>NC<sub>2</sub>OC<sub>14</sub>][SCN] as white solids.

<sup>1</sup>H and <sup>13</sup>C NMR spectrometry analysis revealed the absence of organic impurities in the ILs, and the proton chemical shifts cannot provide information regarding the presence or the absence of hydrogen bonding between the anion and cation of the ILs. The IR spectra for all the ILs show only weak absorption bands in the 3000–3100 cm<sup>-1</sup> region, indicating that the interaction between the cation and anion of the ILs via hydrogen bonds is rather limited. In this case, only the cation-anion Coulombic attraction ensures the cohesion of the salts. In addition, the IR spectra for the hydrated ILs indicated the presence of water traces identified as the large and intense band in the ca. 3400 cm<sup>-1</sup> region corresponding to OH stretching. All the characterization data were consistent with the expected structures and compositions.

**Table 2**

Melting temperature (T<sub>m</sub>), glass transition temperature (T<sub>g</sub>) and temperature of decomposition (T<sub>dec</sub>) for AGB and GB-ILs.

ILs	T <sub>m</sub> ± 0.4/°C	T <sub>g</sub> ± 0.4/°C	T <sub>dec</sub> ± 0.4/°C
[Bu <sub>3</sub> NC <sub>2</sub> ][Sac]	–	–	200.5
[Bu <sub>3</sub> NC <sub>4</sub> ][Sac]	95.0	–	290.3
[Bu <sub>3</sub> PC <sub>2</sub> ][Sac]	–	–43.7	208.6
[Bu <sub>3</sub> PC <sub>4</sub> ][Sac]	58.0	–	304.1
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>12</sub> ][Doc]	–	–32.4	273.2
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>14</sub> ][Doc]	–	–33.6	276.5
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>12</sub> ][SCN]	96.0	–	172.9
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>14</sub> ][SCN]	98.0	–	178.4

### 3.2. Thermal properties

The data of melting point, glass transition temperature, and the decomposition temperature of 10% of weight loss of AGB-ILs and GB-ILs are listed in Table 2. Among the ILs synthesized in this work, four of them, namely the [Bu<sub>3</sub>NC<sub>2</sub>][Sac], [Bu<sub>3</sub>PC<sub>2</sub>][Sac], [Me<sub>3</sub>NC<sub>2</sub>OC<sub>12</sub>][Doc] and [Me<sub>3</sub>NC<sub>2</sub>OC<sub>14</sub>][Doc] are liquid at room temperature. They have no melting point even when cooled from room temperature to –80 °C, whereas a glass transition temperature was only observed for [Bu<sub>3</sub>PC<sub>2</sub>][Sac], [Me<sub>3</sub>NC<sub>2</sub>OC<sub>12</sub>][Doc] and [Me<sub>3</sub>NC<sub>2</sub>OC<sub>14</sub>][Doc] at –43.0 °C, 32.0 °C and 33.0 °C (uncertainty of 0.4 °C obtained from two replicates), respectively. Although the melting point of [Bu<sub>3</sub>PC<sub>4</sub>][Sac] was higher than room temperature (58 °C), it showed a lower melting point than the corresponding ammonium salt [Bu<sub>3</sub>NC<sub>4</sub>][Sac] (95 °C). This indicates that the phosphonium cation gives a relatively low melting point when compared to the corresponding ammonium congener. Considering the effect of counterion on the transition temperature, we found that its size seems to modulate the melting temperatures of the GB-ILs, thus justifying that salts containing the smallest thiocyanate anion displayed high melting temperatures.

The thermal stability of the ILs was studied by thermogravimetric analysis (TGA) over the temperature range of 30–500 °C, as it is indicated in section 2.2. Thermal degradation temperature (T<sub>dec</sub>) of all these salts lies in the range 200–304 °C. For the AGB-ILs, the results show that the stability is enhanced with increasing the alkyl chain length bearing ester group. For example, with [Bu<sub>3</sub>NC<sub>n</sub>][Sac] or [Bu<sub>3</sub>PC<sub>n</sub>][Sac] (n = 2 or 4), when the number of carbon atoms n increases from 2 to 4, there is an increase on the degradation temperature from 200 to 290 °C and 209–304 °C, respectively (Table 2). These results are in good agreement with those reported by Tsunashima and Sugiya (2007) and Tsunashima et al. (2009) describing the thermogravimetric analysis data of alkyl substituted phosphonium ILs.

Considering the saccharinate-based ILs, the comparison of thermogravimetric traces of ammonium-based ILs with those for the corresponding phosphonium show that the thermal degradation temperature of phosphonium depends on the number of the obtained alkyl chain bearing the ester group. The phosphonium-based ILs decompose at slightly higher temperatures ([Bu<sub>3</sub>PC<sub>2</sub>][Sac], T<sub>dec</sub> = 208.6 ± 0.4 °C, [Bu<sub>3</sub>PC<sub>4</sub>][Sac], T<sub>dec</sub> = 304.1 ± 0.4 °C) than the corresponding ammonium congeners ([Bu<sub>3</sub>NC<sub>2</sub>][Sac], T<sub>dec</sub> = 200.5 ± 0.4 °C, [Bu<sub>3</sub>NC<sub>4</sub>][Sac], T<sub>dec</sub> = 290.3 ± 0.4 °C). Thus, the relative cation thermal stabilities can be suggested as [Bu<sub>3</sub>NC<sub>2</sub>]<sup>+</sup> < [Bu<sub>3</sub>PC<sub>2</sub>]<sup>+</sup> < [Bu<sub>3</sub>NC<sub>4</sub>]<sup>+</sup> < [Bu<sub>3</sub>PC<sub>4</sub>]<sup>+</sup>.

For GB-ILs, the decomposition temperature was found to be dependent of the nature of the anions, as it has been reported in numerous previous works (Cao and Tiancheng, 2014; Parajó et al., 2018). The thiocyanate anion reduced the thermal stability to near the 100 °C, a value below the one obtained for the corresponding ILs with docusate counterion. In this sense, the relative anion thermal stabilities appear as follows [SCN]<sup>-</sup> < [Doc]<sup>-</sup>.

**Table 3**EC<sub>50</sub>, EC<sub>20</sub>, EC<sub>10</sub> values (mg.L<sup>-1</sup>) and corresponding 95% confidence limits within brackets, obtained for 5, 15 and 30 min of exposure of *Aliivibrio fischeri*.

IL	Time (min)	EC <sub>50</sub> (mg.L <sup>-1</sup> )	EC <sub>20</sub> (mg.L <sup>-1</sup> )	EC <sub>10</sub> (mg.L <sup>-1</sup> )
[Bu <sub>3</sub> PC <sub>4</sub> ] [Sac]	5	192.34 (101.83; 282.85)	36.11 (2.27; 69.96)	–
	15	64.31 (43.67; 84.95)	20.34 (8.53; 32.14)	10.36 (2.18; 18.54)
	30	<b>32.03 (28.01; 36.04)</b>	<b>15.83 (12.47; 19.19)</b>	<b>10.48 (7.47; 13.48)</b>
[Bu <sub>3</sub> PC <sub>2</sub> ] [Sac]	5	32.00 (29.16; 34.84)	19.77 (16.97; 22.57)	14.91 (12.05; 17.77)
	15	16.44 (15.15; 17.74)	12.65 (11.43; 13.87)	10.85 (9.55; 12.14)
	30	<b>13.81 (12.23; 15.40)</b>	<b>11.22 (10.26; 13.18)</b>	<b>9.93 (8.37; 11.50)</b>
[Bu <sub>3</sub> NC <sub>4</sub> ] [Sac]	5	1904.43 (475.78; 3333.08)	–	–
	15	628.04 (439.27; 816.82)	81.93 (31.54; 132.33)	24.86 (2.04; 47.69)
	30	<b>401.14 (321.01; 481.27)</b>	<b>86.82 (52.00; 121.64)</b>	<b>35.43 (15.09; 55.77)</b>
[Bu <sub>3</sub> NC <sub>2</sub> ] [Sac]	5	248.48 (207.94; 289.03)	92.51 (66.57; 118.46)	51.87 (30.16; 73.57)
	15	103.51 (93.23; 113.78)	59.34 (49.68; 69.01)	42.84 (33.31; 52.38)
	30	<b>68.06 (65.15; 70.98)</b>	<b>46.72 (43.34; 50.11)</b>	<b>37.48 (33.83; 41.13)</b>
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>12</sub> ] [Doc]	5	4.61 (3.92; 5.31)	1.93 (1.43; 2.43)	1.15 (0.71; 1.59)
	15	3.47 (2.95; 3.98)	1.55 (1.15; 1.96)	0.97 (0.61; 1.33)
	30	<b>3.19 (2.69; 3.69)</b>	<b>1.48 (1.07; 1.88)</b>	<b>0.94 (0.58; 1.29)</b>
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>12</sub> ] [SCN]	5	1.41 (1.22; 1.59)	0.69 (0.53; 0.85)	0.46 (0.32; 0.59)
	15	0.77 (0.65; 0.88)	0.38 (0.29; 0.48)	0.26 (0.17; 0.34)
	30	<b>0.61 (0.52; 0.69)</b>	<b>0.34 (0.26; 0.42)</b>	<b>0.24 (0.17; 0.34)</b>
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>14</sub> ] [Doc]	5	3.19 (2.69; 3.69)	1.48 (1.07; 1.88)	0.94 (0.58; 1.29)
	15	1.39 (0.86; 1.92)	0.28 (0.07; 0.48)	–
	30	<b>1.37 (0.97; 1.74)</b>	<b>0.35 (0.16; 0.53)</b>	<b>0.16 (0.04; 0.27)</b>
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>14</sub> ] [SCN]	5	1.61 (1.27; 1.94)	0.97 (0.63; 1.31)	0.72 (0.38; 1.07)
	15	1.10 (0.84; 1.35)	0.58 (0.35; 0.81)	0.40 (0.18; 0.61)
	30	<b>0.90 (0.68; 1.12)</b>	<b>0.45 (0.26; 0.64)</b>	<b>0.30 (0.13; 0.47)</b>

### 3.3. Ecotoxicity

The idea that ILs deriving from natural products such as betaine, are mandatorily biocompatible and environmentally-friendly was challenged in this work, by addressing the ecotoxicity of (A)GB-ILs towards two standard organisms, *Aliivibrio fischeri* and *Raphidocelis subcapitata*. The experimental data from bacteria and microalgae were used for the estimation of Effective Concentrations, which serve as the basis for the comparative analysis on the sensitivity of these organisms to different ILs. For the bacteria, it was possible to derive EC<sub>50</sub>, EC<sub>20</sub> and EC<sub>10</sub> at three different times of exposure, namely 5, 15 and 30 min (Table 3); and for the microalgae, the same EC<sub>50</sub>, EC<sub>20</sub> and EC<sub>10</sub> values were estimated, but after 72 h of incubation (Table 4).

Due to its simplicity and cost-efficiency, the Microtox test is one of the most common toxicological bioassays and has been widely applied and validated on the determination of the toxicity of various ILs (Hernández-Fernández et al., 2015; Luis et al., 2007; Romero et al., 2008; Ventura et al., 2014). By analysing both Tables 3 and 4, similar tendencies were found for the *Aliivibrio fischeri* and *Raphidocelis subcapitata*, but as expected from previous studies (Santos et al., 2015), microalgae showed higher sensitivity for the set of ILs analysed (see Tables 3 and 4). There are indeed differences up to more than two orders of magnitude in the case of [Bu<sub>3</sub>NC<sub>4</sub>][Sac], or of two times in the case of [Me<sub>3</sub>NC<sub>2</sub>OC<sub>12</sub>][SCN].

The least toxic IL was [Bu<sub>3</sub>NC<sub>4</sub>][Sac]. It is indeed, the only IL that

**Table 4**EC<sub>50</sub>, EC<sub>20</sub>, EC<sub>10</sub> (mg.L<sup>-1</sup>) and corresponding 95% confidence limits within brackets for the growth response of *Raphidocelis subcapitata* to the (A)GB-ILs.

IL	EC <sub>50</sub> (mg.L <sup>-1</sup> )	EC <sub>20</sub> (mg.L <sup>-1</sup> )	EC <sub>10</sub> (mg.L <sup>-1</sup> )
[Bu <sub>3</sub> PC <sub>4</sub> ] [Sac]	1.30 (0.81; 1.79)	0.19 (0.06; 0.33)	0.06 (0.00; 0.12)
[Bu <sub>3</sub> PC <sub>2</sub> ] [Sac]	0.43 (0.26; 0.59)	0.08 (0.03; 0.12)	0.03 (0.00; 0.05)
[Bu <sub>3</sub> NC <sub>4</sub> ] [Sac]	12.83 (9.88; 15.78)	7.83 (4.70; 10.96)	5.86 (2.50; 9.23)
[Bu <sub>3</sub> NC <sub>2</sub> ] [Sac]	4.71 (4.13; 5.29)	2.81 (2.20; 3.43)	2.08 (1.46; 2.69)
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>12</sub> ] [Doc]	0.58 (0.21; 0.96)	–	–
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>12</sub> ] [SCN]	0.27 (0.19; 0.34)	0.07 (0.04; 0.10)	0.03 (0.01; 0.05)
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>14</sub> ] [Doc]	0.74 (0.64; 0.84)	0.41 (0.31; 0.50)	0.29 (0.19; 0.38)
[Me <sub>3</sub> NC <sub>2</sub> OC <sub>14</sub> ] [SCN]	0.54 (0.32; 0.77)	0.12 (0.03; 0.21)	0.05 (0.00; 0.10)

could be considered as non-toxic for the bacteria (EC<sub>50</sub>, at 30 min = 401.14 mg.L<sup>-1</sup>). However, the same IL showed appreciable toxicity to the microalgae (EC<sub>50</sub> = 12.83 mg.L<sup>-1</sup>). Strangely, the similar ammonium IL, [Bu<sub>3</sub>NC<sub>2</sub>][Sac], showed a higher toxicity, consistently for both for bacteria as microalgae (see Tables 3 and 4). All tested ILs could be labeled as harmful to the aquatic environment following UN standard labelling guidelines (e.g. UN, 2011). Under these guidelines, the bacteria are not straightforwardly considered as indicators, but microalgae test outcomes - which actually were more sensitive, thus reflecting more environmentally protective labelling - suggest that even the least toxic IL ([Bu<sub>3</sub>NC<sub>4</sub>][Sac]) can be harmful to aquatic life (10 < EC<sub>50</sub> < 100 mg L<sup>-1</sup>); [Bu<sub>3</sub>PC<sub>4</sub>][Sac] and [Bu<sub>3</sub>NC<sub>2</sub>][Sac] are suggested as toxic to aquatic life (1 < EC<sub>50</sub> < 10 mg L<sup>-1</sup>) and all other ILs can be very toxic to aquatic life (EC<sub>50</sub> < 1 mg L<sup>-1</sup>).

Our results show that ILs derived from natural products should not be considered as “green” or “greener”, *per se*, without a corresponding study of their (eco)toxicity. For example, the non-volatility associated to the benign character of ILs, can increase their toxic effect in the aquatic environment (Matzke et al., 2010). Structural modifications are responsible for changings on the ecotoxicological profile and should be understood in order to allow a more biocompatible development of ILs. Nowadays, this is of extreme importance, and is under the scope of REACH Directive (EU, 2007). Taking a look at the structure, [Me<sub>3</sub>NC<sub>2</sub>OC<sub>12</sub>][Doc] and [Me<sub>3</sub>NC<sub>2</sub>OC<sub>14</sub>][Doc] have longer alkyl chains than [Me<sub>3</sub>NC<sub>2</sub>OC<sub>12</sub>][SCN] and [Me<sub>3</sub>NC<sub>2</sub>OC<sub>14</sub>][SCN], and therefore they were expected to be more toxic (Petkovic et al., 2011). However, results show that ILs with [Doc] are less toxic to the tested organisms. Longer cation alkyl chains are normally more toxic, due to the “side-chain” effect, because of the increase in lipophilicity caused by the elongation of the alkyl chain, which promotes higher reactivity with the biological membranes and embedded proteins (Stolte et al., 2007). However, this only happens until a certain threshold. Above this threshold, regardless the number of carbons added, there is no increment in the toxicity, this being assumed as a “cut-off” effect (Matzke et al., 2010). This effect could justify the lower toxicity of [Me<sub>3</sub>NC<sub>2</sub>OC<sub>12</sub>][Doc] and [Me<sub>3</sub>NC<sub>2</sub>OC<sub>14</sub>][Doc], in relation to [Me<sub>3</sub>NC<sub>2</sub>OC<sub>12</sub>][SCN] and [Me<sub>3</sub>NC<sub>2</sub>OC<sub>14</sub>][SCN]. This effect was already observed for several ILs, and could be related to kinetic aspects or insufficient solubility (Matzke et al., 2010). As expected, the EC values for

*Aliivibrio fischeri* are highly dependent on the cation studied (Ventura et al., 2014). In the case of microalgae, the differences in the EC<sub>50</sub> values are less pronounced.

A more direct interaction between the cation rather than the anion and the biological membranes is expected since these are negatively charged. However, in addition to the role of the cation, the anion moiety has been also shown to be relevant in the toxicity of ILs (Ventura et al., 2014), by altering their hydrophobicity (Stolte et al., 2007). Considering the anions tested, [SCN]<sup>-</sup>, [Doc]<sup>-</sup> and [Sac]<sup>-</sup>, it is clear that the ILs ecotoxicity decreases with the increase of the alkyl chain length of the anion ([SCN]<sup>-</sup> is the most toxic and [Sac]<sup>-</sup> the least one).

The effect of the presence of a benzyl group in the cation was addressed by Santos et al. (2015), who found that the introduction of a benzyl group decreased the toxicity of cholinium-based ILs to *Aliivibrio fischeri* and *Raphidocelis subcapitata*, but increased the toxicity to *Lemna minor* and *Daphnia magna*. For example, Ventura et al. (2013) showed that aromatic propyl-imidazolium and pyridinium were more toxic than the non-aromatic equivalents, regardless the species tested. Benzene shows a narcotic toxicity mode of action through interaction with the cellular membranes (Enoch et al., 2008). However, its toxic role in the anion could be reduced because the cellular membranes are negatively charged. This could help to explain the smaller toxicity in the ILs with [Sac]<sup>-</sup>. Also, regarding specifically the [Sac]<sup>-</sup> anion, the toxicity of [Bu<sub>3</sub>PC<sub>4</sub>]<sup>+</sup> was higher than the toxicity of [Bu<sub>3</sub>NC<sub>4</sub>]<sup>+</sup>, which means that the nitrogen central atom induces lower toxicity than the phosphorous central atom, as already reported (Carvalho et al., 2014).

#### 4. Conclusion

In this work, synthesis, characterization and ecotoxicity of eight ILs based on Analogues of Glycine-Betaine and Glycine-Betaine - (A)GB-ILs - were reported. The effect of the central heteroatoms and the alkyl chain length on the physical properties of AGB-ILs were studied. The decomposition temperatures of GB-ILs were found to be dominated by the nature of the anions.

Regarding the ILs ecotoxicity, the freshwater microalgae *Raphidocelis subcapitata* was proven to be more sensitive than the widely tested luminescent marine bacteria *Aliivibrio fischeri*. Therefore, the microalgae seem to be more suitable for using in the assessment of the ecotoxicity of ILs whenever environmental protection frameworks are a critical arena of the study.

This work allowed us to infer about the effect of different anions, cations and on how the natural-based compounds applied on the design of ILs can affect the biocompatibility and benignity of new products and processes. More generally, this work is a further example where the overgeneralizations about the ILs “non-toxic” and “greener” nature are wrong and need to be eliminated from literature.

#### Acknowledgements

This work was developed within the scope of the projects EM2013/031 and GRC ED431C 2016/001 (Xunta de Galicia, Spain) and by the network ReGaLs R2014/015 (Xunta de Galicia, Spain). This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, FCT Ref. UID/CTM/50011/2019, financed by national funds through the FCT/MCTES. Thanks are due for the financial support to CESAM (UID/AMB/50017/2019), to FCT/MCTES through national funds. This work is a contribution to the project PTDC/ATP-EAM/5331/2014, funded by FEDER, through COMPETE2020 - POCI, and by national funds through FCT/MCTES. J.L. Pereira is funded by national funds (OE) through FCT, under a framework contract (art. 23, Decree-Law 57/2016, changed by Law 57/2017); I.P.E. Macário is a recipient of an individual research grant by FCT (SFRH/BD/123850/2016) financed by the FCT/MEC and co-financed by FEDER program. S.P.M. Ventura acknowledges the financial support from FCT by the IF project IF/00402/2015. J.J. Parajó thanks to COST Action CM1206 EXIL.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecoenv.2019.109580>.

#### References

- Alvarez-Guerra, M., Irabien, A., 2011. Design of ionic liquids: an ecotoxicity (*Vibrio fischeri*) discrimination approach. *Green Chem.* 13, 1507. <https://doi.org/10.1039/c0gc00921k>.
- Amde, M., Liu, J.F., Pang, L., 2015. Environmental application, fate, effects, and concerns of ionic liquids: a review. *Environ. Sci. Technol.* 49, 12611–12627. <https://doi.org/10.1021/acs.est.5b03123>.
- Bado-Nilles, A., Diallo, A.O., Marlaire, G., et al., 2015. Coupling of OECD standardized test and immunomarkers to select the most environmentally benign ionic liquids option-Towards an innovative “safety by design” approach. *J. Hazard Mater.* 283, 202–210. <https://doi.org/10.1016/j.jhazmat.2014.09.023>.
- Biczak, R., Pawłowska, B., Bałczewski, P., Rychter, P., 2014. The role of the anion in the toxicity of imidazolium ionic liquids. *J. Hazard Mater.* 274, 181–190. <https://doi.org/10.1016/j.jhazmat.2014.03.021>.
- Bubalo, M.C., Radošević, K., Redovniković, I.R., et al., 2014. A brief overview of the potential environmental hazards of ionic liquids. *Ecotoxicol. Environ. Saf.* 99, 1–12. <https://doi.org/10.1016/j.ecoenv.2013.10.019>.
- Cao, Y., Tiancheng, M., 2014. Comprehensive Investigation on the Thermal Stability of 66 Ionic Liquids by Thermogravimetric Analysis. *Ind. Eng. Chem Res.* 53, 8651–8664. <https://doi.org/10.1021/ie5009597>.
- Carvalho, P.J., Ventura, S.P.M., Batista, M.L.S., et al., 2014. Understanding the impact of the central atom on the ionic liquid behavior: phosphonium vs ammonium cations. *J. Chem. Phys.* <https://doi.org/10.1063/1.4864182>.
- Craig, S., 2004. Betaine in human nutrition. *Am. J. Clin. Nutr.* 80, 539–549. <https://doi.org/10.1056/NEJMra1112830>.
- Enoch, S.J., Hewitt, M., Cronin, M.T.D., et al., 2008. Classification of chemicals according to mechanism of aquatic toxicity: an evaluation of the implementation of the Verhaar scheme in Toxtree. *Chemosphere* 73, 243–248. <https://doi.org/10.1016/j.chemosphere.2008.06.052>.
- EU, 2007. Corrigendum to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).
- Ferreira, Ana M., Helena Passos, Okafuji, Akiyoshi, Freire, Mara G., João, A., Coutinho, P., Ohno, Hiroyuki, 2017. Designing the thermal behaviour of aqueous biphasic systems composed of ammonium-based zwitterions. *Green Chem.* 19, 4012–4016.
- Freire, M.G., Santos, L.M.N.B.F., Fernandes, A.M., et al., 2007. An overview of the mutual solubilities of water-imidazolium-based ionic liquids systems. *Fluid Phase Equilib.* 261, 449–454. <https://doi.org/10.1016/j.fluid.2007.07.033>.
- De Gaetano, Y., Mohamadou, A., Boudesocque, S., et al., 2015. Ionic liquids derived from esters of Glycine Betaine: synthesis and characterization. *J. Mol. Liq.* 207, 60–66. <https://doi.org/10.1016/j.molliq.2015.03.016>.
- Geis, S.W., Fleming, K.L., Korthals, E.T., et al., 2000. Modifications to the algal growth inhibition test for use as a regulatory assay. *Environ. Toxicol. Chem.* 19, 36–41. <https://doi.org/10.1002/etc.5620190105>.
- Goursaud, F., Berchel, M., Guilbot, J., et al., 2008. Glycine betaine as a renewable raw material to “greener” new cationic surfactants. *Green Chem.* 10, 318–328. <https://doi.org/10.1039/b713429k>.
- Gouveia, W., Jorge, T.F., Martins, S., et al., 2014. Toxicity of ionic liquids prepared from biomaterials. *Chemosphere* 104, 51–56. <https://doi.org/10.1016/j.chemosphere.2013.10.055>.
- Hernández-Fernández, F.J., Bayo, J., Pérez de los Rios, A., et al., 2015. Discovering less toxic ionic liquids by using the Microtox toxicity test. *Ecotoxicol. Environ. Saf.* 116, 29–33. <https://doi.org/10.1016/j.ecoenv.2015.02.034>.
- Higgins, T.P., Davey, M., Trickett, J., Kelly, D.P., Murrell, J.C., 1996. Metabolism of methanesulfonic acid involves a multicomponent monooxygenase enzyme. *Micriobiology* 142, 251–260. <https://doi.org/10.1099/13500872-142-2-251>.
- Jordan, A., Gathergood, N., 2015. Biodegradation of ionic liquids—a critical review. *Chem. Soc. Rev.* 44, 8200–8237. <https://doi.org/10.1039/c5cs00444f>.
- Kumar, V., Malhotra, S.V., 2009. Study on the potential anti-cancer activity of phosphonium and ammonium-based ionic liquids. *Bioorg. Med. Chem. Lett* 19, 4643–4646. <https://doi.org/10.1016/j.bmcl.2009.06.086>.
- Li, X.Y., Zhou, J., Yu, M., et al., 2009. Toxic effects of 1-methyl-3-octylimidazolium bromide on the early embryonic development of the frog *Rana nigromaculata*. *Ecotoxicol. Environ. Saf.* 72, 552–556. <https://doi.org/10.1016/j.ecoenv.2007.11.002>.
- Lovelock, K.R.J., Deyko, A., Licence, P., Jones, R.G., 2010. Vaporisation of an ionic liquid near room temperature. *Phys. Chem. Chem. Phys.* 12, 8993–8991.
- Luis, P., Ortiz, I., Aldaco, R., Irabien, A., 2007. A novel group contribution method in the development of a QSAR for predicting the toxicity (*Vibrio fischeri* EC<sub>50</sub>) of ionic liquids. *Ecotoxicol. Environ. Saf.* 67, 423–429. <https://doi.org/10.1016/j.ecoenv.2006.06.010>.
- Macário, I.P.E., Jesus, F., Pereira, J.L., et al., 2018. Unraveling the ecotoxicity of deep eutectic solvents using the mixture toxicity theory. *Chemosphere* 212, 890–897. <https://doi.org/10.1016/j.chemosphere.2018.08.153>.
- Matzke, M., Arning, J., Ranke, J., et al., 2010. Design of Inherently Safer Ionic Liquids: Toxicology and Biodegradation. *Handb. Green Chem.* Wiley-VCH, pp. 233–298.
- Matzke, M., Stolte, S., Thiele, K., et al., 2007. The influence of anion species on the toxicity of 1-alkyl-3-methylimidazolium ionic liquids observed in an (eco)

- toxicological test battery. *Green Chem.* 9, 1198. <https://doi.org/10.1039/b705795d>.
- Messadi, A., Mohamadou, A., Boudesocque, S., et al., 2013. Syntheses and characterisation of hydrophobic ionic liquids containing trialkyl(2-ethoxy-2-oxoethyl)ammonium or N-(1-methylpyrrolidyl-2-ethoxy-2-oxoethyl)ammonium cations. *J. Mol. Liq.* 184, 68–72. <https://doi.org/10.1016/j.molliq.2013.04.023>.
- OECD, 2011. *Oecd guidelines for the testing of chemicals, Section 2*.
- Oliveira, M.V.S., Vidal, B.T., Melo, C.M., et al., 2016. (Eco)toxicity and biodegradability of protic ionic liquids. *Chemosphere* 147, 460–466. <https://doi.org/10.1016/j.chemosphere.2015.11.016>.
- Parajó, J.J., Villanueva, M., Otero, I., Fernández, J., Salgado, J., NaFoMat, 2018. Thermal stability of aprotic ionic liquids as potential lubricants. Comparison with synthetic oil bases. *J. Chem. Thermodynamics* 116, 185–196. <https://doi.org/10.1016/j.jct.2017.09.010>.
- Pawłowska, B., Biczak, R., 2016. Evaluation of the effect of tetraethylammonium bromide and chloride on the growth and development of terrestrial plants. *Chemosphere* 149, 24–33. <https://doi.org/10.1016/j.chemosphere.2016.01.072>.
- Pereira, Matheus M., Almeida, Mafalda R., Gomes, Joana, Rufino, A.F.C.S., Rosa, Marguerita E., Coutinho, João A.P., Mohamadou, Aminou, Freire, Mara G., 2018. Glycine-Betaine ionic liquid analogues as novel phase-forming components of aqueous biphasic systems. *Biotechnol. Prog.* 34, 1205–1212.
- Petkovic, M., Seddon, K.R., Rebelo, L.P.N., Pereira, C.S., 2011. Ionic liquids: a pathway to environmental acceptability. *Chem. Soc. Rev.* 40, 1383–1403. <https://doi.org/10.1039/c004968a>.
- Pretti, C., Chiappe, C., Pieraccini, D., et al., 2006. Acute toxicity of ionic liquids to the zebrafish (*Danio rerio*). *Green Chem.* 8, 238–240. <https://doi.org/10.1039/B511554J>.
- Ranke, J., Stolte, S., Störmann, R., et al., 2007. Design of sustainable chemical products - the example of ionic liquids. *Chem. Rev.* 107, 2183–2206. <https://doi.org/10.1021/cr050942s>.
- Romero, A., Santos, A., Tojo, J., Rodríguez, A., 2008. Toxicity and biodegradability of imidazolium ionic liquids. *J. Hazard Mater.* 151, 268–273. <https://doi.org/10.1016/j.jhazmat.2007.10.079>.
- Ruß, C., König, B., 2012. Low melting mixtures in organic synthesis – an alternative to ionic liquids? *Green Chem.* 14, 2969–2982. <https://doi.org/10.1039/b000000x>.
- Salgado, J., Parajó, J.J., Teijeira, T., et al., 2017. New insight into the environmental impact of two imidazolium ionic liquids. Effects on seed germination and soil microbial activity. *Chemosphere* 185, 665–672. <https://doi.org/10.1016/j.chemosphere.2017.07.065>.
- Santi, E., Viera, I., Mombrú, A., et al., 2011. Synthesis and characterization of heteroleptic copper and zinc complexes with saccharinate and aminoacids. Evaluation of SOD-like activity of the copper complexes. *Biol. Trace Elem. Res.* 143, 1843–1855. <https://doi.org/10.1007/s12011-011-8992-2>.
- Santos, J.I., Gonçalves, A.M.M., Pereira, J.L., et al., 2015. Environmental safety of cholinium-based ionic liquids: assessing structure-ecotoxicity relationships. *Green Chem.* 17, 4657–4668. <https://doi.org/10.1039/c5gc01129a>.
- Silva, F.A., Coutinho, J.A.P., Ventura, S.P.M., 2019. *Aquatic Toxicology of Ionic Liquids (ILs) in Encyclopedia of Ionic Liquids, 1<sup>st</sup> Edition*. Springer Nature.
- Stein, J., 1973. *Handbook of Phycological Methods—Culture, Methods and Growth Measurements*. (London).
- Stolte, S., Matzke, M., Jürgen, A., et al., 2007. Effects of different head groups and functionalised side chains on the aquatic toxicity of ionic liquids. *Green Chem.* 9, 1170–1179. <https://doi.org/10.1039/b711119c>.
- Stolte, S., Stepnowski, P., 2011. Editorial [hot topic: ionic liquids: analytical and environmental issues (guest editors: piotr Stepnowski and stefan Stolte)]. *Curr. Org. Chem.* 15, 1871. <https://doi.org/10.2174/138527211795703621>.
- Tsunashima, K., Niwa, E., Kodama, S., et al., 2009. Thermal and transport properties of ionic liquids based on benzyl-substituted phosphonium cations. *J. Phys. Chem. B* 113, 15870–15874. <https://doi.org/10.1021/jp908356c>.
- Tsunashima, K., Sugiya, M., 2007. Physical and electrochemical properties of low-viscosity phosphonium ionic liquids as potential electrolytes. *Electrochem. Commun.* 9, 2353–2358. <https://doi.org/10.1016/j.elecom.2007.07.003>.
- UN, 2011. Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fourth Rev. United Nations <https://doi.org/10.1265/jjh.65.5>.
- Ventura, S.P.M., E Silva, F.A., Gonçalves, A.M.M., et al., 2014. Ecotoxicity analysis of cholinium-based ionic liquids to *Vibrio fischeri* marine bacteria. *Ecotoxicol. Environ. Saf.* 102, 48–54. <https://doi.org/10.1016/j.ecoenv.2014.01.003>.
- Ventura, S.P.M., Gonçalves, A.M.M., Sintra, T., et al., 2013. Designing ionic liquids: the chemical structure role in the toxicity. *Ecotoxicology* 22, 1–12. <https://doi.org/10.1007/s10646-012-0997-x>.
- Zeisel, S.H., Mar, M.-H., Howe, J.C., Holden, J.M., 2003. Concentrations of choline-containing compounds and betaine in common foods. *J. Nutr.* 133, 1302–1307. <https://doi.org/10.1080/07347330903549820>.