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Ecotoxicity analysis of cholinium-based ionic liquids to *Vibrio fischeri* marine bacteria

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ABSTRACT

Cholinium-based ionic liquids are quaternary ammonium salts with a wide range of potential industrial applications. Based on the fact that the cholinium is a complex B vitamin and widely used as food additive, the cholinium-based ionic liquids are generically regarded as environmentally "harmless" and thus, accepted as "non-toxic", although their ecotoxicological profile is poorly known. This work provides new ecotoxicological data for ten cholinium-based salts and ionic liquids, aiming to extend the surprisingly restricted body of knowledge about the ecotoxicity of this particular family and to gain insight on the toxicity mechanism of these compounds. The results reported here show that not all the cholinium tested can be considered harmless towards the test organism adopted. Moreover, the results suggest that the cholinium family exhibits a different mechanism of toxicity as compared to the imidazolium ionic liquids previously described in the literature.

1. Introduction

Ionic liquids (ILs) are salts with low melting points, liquid at or close to room temperature. The reason for their low melting points lies in the asymmetry of their ionic structure and the dispersion of their charges, which is responsible for lower intermolecular attractions and, thus decreasing the energy of the lattice (Ranke et al., 2004). They were initially proposed as alternatives to the common organic solvents (Earle and Seddon, 2002) but, as soon as their chemistry has developed, unexpected opportunities in different areas emerged. They possess a series of unusual properties, namely high chemical and thermal stability, high ionic conductivity and a wide electrochemical potential window (Haerens et al., 2009; Wasserscheid and Keim, 2000; Zhao et al., 2011) very useful for multiple industrial and chemical applications. Besides those properties, ILs are known for their non-flammability, non-explosiveness and very low vapor pressures (Deetlefs and Seddon, 2006; Earle et al., 2006; Lovelock et al., 2010), which drastically reduces their potential to constitute atmospheric pollutants. Due to their ionic character most of them are however soluble in water (Anthony et al., 2001; Freire et al., 2007), which can be responsible for their potential release into aquatic ecosystems, making them a potential environmental problem (Matzke et al., 2010). In the last years, the attention towards the knowledge of (eco)toxicological hazard potential of several chemical compounds has been boosted by the yield of regulatory demands, *e.g.* those defined by the European Union regulation for the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) (EC, 2007). The number of studies involving the toxicological scrutiny (cytotoxicity, (eco)toxicity, phytotoxicity and antimicrobial activity), as well as the environmental fate analysis of ILs is growing (Coleman and Gathergood, 2010; Frade and Afonso, 2010; Pham et al., 2009; Ranke et al., 2007b). Results reported in different works (Pretti et al., 2009; Stolte et al., 2007a) suggest that the ILs' (eco)toxicity varies widely with organisms and across trophic levels (Matzke et al., 2010; Ventura et al., 2010). In general, the authors consistently conclude that by changing the IL cation core it is possible to induce significant changes in the ecotoxicity (Docherty and Kulpa, 2005; Garcia et al., 2005; Matzke et al., 2010; Pham et al., 2009; Stolte et al., 2007b; Ventura et al., 2012b). Besides the cation core, other IL features, such as the cation alkyl chain length and anion type were also investigated. Several works have shown that the elongation of the cation alkyl side chain is responsible for an increase on the ecotoxicity ("side chain effect") (Matzke et al., 2010), a phenomenon which is only valid until a certain number of carbons ("cut-off effect") (Matzke et al., 2010; Ventura et al., 2012b). When the cation side chain is functionalized, the large majority of the reports describe a decrease in the ILs' toxicity,

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when compared with the respective non-functionalized chains (Coleman et al., 2012; Gore et al., 2013; Matzke et al., 2010; Morrissey et al., 2009; Myles et al., 2010; Stolte et al., 2007a, 2007b; Ventura et al., 2013b). This decrease was justified by the higher hydrophilicity of the ILs. Indeed, the toxicity of these compounds was correlated with several parameters such as lipophilicity (Costello et al., 2009; Ranke et al., 2007a), octanol–water partition coefficients (Ventura et al., 2011) and solubility in water (Ranke et al., 2007a, 2009; Ventura et al., 2013a). The anion moiety was also addressed in some studies although to a lower extent (Matzke et al., 2010; Stolte et al., 2007b). Since the ILs toxicity seems to be associated with their lipophilic nature, most studies dealing with ILs are based on hydrophobic (Frade and Afonso, 2010; Matzke et al., 2010; Pham et al., 2009; Ranke et al., 2007b; Stolte et al., 2012a; Ventura et al., 2010, 2013a) and halogenate anions (Frade and Afonso, 2010; Matzke et al., 2010; Pham et al., 2009; Ranke et al., 2007b). Recently, efforts to fulfill the gap of ecotoxicity data considering the effect of distinct hydrophilic anions (Ventura et al., 2012a; Ventura et al., 2012b) have been developed.

Cholinium, also known as choline, is the *N*,*N*,*N*-trimethylethanolammonium cation, an essential nutrient (Zeisel and da Costa, 2009) is receiving a considerable attention (Domínguez de María and Maugeri, 2011; Gorke et al., 2010) due to its claimed "benign" (Petkovic et al., 2011), biocompatible (Petkovic et al., 2010; Sekar et al., 2012), "environmentally friendly" or "non-toxic" nature (Gorke et al., 2010; Li et al., 2012; Nockemann et al., 2007a; Petkovic et al., 2010). This family is derived from quaternary ammonium salts described as important structures in living processes, used as precursors for the synthesis of vitamins (*e.g.* vitamin B complexes and thiamine) and enzymes that participate in the carbohydrate metabolism (Meck and Williams, 1999; Zeisel, 1999). Cholinium salts with low melting points have been the subject of some studies (Hamzé et al., 2005; Pernak and Chwala, 2003) and in this context, the concept of cholinium (or choline) based-ILs appeared. There is an increasing number of ILs based on this particular cation and the interest in these compounds is being fueled by the widespread idea of their "high biodegradability nature" and "non-toxic profile" (Hou et al., 2013; Meck and Williams, 1999; Shahriari et al., 2013). In terms of potential applications, cholinium-based ILs have been used not only as catalysts or solvents in organic synthesis (Abbott et al., 2005, 2006; Abello et al., 2004; Morales et al., 2004), but also as solvents in the preparation of novel polymorphous materials (Abbott et al., 2006; Cooper et al., 2004; Liu et al., 2005; Parnham et al., 2006). More recently, some choline-based ILs were used as eutectic solvents for lipase activation in the enzymatic preparation of biodiesel (Zhao et al., 2011), in carbon dioxide capture (Leron and Li, 2013), in the separation of alcohols from model oils (Guo et al., 2013) and finally, in the separation of biomolecules (Domínguez de María and Maugeri, 2011; Gorke et al., 2010; Pereira et al., 2013; Shahriari et al., 2013). Despite the crescent number of potential applications for the choline family, the number of works dealing with the toxicity of cholinium compounds is still scarce and of limited focus – most of these works report exclusively on cytotoxicity using mammalian cell lines, which are of poor environmental representativeness (Frade et al., 2013; Suresh et al., 2012; Vijayaraghavan et al., 2010; Weaver et al., 2010b). Meanwhile, just some a few studies report on environmental effects of cholinium-based ILs targeting specifically the aquatic compartment (Couling et al., 2006; Gouveia and Araújo, 2012; Nockemann et al., 2007b; Pretti et al., 2009; Stolte et al., 2012b), which is of particular relevance having into account the potential application of these compounds as solvents. Because the number of different structures being considered in ILs (including the cholinium family) development is increasing every day, the experimental assessment of the variant's ecotoxicity is becoming unfeasible (too time-demanding and costly). In this way, there is a growing interest in the application of mathematical models (Alvarez-Guerra and Irabien, 2011; Torrecilla et al., 2009; Yan et al., 2012) relating chemical structure variation to biological activity. These can be extraordinarily useful tools to be used in the proper design of "optimal" ILs from a technical and environmental point of view, minimizing the array of ILs undergoing mandatory prospective risk assessment before licensing.

This study addresses the evaluation of the "benign" nature of ten cholinium-based salts and ILs (Meck and Williams, 1999; Nockemann et al., 2007a; Shahriari et al., 2013; Stasiewicz et al., 2008; Zeisel, 1999), covering a wide range of anion hydrophobicities and distinct alkyl chains were studied and their ecotoxicity compared with other ILs, organic solvents and pesticides. For that purpose, new ecotoxicological data based on the luminescence response of the marine bacteria *Vibrio fischeri* (Standard Microtox® liquid-phase assays, here abbreviated as Microtox® test) are reported and discussed. The Microtox® test was selected in this study because it is a quick, simple, cost-effective, sensitive, widely used and the accepted method (Johnson, 2005) in the ecotoxicity assessments. The mechanism of toxicity of these compounds is also discussed.

2. Materials and methods

Three ILs were tested in this work: cholinium bicarbonate [Chol][Bic] (80 wt%), cholinium bitartrate [Chol][Bit] (99 wt%), and cholinium chloride [Chol]Cl (98 wt%), all purchased from Sigma-Aldrich. Cholinium acetate [Chol][Ac] (98 wt%), cholinium dihydrogenophosphate [Chol][DHPhosp] (> 98 wt%) and cholinium dihydrogenocitrate [Chol][DHCit] (98 wt%) were purchased from Iolitec (Ionic Liquid Technologies, Germany). Cholinium salicylate [Chol][Sal] (95 wt%) and benzyldimethyl(2-hydroxyethyl)ammonium chloride [BzChol]Cl (97 wt%) were acquired at Fluka, while cholinium propanoate [Chol][Prop] (> 99 wt%) and cholinium butanoate [Chol][But] (> 99 wt%) were kindly supplied by Prof. Robin D. Rogers from the University of Alabama (for more details about the chemical structures and abbreviation names of the ILs see Supporting Information file, Fig. A1). With the exception of cholinium bicarbonate, the compounds were washed with ultra-pure water before their use in the Microtox® tests, and then dried under constant stirring at high vacuum and moderate temperature (≈ 353 K) for a minimum of 48 h. This treatment allows the removal of water and other volatile compounds. The cholinium bicarbonate was used without the drying step, being the initial water content considered in the preparation of the aqueous solution of this specific IL. Afterwards, their purity was checked by ^{1}H and ^{13}C NMR. The water used was ultrapure water, double distilled, passed through a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus. Different stock solutions were prepared with certain concentrations, described in Table A1. Those were determined by conductivity using a Mettler Toledo S47 SevenMulti™ dual meter pH/conductivity equipment, coupled with an InLab®741 Conductivity Probe as electrode, according to a recently method proposed by our group (Neves et al., 2013).

Standard Microtox® liquid-phase assays were used to evaluate the inhibition of *V. fischeri* (strain NRRL B-11177) luminescence following exposure to each compound at 15 °C. The indications of the manufacturer on the standard 81.9 test protocol were generally followed (Azur Environmental, 1998). Briefly, the bacteria were exposed to a range of diluted aqueous solutions (0–81.9 % relative to the previously prepared stock solution, using a geometric factor of 2; Table A1) of each IL. After 5, 15 and 30 min of exposure to the IL, the bioluminescence emission of *V. fischeri* in each dilution treatment was measured and compared with the bioluminescence emission of a blank control sample allowing the estimation of the corresponding 5 min-, 15 min- and 30 min-EC_x values (EC_x being the estimated concentration yielding *x* % effect) – the decrease in luminescence as concentrations increase constitutes an integrated measure of the physiological impairment of the bacteria hence demonstrates the toxic effect of the tested compound. EC_{10}, EC_{20} and EC_{50} values, plus the corresponding 95 % confidence intervals were estimated for each IL tested by non-linear regression, using the least-squares method to fit the data to the logistic equation.

3. Results and discussion

3.1. Ecotoxicity evaluation of cholinium-based ILs

This work reports a set of ecotoxicity parameters (EC_{10}, EC_{20} and EC_{50}) for ten distinct cholinium-based ILs and salts towards the bioluminescence of the bacteria *V. fischeri*. Usually, only the EC_{50} parameter describing a 50 % of reduction in the luminescence of the

bacteria is reported. However, EC_{10} and EC_{20} provide intermediate toxicity references hence a more complete ecotoxicological characterization of these compounds. Furthermore EC_{10} and EC_{20} are thresholds for the estimation of the lowest observed effect concentration, and EC_{10} in particular can be used as a reliable surrogate of the no-observed effect concentration, both relevant measures for the environmental risk assessment of chemicals (EC, 2003). Tables 1–3 present the ecotoxicity values for the ten compounds studied at 5, 15 and 30 min of exposure time. These results allow a comprehensive and more systematic discussion of the impact of their chemical structures on the ecotoxicity and, in particular, it will allow an evaluation of two widespread ideas in the ILs field: (i) the generalization of the "biocompatible" (Petkovic et al., 2010) or "non-toxic" (Petkovic et al., 2010) character associated with the entire cholinium family based on a reduced number of compounds and organisms tested (Meck and Williams, 1999; Petkovic et al., 2011; Weaver et al., 2010a) and (ii) the effect of distinct hydrophilic anions upon the ecotoxicity, often neglected or not considered by some authors (Ismail Hossain et al., 2011; Muhammad et al., 2012) as a relevant topic.

Table 1
Initial concentrations (mg L^{-1}) of the cholinium stock solutions prepared for the Microtox® measurements.

Ionic liquid	Concentration (mg L^{-1})
[Chol][DHCit]	1.65×10^3
[Chol]Cl	1.69×10^3
[Chol][Bit]	5.90×10^2
[Chol][But]	4.04×10^3
[Chol][Prop]	3.58×10^3
[Chol][DHPhosp]	2.01×10^3
[Chol][Bic]	2.45×10^4
[Chol][Ac]	5.39×10^3
[Chol][Sal]	1.50×10^3
[Bzchol]Cl	3.40×10^3

Table 2
EC_{10} values (mg L^{-1}) estimated following 5, 15 and 30 min of exposure of the luminescent marine bacteria *Vibrio fischeri* to the cholinium-based ILs, with the corresponding 95% confidence lower and upper limits (within brackets).

Ionic liquid	EC_{10} (mg L^{-1}) 5 min (lower limit; upper limit)	EC_{10} (mg L^{-1}) 15 min (lower limit; upper limit)	EC_{10} (mg L^{-1}) 30 min (lower limit; upper limit)
[Chol][DHCit]	5.81 (0.00; 11.69)	15.54 (7.80; 23.29)	17.48 (7.81; 27.15)
[Chol]Cl	81.42 (38.30; 124.54)	82.68 (23.96; 141.41)	75.35 (39.33; 111.37)
[Chol][Bit]	20.82 (14.28; 27.36)	25.58 (19.44; 31.72)	24.44 (19.27; 29.60)
[Chol][But]	124.63 (78.04; 171.23)	149.92 (78.58; 221.26)	193.75 (80.23; 307.28)
[Chol][Prop]	93.94 (62.63; 125.25)	56.78 (21.24; 92.33)	59.06 (20.28; 97.84)
[Chol][DHPhosp]	17.94 (9.33; 26.54)	23.34 (12.92; 33.75)	44.34 (25.71; 62.97)
[Chol][Bic]	2685.27 (0.00; 6489.44)	1132.71 (0.00; 2830.37)	741.98 (12.47; 1471.49)
[Chol][Ac]	200.73 (142.52; 258.94)	147.47 (105.85; 189.10)	136.56 (104.20; 168.93)
[Chol][Sal]	98.47 (6.30; 190.64)	44.88 (0.00; 100.95)	24.23 (0.00; 49.94)
[Bzchol]Cl	590.02 (159.18; 1020.85)	365.86 (219.52; 512.20)	357.17 (225.47; 488.88)

Table 3
EC_{20} values (mg L^{-1}) estimated following 5, 15 and 30 min of exposure of the luminescent marine bacteria *Vibrio fischeri* to the cholinium-based ILs, with the respective 95% confidence lower and upper limits (within brackets).

Ionic liquid	EC_{20} (mg L^{-1}) 5 min (lower limit; upper limit)	EC_{20} (mg L^{-1}) 15 min (lower limit; upper limit)	EC_{20} (mg L^{-1}) 30 min (lower limit; upper limit)
[Chol][DHCit]	10.48 (2.42; 18.55)	21.00 (13.257; 28.741)	23.11 (13.69; 32.54)
[Chol]Cl	175.22 (109.55; 240.88)	167.55 (82.09; 253.02)	148.08 (97.10; 199.06)
[Chol][Bit]	26.64 (20.36; 32.91)	30.37 (24.62; 36.11)	28.74 (24.15; 33.33)
[Chol][But]	250.64 (182.00; 319.29)	284.98 (184.90; 385.06)	339.43 (191.49; 487.37)
[Chol][Prop]	194.56 (147.22; 241.90)	129.01 (69.16; 188.85)	128.83 (65.30; 192.36)
[Chol][DHPhosp]	61.82 (40.72; 82.92)	73.49 (50.16; 96.81)	114.08 (79.83; 148.33)
[Chol][Bic]	–	–	–
[Chol][Ac]	388.16 (306.24; 470.07)	281.72 (223.22; 340.23)	246.17 (203.01; 289.33)
[Chol][Sal]	171.18 (55.21; 287.15)	93.72 (8.29; 179.14)	56.18 (12.10; 100.26)
[Bzchol]Cl	1133.66 (625.24; 1642.09)	668.07 (485.88; 850.26)	606.57 (449.75; 763.39)

The results here reported suggest that the increasing trend of ecotoxicity observed for the cholinium-based ILs investigated is independent of the parameter analyzed (EC_{10}, EC_{20} or EC_{50}), and can be described as follows:

$$[Chol][Bic] \lll [BzChol]Cl \lll [Chol][But] < [Chol][Ac] < [Chol]Cl < [Chol][Prop] < [Chol][DHPhosp] < [Chol][Sal] < [Chol][Bit] \approx [Chol][DHCit]$$

being [Chol][DHCit] the most toxic and [Chol][Bic] the less toxic of the compounds studied. In fact, the toxicity of [Chol][Bic] is so low (Table 1) that the estimation of EC_{20} and EC_{50} values was prevented due to the lack of data on the high-effect fraction of the concentration–response curve (note that concentrations as high as 10 and 20 g L^{-1} were tested). Indeed, maximum luminescence inhibition was of 35 % noticed at 20 g L^{-1} following 30 min exposure to [Chol][Bic]. Similar level of luminescence inhibition (specifically 40 %) was obtained following exposure for 30 min to 0.7 g L^{-1} of the immediate least toxic follower [BzChol]Cl. This two-order of magnitude distance between equivalent effect levels demonstrates clearly the distance between [Chol][Bic] and the remaining compounds tested, a trend that would not be unraveled by the comparative analysis of the EC_{10} table. Due to the limitation there is a high level of uncertainty associated with [Chol][Bic] EC_{10} estimation, translating into an erroneous view that this compound's toxicity is only half of that elicited by [BzChol]Cl. On the basis of this limitation, [Chol][Bic] will not be considered for further discussion considering (Tables 2 and 3). However, and despite the uncertainty of the outcome, an estimation of the EC_{50} for [Chol][Bic] was assessed. The results indicate that the anion has an important impact on the ecotoxicity of the cholinium-based ILs studied, as previously suggested by us for other IL families (Ventura et al., 2012a, 2012b, 2013a) and also, briefly mentioned by other authors (Ismail Hossain et al., 2011; Stolte et al., 2007b). Moreover, it seems that the inclusion of a benzyl group in one of

Table 4
EC_{50} values (mg L^{-1}) estimated following 5, 15 and 30 min of exposure of the luminescent marine bacteria *Vibrio fischeri* to the cholinium-based ILs, with the respective 95% confidence lower and upper limits (within brackets).

Ionic liquid	EC_{50} (mg L^{-1}) 5 min (lower limit; upper limit)	EC_{50} (mg L^{-1}) 15 min (lower limit; upper limit)	EC_{50} (mg L^{-1}) 30 min (lower limit; upper limit)
[Chol][DHCit]	28.75 (15.78; 41.73)	35.10 (27.58; 42.62)	37.23 (28.60; 45.85)
[Chol]Cl	648.44 (524.84; 772.05)	559.61 (411.13; 708.08)	469.34 (383.76;554.91)
[Chol][Bit]	40.563 (34.455; 46.670)	40.70 (34.78; 46.62)	37.90 (32.73; 43.08)
[Chol][But]	826.26 (705.29; 947.22)	853.33 (691.14; 1015.52)	884.10 (670.60; 1097.61)
[Chol][Prop]	674.48 (585.75; 763.20)	523.77 (392.43; 655.11)	487.90 (351.53; 624.27)
[Chol][DHPhosp]	511.20 (426.68; 595.72)	520.92 (440.69; 601.16)	572.72 (487.50; 657.94)
[Chol][Bic]	––	––	––
[Chol][Ac]	1196.71 (1058.07; 1335.35)	850.74 (752.98; 948.49)	673.21 (606.51; 739.90)
[Chol][Sal]	440.06 (276.11; 604.02)	329.54 (171.28; 487.80)	236.11 (137.41; 334.80)
[Bzchol]Cl	3457.30 (2339.71; 4574.89)	1867.75 (1585.25; 2150.25)	1498.31 (1280.38; 1716.24)

the cation alkyl chains reduces its toxicity with [BzChol]Cl being one of the less toxic compounds studied.

The results present a decreasing order of the EC_x values with the increase of the exposure time from 5 to 30 min. This can be justified by the necessity of long periods of time for the toxic mechanism to occur (Ventura et al., 2012b). However, this behavior is not observed for the ILs with a fast toxic action, *i.e.* for the most toxic cholinium-based ILs (with the lowest EC_x values), such as [Chol][DHCit] and [Chol][Bit].

According to the results reported (EC_{50} values at 30 min of exposure time – Table 4), it is possible to categorize these cholinium compounds as belonging to the Category: Acute III according to the European Classification (EU, 2011) and as (1) "moderately toxic" ([Chol][DHCit] and [Chol][Bit] with 10 mg L^{-1} $< EC_{50} <$ 100 mg L^{-1}), (2) "practically harmless" ([Chol][Sal], [Chol]Cl, [Chol][Prop], [Chol][DHPhosp], [Chol][Ac] and [Chol][But] with 100 mg $L^{-1} < EC_{50} <$ 1000 mg L^{-1}) and (3) "harmless" ([BzChol]Cl with $EC_{50} >$ 1000 mg L^{-1}) ILs, according to Passino's classification (Passino and Smith, 1987). Contrarily to the widespread notion that cholinium-based ILs are "non-toxic" (Meck and Williams, 1999; Nockemann et al., 2007a; Shahriari et al., 2013; Stasiewicz et al., 2008; Stolte et al., 2012a; Zeisel, 1999), the results here reported indicate in one hand that this family is not devoid of toxicity, and in the other hand, that the investigated anions have a key role in their toxicity. Recently, Hernando et al. (2007) proposed a new, more restricted and specific classification for Microtox® aquatic toxicity tests. This has been used to classify organic compounds such as pesticides, herbicides and drugs. Considering the results for our cholinium compounds, and in accordance to this classification, [Chol][DHCit] and [Chol][Bit] are considered as "harmful", being the remaining compounds classified as "not harmful" (Hernando et al., 2007).

The comparison of our results with the literature data is limited due to the lack of information concerning the toxicity behavior of this family of compounds (Matzke et al., 2010). There are some data available for other specific cholinium-based ILs (Hou et al., 2013; Nockemann et al., 2007a; Petkovic et al., 2010; Stolte et al., 2012a), but these results were not specific for the organism here studied and, as previously discussed by us (Ventura et al., 2010, 2012a), the organism's physiology are important factors to be taken into account in the discussion of the (eco)toxicity of a given group of xenobiotics. If this is taken into account, our results are in agreement with those by Petkovic et al. (2010). Hou et al. (2013) reported data for cholinium amino-acid ILs showing that these compounds prepared from renewable sources can be advantageous in terms of toxicity. An attempt to understand the toxic mechanism of the studied compounds was conducted by investigating the correlation of the EC_{50} endpoint with the octanol–water partition coefficients (K_{ow}) (Chemspider) of the ILs. This parameter is useful for the environmental risk assessment of chemicals, since the partition coefficients in 1-octanol–water systems display similarities to the partition of compounds between water and the biological matrices. The 1-octanol is one solvent with amphiphilic properties, similar to a generalized lipid phase in terms of their dielectric properties (Turner and Williamson, 2005). Correlations between environmental parameters for natural systems and K_{ow} values have been successfully obtained because of the 1-octanol ability to mimic the behavior of a lipid phase (Ranke et al., 2007b; Ventura et al., 2011). Despite the lack of K_{ow} values for four of the cholinium compounds here investigated, namely [Chol][Sal], [Chol][But], [BzChol]Cl and [Chol][Bic], a relationship between the logarithm functions of K_{ow} (log K_{ow}) and the EC_{50} (at 30 min) parameter was established and depicted in Fig. 1. These results suggest that the cholinium-based ILs with higher affinity for water (lower log K_{ow}) present lower toxicities (higher log EC_{50}). The toxicity of the cholinium compounds studied seems thus to correlate with their hydrophobicity/lipophilicity as previously observed for the other IL families studied, suggesting that the mechanisms related with the membrane permeation must play an important role on their toxicity. However, as discussed below, our results suggest that some compounds of the cholinium family may present mode of toxic action distinct from that expected. In fact, in Section 3.2., some of these differences are addressed in depth; the discussion reveals that the common justification based on membrane interactions as the principal promoter of toxicity is only valid for the most common cations, cholinium being an exception (Gal et al., 2012; Ma et al., 2010).

3.2. Comparison of the ecotoxicity of cholinium compounds with common ILs and organic solvents

The EC_{50} values following a 30 min exposure-period (Table 4) were compared with the ecotoxicity of other IL families, including imidazolium (1-ethyl-3-methylimidazolium chloride [C_2mim]Cl, 1-butyl-3-methylimidazolium chloride [C_4mim]Cl, 1-hexyl-3-methylimidazolium chloride [C_6mim]Cl), pyridinium (1-butylpyridinium chloride [C_4pyr]Cl), pyrrolidinium (1-butyl-1-methylpyrrolidinium chloride [C_4mpyrr]Cl) and a quaternary ammonium (dimethylethylbutylammonium chloride [$N_{1,1,2,4}$]Cl). Moreover, this comparison was also performed taking into account the toxicity of various organic solvents commonly used in industry. These results are depicted in Fig. 2, where their disposition follows a decreasing order of ecotoxicity (equivalent to an increase in the EC_{50} endpoint at 30 min of exposure time). Passino and Smith classification (Passino and Smith, 1987) discussed above was also included in Fig. 2.

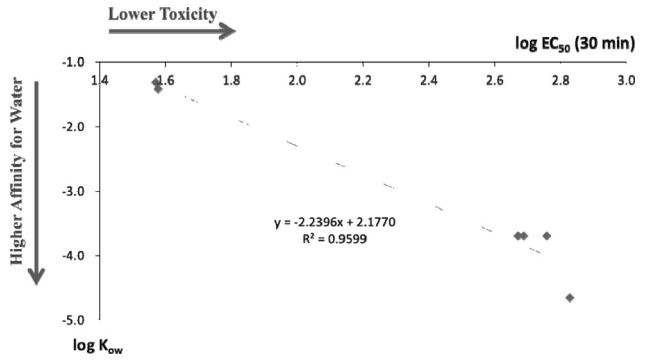

Fig. 1. Correlation between the logarithm of EC_{50} at 30 min of exposure time and the octanol–water partition coefficients (K_{ow}) for some of the cholinium compounds studied (Chemspider).

An evaluation of the cation effect based on the compounds containing a common chloride anion shows that their ecotoxicity increases in the following order: [C_4mpyrr]Cl⩽[$N_{1,1,2,4}$]Cl < [C_2mim]Cl⩽[C_4mim]Cl < [Chol]Cl < [C_4pyr]Cl⩽[C_6mim]Cl.

A large difference in toxicity is identified between the cations studied, ranging from "harmless" with an EC_{50} of 7980 mg L^{-1} for [C_4mpyrr]Cl to the "moderately toxic" with an EC_{50} of 18 mg L^{-1} for the [C_6mim]Cl. Among the cations studied and, in spite of [Chol]Cl being classified as "practically harmless", only the pyridinium-based IL and the imidazolium with the longest alkyl chain (C_6) present lower EC_{50} values than the [Chol]Cl. It is particularly curious to notice that, contrarily to what is generally accepted as a heuristic rule based in the toxicities of imidazolium based ILs (Garcia et al., 2005; Matzke et al., 2010; Samori et al., 2007, 2010; Ventura et al., 2012b), the oxygenation of the cation alkyl chains in quaternary ammonium does not contribute to a reduction of the toxicity. Herein, [$N_{1,1,2,4}$]Cl, in spite of its longer alkyl chains and absence of hydroxyl groups (which means its higher hydrophobicity), is actually less toxic than the [Chol]Cl. The same is true for a number of other cations in this list that, although more hydrophobic than the cholinium (*e.g.* [C_2mim]Cl, [C_4mim]Cl and [C_4mpyrr]Cl), present a lower toxicity. In fact, the higher toxicity of the cholinium cation when compared with other

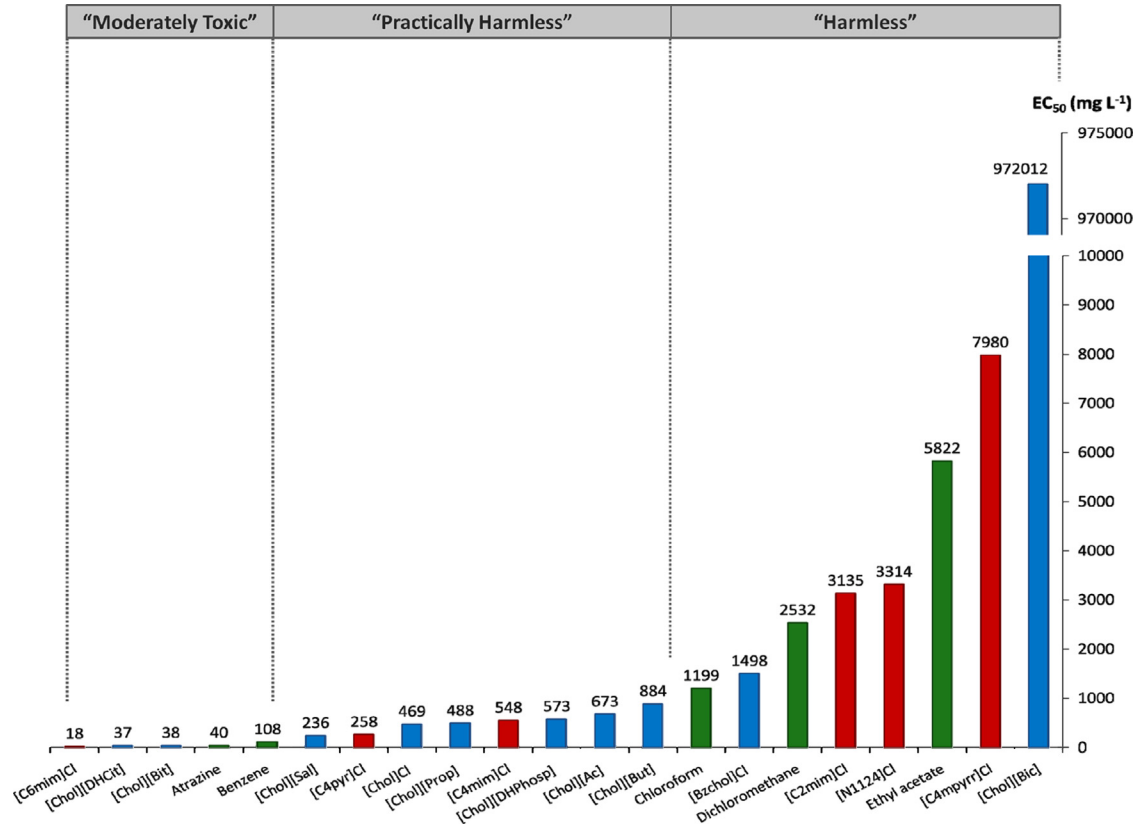

Fig. 2. *V. fischeri* luminescence EC_{50} estimated at 30 min of exposure for cholinium-based ILs (blue bars), other IL families (red bars) and other chemical compounds (green bars). Values for [C_2mim]Cl, [C_4mim]Cl, [C_4pyr]Cl, [$N_{1,1,2,4}$]Cl were retrieved Stolte et al. (2007b); [C_4mpyrr]Cl from UFT (2013); atrazine from Tchounwou et al. (2000); benzene, chloroform, dichlorometane, ethyl acetate from Kaiser and Palabrica (1991). The estimated EC_{50} for [Chol][Bic] is presented for consistency in the related comparative discussion, but the high uncertainty of the estimate (35 % luminescence inhibition was the highest effect that could be measured; see text for details) prevents either further uses of this value or feasible conclusions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

quaternary ammonium or other IL cations is clearly evidencing that the cation toxicity mechanism is different from those previously proposed for imidazolium-based ILs that seems to be mostly related with membrane interactions, namely membrane binding, insertion and disruption (Gal et al., 2012; Ma et al., 2010).

In order to provide a more complete framework for the cholinium-based ILs ecotoxicity, Fig. 2 also reports the EC_{50} values of some pesticides and organic solvents. The results depicted in Fig. 2 suggest that the toxicity of the two most toxic cholinium compounds, [Chol][DHCit] and [Chol][Bit], is comparable to that of the pesticide atrazine. Finally, it is demonstrated that the EC_{50} value of most cholinium-based ILs is comprised between the ecotoxicity of the pesticide and the organic solvents (dichloromethane and ethyl acetate) here used for comparison, being the [BzChol]Cl and [Chol][Bic] the single exceptions.

4. Conclusions

The present work assesses the ecotoxicity of ten cholinium-based ILs and salts towards the bioluminescent marine bacteria *V. fischeri*. The results show that while most of these compounds can be classified as "practically harmless", their ecotoxicity is actually higher than that of common organic solvents such as chloroform of dichloromethane, and some have toxicities comparable to reference pesticides such as atrazine. Also, the toxicities here reported underline the important role of an underestimated IL structural feature in the toxicity of the cholinium (and ionic liquids in general): the anion. The correlation between the toxicity and the anion hydrophobicity suggests that the permeation through the cell membrane plays an important role in the toxicity. However, the higher toxicity of the cholinium cation when compared to other quaternary ammonium or other IL cations indicates that the controlling cation toxicity mechanism is different than previously proposed.

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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.2014.01.003.

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