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(Eco)toxicity and biodegradability of protic ionic liquids

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HIGHLIGHTS

- The toxicity and biodegradability of four PILs was tested.
- The elongation of the alkyl chain tends to increase the toxic effect of PILs.
- The PILs m-2-HEAA and m-2-HEAP are the less and the most toxic.
- The four PILs have low biodegradability.

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ABSTRACT

Ionic liquids (ILs) are often claimed to be "environmentally friendly" compounds however, the knowledge of their potential toxicity towards different organisms and trophic levels is still limited, in particular when protic ionic liquids (PILs) are addressed. This study aims to evaluate the toxicity against various microorganisms and the biodegradability of four PILs namely, N-methyl-2-hydroxyethylammonium acetate, m-2-HEAA; N-methyl-2-hydroxyethylammonium propionate, m-2-HEAPr; N-methyl-2-hydroxyethylammonium butyrate, m-2-HEAB; and N-methyl-2-hydroxyethylammonium pentanoate, m-2-HEAP. The antimicrobial activity was determined against the two bacteria, *Sthaplylococcus aureus* ATCC-6533 and *Escherichia coli* CCT-0355; the yeast *Candida albicans* ATCC-76645; and the fungi *Fusarium* sp. LM03. The toxicity of all PILs was tested against the aquatic luminescent marine bacterium *Vibrio fischeri* using the Microtox® test. The impact of the PILs was also studied regarding their effect on lettuce seeds (*Lactuta sativa*). The biodegradability of these PILs was evaluated using the ratio between the biochemical oxygen demand (BOD) and the chemical oxygen demand (COD). The results show that, in general, the elongation of the alkyl chain tends to increase the negative impact of the PILs towards the organisms and biological systems under study. According to these results, m-2-HEAA and m-2-HEAP are the less and most toxic PILs studied in this work, respectively. Additionally, all the PILs have demonstrated low biodegradability.

1. Introduction

Ionic liquids (ILs) are molten salts at low temperature which properties can be tuned for a specific application, by the adequate cation/anion/alkyl chain combination (Hussey, 1988). Because of their unique properties, including non-volatility and non-flammability, variable solubility, high chemical and thermal stability (Domanska, 2006), high ionic conductivity and wide electrochemical potential window, ILs have been widely studied, used and recognized as promising alternatives for various applications, some of them with high industrial potential (Wasserscheid and Keim, 2000). In this scenario, ILs have been applied as promising alternatives in organic synthesis (Wang et al., 2003), catalysis (Souza et al., 2003), electrochemical (Jiang et al., 2004), biocatalysis

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(Ventura et al., 2012b; Sintra et al., 2014), enzyme immobilization (Souza et al., 2013b; Oliveira et al., 2014) and in various extraction processes (Freire et al., 2012; Ventura et al., 2012a, 2012c).

Recently, the protic ILs (PILs) have been the principal focus of several studies (Chen et al., 2014; Huang et al., 2014; Kusano et al., 2014; Peric et al., 2014; Santos et al., 2014). They are synthesized by proton transfer from a Brønsted acid to a Brønsted base (Anouti et al., 2008; Mirjafari et al., 2013). A key property of PILs is their capacity to promote hydrogen bonds, in which proton acceptance and proton donation are included (Austen et al., 2012). The interest in this class of ILs stems from their simple synthesis, low cost of preparation and purification, and also their claimed biodegradable nature (Hussey, 1988). They have been applied in organic synthesis (Hangarge et al., 2002), chromatography techniques (Poole, 2004), as proton conducting electrolytes (Menne et al., 2013), catalysts (Jiang et al., 2004), and solvents (Achinivu et al., 2014). PILs are normally considered as of good technical performance (Greaves et al., 2006), however the current European Union environmental legislation concerning the registration, evaluation, authorization and restriction of chemicals (REACH, 2006) imposes the safety assessment of new chemicals in which (eco)toxicological and biodegradation demands are included. Despite the widespread idea that PILs should be less toxic than the aprotic ILs (Peric et al., 2014) considering the aquatic compartment, they are poorly studied and new structures require new and adequate tests. Peric and collaborators (Peric et al., 2011, 2013, 2014, 2015) are one of the most active groups in the study of the toxicological profile of PILs. The authors have performed tests to assess the toxicity of different chemical structures considering aquatic and terrestrial organisms and also on their biodegradability profile (Peric et al., 2013). They found that one of the most sensitive species studied considering the evaluation of the toxicity of different PILs was the macrophyte *Lemna minor* (Peric et al., 2013). These PILs were also found to be more toxic for *Lemna minor* than the aliphatic ILs studied in the same work (Peric et al., 2013), which the authors attributed to the higher hydrophilicity of PILs. As a result of their investigation, the authors classified 2-hydroxytriethanolamine pentanoate (2-HDEAP) as harmful regarding the three plants analyzed, namely Allium cepa (onion), Lolium perenne (grass) and the dicotyledonae Raphanus sativus (radish) (Peric et al., 2014). Moreover, the authors have also performed tests considering the acetylcholinesterase inhibition (Peric et al., 2013) which are normally attributed to the study of the potential biochemical toxic mechanisms imposed by toxicants. These results have highlighted the incapacity of the PILs tested to inhibit acetylcholinesterase, being the contrary conclusion obtained for the aprotic ILs under study (Peric et al., 2013).

Despite the lack of current data in the biodegradability of PILs, there are some results already being provided in the open literature, as recently reviewed by Gathergood and collaborators (Jordan and Gathergood, 2015). In this work, the authors mentioned that the majority of the PILs being classified as biodegradable, are those synthesized from analogues of cholinium salts (Jordan and Gathergood, 2015). Thus, the low toxicity and good biodegradability of shorter PILs (Peric et al., 2013) claimed still for a very limited number of chemical structures, allowed the general conclusion that together with their low production costs, simple preparation and still numerous applications, they are environmental benign alternatives considering the ILs class.

The main objective of this work is the toxicity and biodegradability evaluation of four PILs, in which we have applied experimental tests to determine *i)* their antimicrobial activity, towards a Gram-negative and a Gram-positive bacteria, one fungus and one yeast, *ii)* their toxicity *via* Microtox®, *iii)* their phytotoxicity tested in lettuce seeds and *iv)* their inherent biodegradation in water. Their toxic effect was studied regarding six biological systems, five microorganisms, the bacteria *Vibrio fischeri*, *Escherichia coli* CCT-0355 and *Staphylococcus aureus* ATCC-6533, the yeast *Candida albicans* ATCC-76645 and the mold *Fusarium* sp. LM03 and one plant (*Lactuta sativa*) was assessed. Here, model organisms that represent entire classes were selected being assumed that their reaction to the toxic compound can be extrapolated to other organisms of the same class. The IL concentration which prevented the germination of lettuce seeds in 50% (LD_{50}), analyzed by the final germination percentage (FG) and LD_{50} parameters were also determined considering the phytotoxic tests done.

The biodegradability was tested by the determination of the Chemical Oxygen Demand (COD) which is a parameter that measures the amount of organic matter capable to be oxidized by a chemical *via* when in a liquid sample (expressed in mg O_2 L^{-1}). The Biological Oxygen Demand (BOD) represents the amount of oxygen consumed in the biodegradation of the organic matter in the aquatic environment by biological processes. Behind the COD and BOD parameters, the percentage of oxygen consumed (%O_2) was also determined. This method is recurrently used in wastewater treatment studies (Anastasi et al., 2010). These parameters are being used to evaluate the biodegradation profile of different ILs (Jordan and Gathergood, 2015).

2. Experimental section

2.1. Materials

In this work, four PILs were used, namely the N-methyl-2-hydroxyethylammonium acetate (m-2-HEAA), the N-methyl-2-hydroxyethylammonium propionate (m-2-HEAPr), the N-methyl-2-hydroxyethylammonium butyrate (m-2-HEAB) and the N-methyl-2-hydroxyethylammonium pentanoate (m-2-HEAP), whose chemical structures are depicted in Fig. 1. Those were synthesized at our laboratory, by reacting equimolar amounts of the amine and the respective organic acids, as detailed elsewhere (Matzke et al., 2010). All ILs were used in this study in their pure form (99%). Tetracycline (purity of 95–100%) and miconazole (purity of 99.77%), compounds used as positive controls in the antimicrobial experiments for bacteria and fungus, were purchased at DEG Farmacêutica and Genix Farmacêutica, respectively. NaCl (purity of 99%) from Quimex was used as the negative control in the antimicrobial experiments. The lettuce seeds were purchased in the Central Market from Aracaju, Sergipe, Brazil.

2.2. (Eco)toxicity evaluation

2.2.1. Antimicrobial activity tests

To test the antimicrobial activity of the PILs, a standard protocol validated by us for aprotic ILs (Ventura et al., 2012d) and adopted from literature (Rebros et al., 2009; Biczak et al., 2014) was followed. Briefly, aqueous solutions of miconazole (50 μg L^{-1}) and tetracycline (50 μg L^{-1}) were used as reference compounds and positive control for fungi and bacteria, respectively. The negative control used in this work was an aqueous solution of NaCl at 0.9% (w/v). The microorganisms, *E. coli* CCT-0355 (Gram-negative bacteria), *Staphylococcus aureus* ATCC-6533 (Gram-positive bacteria), *Fusarium* sp. LM03 (mold) and *C. albicans* ATCC-76645 (yeast). These microorganisms were grown in a Bushell-Hass medium (total composition, g L^{-1}: $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; $(NH_4)_2HPO_4$, 1.0; KNO_3, 1.0; and $FeCl_3$, 0.05) until an optical density of 1.0, taking into account the MacFallen scale (Ventura et al., 2012d). Suspensions of 1 mL of these microorganisms were uniformly spread on the plates [samples prepared with Nutrient Agar (total composition (g L^{-1}): meat extract 3.0; peptone 5.0; agar 15;

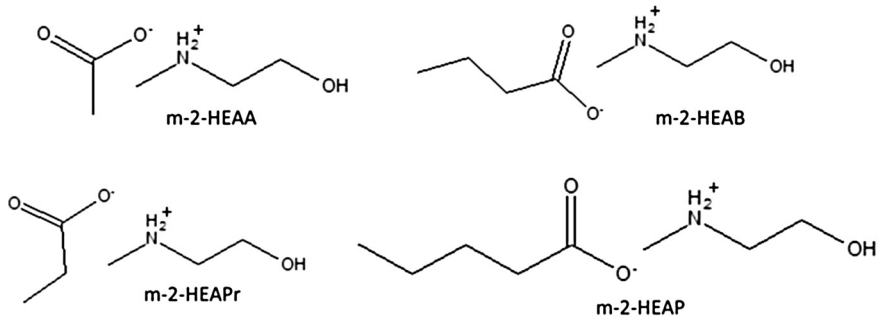

Fig. 1. Chemical structure and respective abbreviation names for the PILs studied.

pH: 6.8 ± 0.2) and Sabouraud medium (total composition (g L^{-1}): peptone 10; dextrose 40; agar 15; pH: 5.6 ± 0.2) medium for bacteria and fungi, respectively], and wells of 6 mm of diameter were punched under a sterilized ambient, with a sterile glass tube. Samples of each one of the PILs (50 μL) were then placed into the wells, previously inoculated by the target microorganisms. The plates were then incubated at 37 °C. The growth inhibition halo was measured using a caliper after 24 or 48 h, depending of the microorganism. Each PIL was tested in triplicate, being the halo of inhibition presented as the average of the replicates (in mm).

2.2.2. Microtox® test

Standard Microtox® liquid-phase assays (Ventura et al., 2010) were used to evaluate the luminescence inhibition of the bacteria *V. fischeri* (strain NRRL B-11177) following exposure to each compound at 15 °C (Johnson, 2005). The bacteria was exposed to a range of geometrically diluted aqueous solutions (normally from 0 to 81.9%, geometric factor = 2) of each PIL, where 100% of IL corresponds to a known concentration of a stock solution (≈1000 mg L^{-1} for all PILs except for m-2-HEAP, whose concentration of the stock solution was 1435 mg L^{-1}) previously prepared (Azur, 1998). After 5 and 15 min of exposure time to each PIL, the light output of the luminescent bacteria was measured and compared with the light output of a blank control sample (diluent solution) for the estimation of the corresponding 5 min- and 15 min-EC_{50} values (Ventura et al., 2012b), EC_{50} being the estimated concentration yielding 50 percent effect, and the corresponding 95 percent confidence intervals were estimated for each PIL through Microtox® Omni™ Software version 4.1.25 (Azur, 1998).

The toxic units (TU) were also determined at 15 min of exposure time by the calculation of the inverse of each EC_{50} value as describe by Eq. (1) (Chang et al., 2013).

$$TU = \frac{1}{EC_{50}} \times 100 \quad (1)$$

2.2.3. Phytotoxic tests

The phytotoxicity study aimed at assessing the toxicity level of the PILs on lettuce seeds (*Lactuta sativa*). The seeds were pretreated by being washed with tap water for 4 h, and then placed on Petri dishes (12 × 12 cm). Each dish contained three paper filters saturated with 9 mL of different aqueous solutions of each PIL studied. Each PIL was tested at six concentrations, 1.0×10^{-7}, 1.0×10^{-6}, 1.0×10^{-5}, 5.0×10^{-3}, 1.0×10^{-3} and 1.0×10^{-2} (g L^{-1}). For the treatments and the control (water), 30 undamaged seeds of identical size were placed evenly on the filter papers placed in the Petri dishes and then incubated in the dark at 28 °C. The control test was used to investigate the stability and reproducibility of the germination capacity of the lettuce seeds in absence of xenobiotics. Three repetitions for each experiment [meaning each lethal dose (LD) concentration selected] were done. After three days of incubation, the final germination percentages (FG - %) were obtained according to standard protocols described in literature (Biczak et al., 2014). The cells were also analyzed with light optic microscope. Cells in interphase and undergoing division were examined to assess the induction of chromosome and nuclear aberrations, such as abnormal anaphases (multipolar, with bridges, delayed, etc.), fragments and loss of chromosomes, C-metaphases, micronuclei and multinucleated cells (Souza et al., 2013a).

2.3. Biodegradability

The chemical and biochemical oxygen demands (abbreviated in this work as COD and BOD) were determined using the experimental methodology described in the Standard Methods Analysis of Water and Wastewater. The biodegradability of the PILs tested at different concentrations was determined after 48 h of incubation (APHA, 2012). The dissolved oxygen was determined using an oximeter. The percentage of oxygen consumed (O_2– %) was calculated from the difference between the value of dissolved oxygen in the beginning and at the end of the incubation period (APHA, 2012).

3. Results and discussion

3.1. (Eco)toxicity evaluation

3.1.1. Antimicrobial activity

The antimicrobial activity test was based on the diffusion test agar recommended by Bauer et al. (1966), where the paper disks are soaked in the compounds of interest and placed on Petri plates previously seeded with the microorganisms of interest.

Fig. 1 shows the results of the antimicrobial activity of all PILs tested against different microorganisms. The criteria to analyze these results are: *i)* how the results obtained for the different PILs compare and depend on the increase in the alkyl chain length and *ii)* how the data obtained for the PILs compares with the growth inhibition halo obtained for the positive controls, tetracycline for the bacteria species and miconazole for yeast and mold. Both criteria can be analyzed in Fig. 2, which shows the behavior obtained for the PILs against all the microorganisms under study and allows the comparison between the results obtained for the PILs and controls. The results suggest that all PILs have activity against the microorganisms under investigation, principally for the yeast and mold [the increase in the alkyl chain becomes more toxic when compared with the results obtained for the positive control (antibiotic)].

The *E. coli* CCT-0355 results show a common behavior of an increase in toxicity when the alkyl chain length is elongated until the chain of four carbons (Fig. 2), followed by the "*cut-off*" effect at

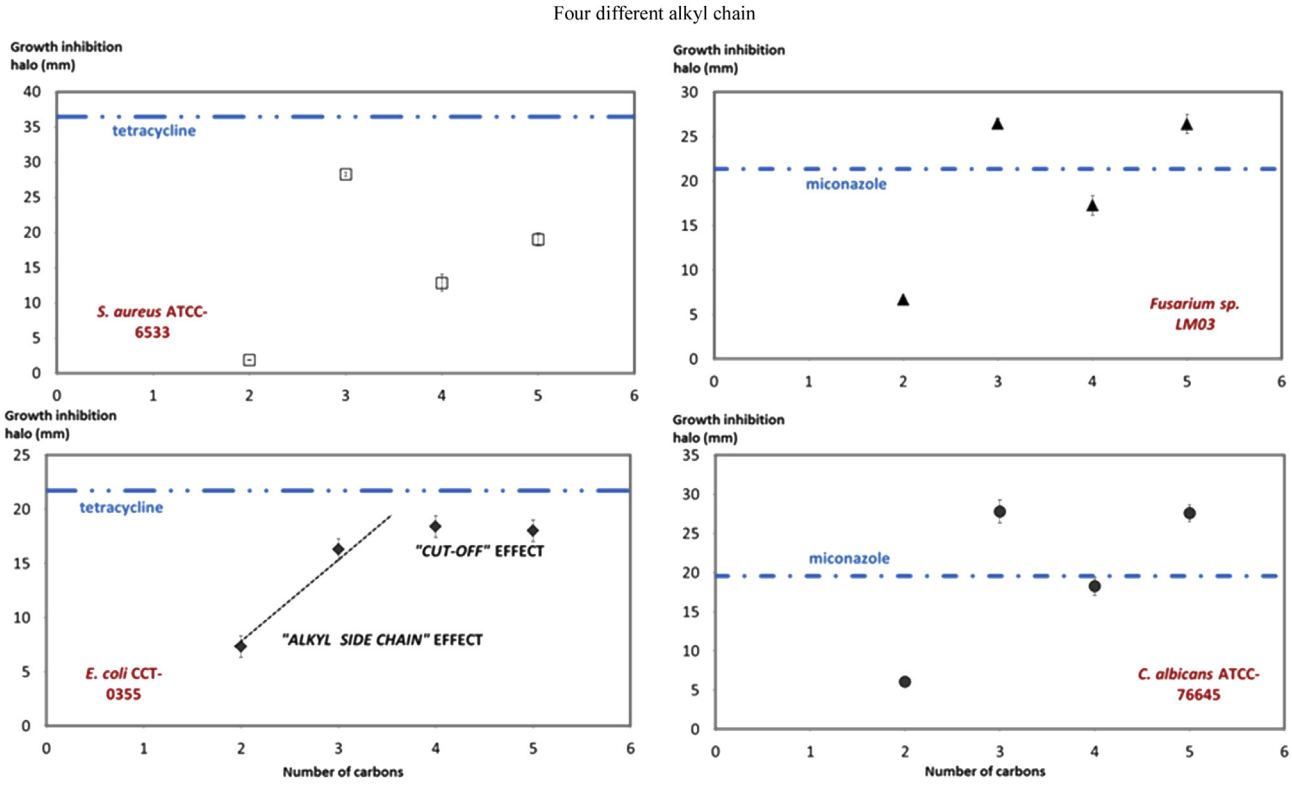

Fig. 2. Growth inhibition halo (in mm) *versus* the number of carbons in the alkyl chain; results of the positive controls (tetracycline and miconazole – blue lines): (□) *S. aureus* ATCC-6533, (◆) *E. coli* CCT-0355, (▲) *Fusarium* sp. LM03, (●) *C. albicans* ATCC-76645.

the butyl chain. The data of *S. aureus* ATCC-6533 shows the lower toxicity of the PILs when compared with the control tetracycline used for the bacteria species, behavior also observed for *E. coli* CCT-0355. However, the "*alkyl side chain*" and "*cut-off*" effects are not as clear as for *E. coli* CCT-0355. The same tendency is not observed when the results of both yeast and fungi are analyzed against the data of miconazole (positive control). These results seem to suggest one more time the same negative impact of the elongation of the alkyl chain in the toxicity of these PILs, which is in close agreement with other authors (Pernak et al., 2001a, 2001b, 2003, Pernak and Chwała, 2003, Pernak et al., 2004; Ventura et al., 2012a). However, it was noticed a strange behavior of the PIL with three carbons (m-2-HEAPr) for all microorganisms, except *E. coli* CCT-0355. Despite the morphologic differences between the microorganisms under study (Salyers and Whitt, 2001; Ventura et al., 2012a), the peculiar difference found for ILs with three and four carbons in the alkyl chain is not new (Cho et al., 2007). In this work (Cho et al., 2007), the same eccentric behavior was reported for imidazolium ILs based on the bromide (Br-) anion tested towards the green alga *Selenastrum capricornutum* ATCC-22662. Despite the effect of the alkyl chain length of the PILs under study in this work, the main antimicrobial results suggest that the effect of PILs against both bacteria is less pronounced when compared with the effect of the positive control tetracycline. Meanwhile, this tendency is not verified for the mold and yeast.

3.1.2. *Microtox® tests*

The bioluminescent bacterium *V. fischeri* and the Microtox® technique have been used as part of a model standard methodology to test the toxicity of different chemicals (Ranke et al., 2007; Matze et al., 2010; Ventura et al., 2012a, 2014). The four PILs were tested in terms of their effect against this marine luminescent bacterium for 5 and 15 min of exposure/toxic action. Table 1 shows the toxicity results for the luminescent bacteria and for two exposure times in the form of EC_{50} (mg L^{-1}) values. From a brief analysis of the results, the toxicity of PILs towards the bacteria is ordered as m-2-HEAA < m-2-HEAPr < m-2-HEAB < m-2-HEAP, tendency evidenced for both 5 and 15 min of exposure. This tendency is clearly explained by the increase in the alkyl chain length of the anion. The same tendency was already claimed for the elongation of the alkyl chains substituted in the cation (Matze et al., 2010; Ventura et al., 2012a). In the same table, the toxic units (TU) are also described. When Microtox® is applied, the TU data can be used as a measure of the toxic action of the chemicals (Chang et al., 2013). According to the results of TU, and supported by the classification normally applied to identify the most and less toxic chemicals, it is safe to describe the PILs tested here as belonging to the Category: Acute III

Table 1
Microtox® EC_{50} values (mg L^{-1}) for the *Vibrio fischeri* after 5 and 15 min of exposure to the PILs, with the respective 95% confidence limits (in brackets).

ILs	EC_{50} (mg L^{-1}) 5min (Lower limit; upper limit)	EC_{50} (mg L^{-1}) 15min (Lower limit; upper limit)	TU[a,b]
m-2-HEAA	900.63 (427.14; 1898.29)	962.54 (220.11; 4209.61)	0. 10
m-2-HEAPr	620.78 (304.29; 1266.62)	887.81 (104.83; 7504.40)	0.11
m-2-HEAB	591.33 (436.91; 799.76)	717.00 (30.33; 780.05)	0.14
m-2-HEAP	456.62 (162.59; 1282.46)	551.61 (142.02; 2142.46)	0.18

[a] The TU data were calculated with the EC_{50} data at 15 min of exposure time.
[b] **Note:** the classification referred in literature describes the following: TU < 1 non-toxic; TU = 1–10 toxic; TU = 10–100 very toxic; TU > 100 – extremely toxic (Chang et al., 2013).

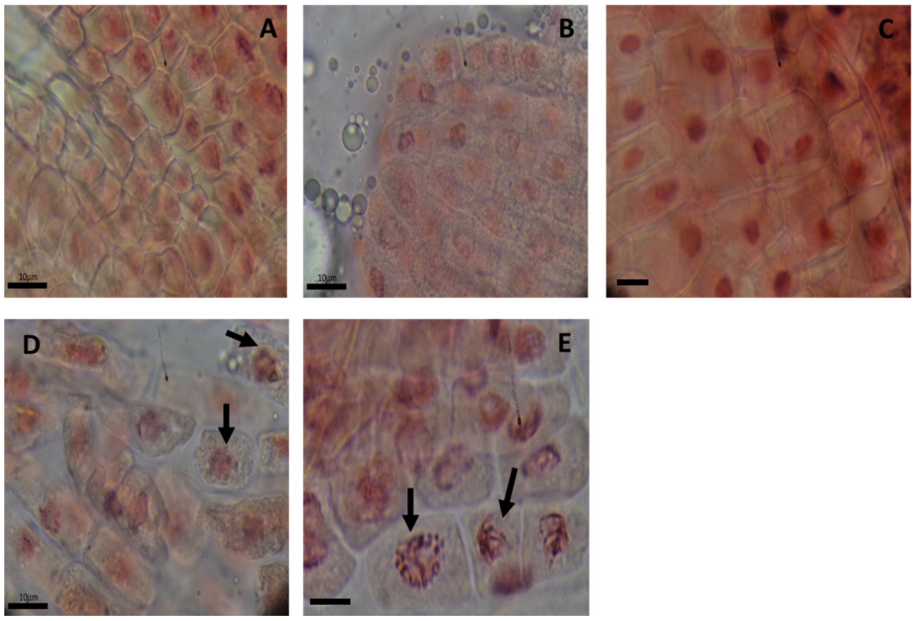

Fig. 3. Mitosis phases of *Lactuta sativa* cells with and without the presence of PILs: (**A**) – control; (**B**) – seeds exposed to m-2-HEAA (1×10^{-6}) (**C**) – seeds exposed to m-2-HEAPr (1×10^{-6}); (**D**) – seeds exposed to m-2-HEAB (1×10^{-5}) and (**E**) – seeds exposed to m-2-HEAP (1×10^{-5}).

according to the European Classification (EU, 2011) and "practically harmless" considering the Passino and Smith (1987) labeling (100 mg L^{-1} < EC_{50} < 1000 mg L^{-1}). The same "practically" harmless nature of PILs was also reported by Peric et al. (2013). Moreover, while these PILs are considered as non-toxic (EC_{50} values higher than 100 mg L^{-1}) based on Microtox® tests, actually m-2-HEAPr and m-2-HEAP are considered toxic considering the antimicrobial tests results, since these two PILs are more toxic than miconazole (positive control) for the *Fusarium* sp. LM03.

3.1.3. Phytotoxicity

The sensitivity of *Lactuta sativa* germination to the four PILs here investigated was also checked by determining the LD_{50} values. The main results suggest a descendent order of phytotoxicity as follows: m-2-HEAA (LD_{50} = 1.85 ± 0.01 > m-2-HEAPr (LD_{50} = 1.18 ± 0.03) > m-2-HEAB (LD_{50} = 1.16 ± 0.01) > m-2-HEAP (LD_{50} = 0.45 ± 0.04). It is clearly seen the decrease of LD_{50} with the increase in the alkyl chain of the PIL and, consequently of the hydrophobic nature of the PIL tested, which is in accordance with recent literature (Studzinska and Buszewski, 2009, Cybulski et al., 2011; Biczak et al., 2014; Bubalo et al., 2014). Actually, the same tendencies are described for the marine luminescent bacteria (see results from Section 3.1.2 of this work).

To further support the phytotoxicity results found for the PILs, images of the mitotic cell division of *Lactuta sativa* seeds, when exposed to different PILs concentrations are presented in Fig. 3. Different conditions were considered, namely the absence of PILs (Fig. 3A) and the presence of each PIL under analysis, from the shorter until the long alkyl chains, i.e. from m-2-HEAA (Fig. 3B) to m-2-HEAP (Fig. 3E). By comparison with Fig. 3A, it is possible to observe that all PILs have some impact in the structure of the cell, although to a less extent for the m-2-HEAA (Fig. 3B) and m-2-HEAPr (Fig. 3C). However, the same behavior is not observed in Fig. 3D and E, in which the seeds were placed in contact with the more hydrophobic PILs, namely m-2-HEAB (four carbons in the alkyl chain) and m-2-HEAP (five carbon atoms in the alkyl chain). Fig. 3D shows a cell having micronuclei in prophase while in Fig. 3E the cell was at interphase with chromosomal loss and presents micronuclei in prophase. The data suggests that the toxicity or the negative impact of the presence of the PILs increases with the elongation of the alkyl chain. These changes in the aspect of the cells are attributed to the cell mutagenicity, as discussed elsewhere (Caritá and Marin-Morales, 2008).

3.2. Biodegradability evaluation

In contrast to chemical degradation, which requires the assistance of an oxidant for catalysis, biodegradation is the microbial breakdown of chemical compounds (Coleman, Gathergood 2010). Table 2 shows that all compounds showed values of BOD/COD lower than 0.3, which is indicating the non-biologically treatable nature of these PILs, thus justifying their poor biodegradable character (Anastasi et al., 2010). Some authors describe the radical size with the toxicity and biodegradability of the compound (Jordan and Gathergood, 2015). The biodegradation potential of ILs in general (protic and aprotic) in aqueous media has been addressed in some works (Wells and Coombe, 2006; Docherty et al., 2007; Romero et al., 2008; Coleman and Gathergood 2010, Peric et al.,

Table 2
Results for the chemical oxygen demand (COD – mg L^{-1}), biochemical oxygen demand (BOD – mg L^{-1}) and percentage of oxygen consumed (O_2 – %).

Ils	COD (mg L^{-1})	BOD (mg L^{-1})	COD/BOD	BOD/COD (% oxygen uptake or biodegradation)	%O_2 (%)
m-2-HEAA	28760.00	271.10	106.10	0.01	30.50
m-2-HEAPr	23280.00	333.00	69.90	0.01	36.70
m-2-HEAB	20540.00	322.20	63.70	0.02	35.30
m-2-HEAP	15060.00	255.00	59.00	0.02	28.00

2011; Stolte et al., 2012; Peric et al., 2013; Jordan and Gathergood, 2015). Wells and Coombe (2006) investigated the biodegradability of quaternary ammonium, imidazolium, phosphonium and pyridinium compounds by measuring the BOD. They observed that the cations with short side chains (C4) were not biodegradable, which is in agreement with our results, meaning that this result seems to be independent of the protic or aprotic nature of ILs. Moreover, the generality of the works reporting biodegradability studies describe the low biodegradable nature of ILs (Jordan and Gathergood, 2015).

4. Conclusions

In this study, the toxicity and biodegradability of four distinct PILs were analyzed. From the main results of this study, it was found that, in general, the elongation of the alkyl chain of the PILs tested is increasing the negative impact of these chemicals for the various microorganisms tested, as expected. This trend was found for the different microorganisms studied, in particular for the marine bacteria and *Lactuta sativa* seeds. Despite the low toxic profile of these PILs against the marine bacteria, this low effect was not verified for all the microorganisms under study in this work, in particular for the yeast and mold, whose effect of some PILs was even more pronounced than the effect promoted by the antibiotic used as positive control. It was then concluded that m-2-HEAPr and m-2-HEAP represent the less and more toxic PILs studied in this work. Regarding the biodegradability study, the results suggest that these four chemicals showed low biodegradability.

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