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TERRESTRIAL ECOTOXICITY OF SHORT ALIPHATIC PROTIC IONIC LIQUIDS

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Abstract—A study of the ecotoxicity of different short aliphatic protic ionic liquids (PILs) on terrestrial organisms was conducted. Tests performed within the present study include those assessing the effects of PILs on soil microbial functions (carbon and nitrogen mineralization) and terrestrial plants. The results show that the nominal lowest-observed-adverse-effect concentration (LOAEC) values were 5,000 mg/kg (dry soil) for the plant test in two species (*Lolium perenne, Allium cepa*), 1,000 mg/kg (dry soil) for the plant test in one species (*Raphanus sativus*), and 10,000 mg/kg (dry soil) for carbon and nitrogen microbial transformation tests (all concentrations are nominal). Most of the median effective concentration values (EC50) were above 1,000 mg/kg (dry soil). Based on the obtained results, these compounds can be described as nontoxic for soil microbiota and the analyzed plants, and potentially biodegradable in soils, as can be deduced from the respirometric experiment. The toxicity rises with the increase of complexity of the PILs molecule (branch and length of aliphatic chain) among the three PILs analyzed. Environ. Toxicol. Chem. 2011;30:2802–2809. © 2011 SETAC

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INTRODUCTION

Ionic liquids are novel solvents of rising interest as greener alternatives to traditional volatile organic solvents, aimed to facilitate so-called sustainable chemistry. As a consequence of their unusual physical properties, reusability, and apparently environmentally friendly nature, ionic liquids have attracted the interest of industry and academia. In the near future, many new ionic liquids will be developed, but with little data relating to their hazard potential [1]. These chemicals are liquids composed entirely of ions; they are salts with a melting point lower than 100°C. The reason for their low melting point lies in the asymmetry of the ions and the important steric hindrance among functional groups [2]. They have a very low vapor pressure, and thus their nonvolatile nature reduces the risk of air pollution and makes them potential green substitutes for volatile organic solvents. Their polarity, hydrophilicity/hydrophobicity, and other properties can be tuned by a suitable combination of cations and anions; therefore, they have been termed *designer solvents* [1,3]. Two main groups of ionic liquids have been identified: aprotic ionic liquids and protic or Brønsted ionic liquids (PILs). The PILs have a proton available for hydrogen bonding. Common and classical ionic liquids, which belong mainly to the group of aprotic ionic liquids, are designed with bulky organic cations, such as imidazolium, pyridinium, pyrrolidinium, and quaternary ammonium, with alkyl chain substituents and different inorganic anions. In the last few years, numerous reports have revealed different applications of ionic liquids in terms of separation, catalysis, photochemistry, electrosynthesis, lubricants, electrolytes for batteries and dye-sensitized solar cells, as cleaning solvents in applications in which large amounts of solvents are used to clean batch processing equipment, and in minimization of CO_2/SO_2 emissions by removal of SO_2 and CO_2 from natural gas [4–6].

Regarding the fate and effects of ionic liquids in the environment, the water solubility of many ionic liquids is not negligible, and the release of ionic liquids into aquatic and terrestrial environments may lead to water and soil pollution and related risks. Several properties of ionic liquids and their effects on aquatic organisms have been investigated [7–12]. However, more research on the effect of protic ionic liquids on soil and sediment organisms is required. Many commonly used ionic liquids are toxic to aquatic and terrestrial organisms, as demonstrated by toxicological research studies concerning ionic liquids undertaken in the past decade [13].

A new group of PILs, with different cations and anions from those previously known, has been designed and could have a lesser environmental impact than the former ones, because the new PILs are based on polysubstituted amines and organic anions. Both the cationic and anionic parts of the molecule are organic and present a relatively low molecular weight [14,15]. These new PILs are 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxydiethanolamine propionate (2-HDEAPr), and 2-hydroxytriethanolamine pentanoate (2-HTEAPe). Considering the interest in these substances as more environmentally sustainable than volatile organic solvents, one must examine their potential toxicity. The current chemical legislation for Registration, Evaluation, Authorization, and Restriction of Chemical Substances (REACH) holds suppliers of chemicals responsible for their products. The REACH criteria must be fulfilled for ionic liquids as well, taking into account their possible commercial use [16].

One of the new PILs, 2-HEAF, was found to dissolve many inorganic salts, hydroxylated compounds, and some insoluble polymers such as polyaniline and polypyrrole [17]. Also, 2-HEAF was analyzed as a potential solvent in the preparation of organosoluble polyanilines with reasonable molecular weights [18,19], for some heterogeneous catalytic hydrogenation processes

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[20,21]. Application and prospects of a two-phase, tuneable solvent system composed of ionic liquids and supercritical fluids with an emphasis on supercritical carbon dioxide have been reviewed [22]. Also, some works have focused on the effect of temperature on the thermodynamics of the mixture of 2-HEAF and short hydroxylic solvents (water, methanol, and ethanol) [23]. Other PILs also derived from 2-hydroxyethylammonium and organic acids have been studied to develop a new process for absorption of CO_2 [24]. Also, the catalytic activity in the aldol condensation processes of the PILs derived from mono-, di-, and triethanolamine and pentanoic acid (e.g., 2- HTEAPe) has been studied, with successful results [20].

The aim of the present study was to evaluate the toxicity of the three new PILs (2-HEAF, 2-HDEAPr, and 2-HTEAPe) to terrestrial organisms by performing different bioassays with plants (onion, grass, and radish) and soil microorganisms involved in the most important biogeochemical cycles (carbon and nitrogen mineralization of organic matter).

MATERIALS AND METHODS

The ecotoxicological analyses included two types of tests to evaluate acute (short-term exposure) and chronic (prolonged exposure or long-term toxicity in small, repeated doses) toxicity and biodegradability. In the assessment of acute toxicity, a test involving terrestrial plants was conducted, whereas in the assessment of chronic toxicity the tests performed involved soil microorganisms responsible for carbon and nitrogen mineralization.

Synthesis of protic ionic liquids

The amine compounds (monoethanolamine, diethanolamine, and triethanolamine) were purchased from Aldrich with 99% purity by mass, and the corresponding acids (formic, acetic, *n*-propionic, and *n*-pentanoic acid) were purchased from Sigma with purity greater than 99.5% by mass. These components were used without any pretreatment. During the course of the experiments, the purity of solvents was monitored as reported by Iglesias et al. [14]. The amine was placed in a three-necked flask made entirely of glass equipped with a reflux condenser, a platinum resistance thermometer for temperature control, and a dropping funnel. The flask was mounted in a thermostatic bath. The corresponding acid was added dropwise to the flask under stirring with a magnetic bar. The progress of the reaction was reflected by a gradual increase in viscosity; slight warmth and vigorous agitation into the reactor ensured this progress. A yellowish mass was obtained when the reaction process and purification (strong agitation and slight heating for the vaporization of residual nonreacted acid for at least 48 h) were completed. To decrease the water content as much as possible, the PIL was dried for 72 h at 50°C and under a vacuum of 20 kPa with stirring before each use, reaching maximum water contents of 0.02 (2-HEAF), 0.01 (2-HDEAPr), and 0.005 (2-HTEAPe)% [14,23].

To confirm the structures of the products, ^{1}H NMR and Fourier transform infrared spectroscopy analysis were performed. The Fourier transform infrared spectrum was taken by a Jasco FT/IR 680 plus model (Jasco) infrared spectrometer, using an NaCl disk. The broad band in the 3,500 to 2,400/cm range exhibits a characteristic ammonium structure for all neutralization products. The OH stretching vibration is embedded in this band. The broad band centered at 1,600/cm is a combined band of the carbonyl stretching and N-H plane bending vibrations. The ^{1}H NMR spectrum was measured using Varian Mercury-400 spectrometer (Varian Analytical Instruments), with $CDCl_3$ as solvent and tetramethylsilane as internal standard and gave the following signals for 2-HEAF, δ: 8.22 ppm (singlet [s], 1H, $H–COO^{-1}$); 6.0 to 6.3 ppm (broad signal, 4H, $–NH_3 + OH$); 3.7 ppm (triplet [t], 2H, $–CH_2–O$); 3.5 ppm (t, 2H, $–CH_2–N$); for 2-HDEAPr, δ: 5.4 to 6.1 ppm (broad signal, 4H, $–NH_2 + OH$); 3.8 ppm (t, 4H, $–CH_2–O$); 3.1 ppm (t, 4H, $–CH_2–N$); 2.4 ppm (multiplet [m], 2H, $–CH_2–COO$); 1.1 ppm (t, 2H, $–CH_3$); for 2-HTEAPe, δ: 4.7 to 5.0 ppm (broad signal, 4H, $–NH + OH$); 3.7 ppm (t, 6H, $–CH_2–O$); 2.8 ppm (t, 6H, $–CH_2–N$); 2.3 ppm (t, 2H, $–CH_2–COO$); 1.6 ppm (t, 2H, $–CH_2–C$); 1.3 ppm (m, 2H, $–CH_2–C$); 0.9 ppm (t, 2H, $–CH_3$).

Soil samples

To perform the tests, a soil sample (air dried and 2 mm-sieved) was obtained from the superficial layer (A horizon) of natural pine wood forest soil sampled in Premià de Dalt, Barcelona (41.52° N, 2.33° E). The soil corresponds to a haplic arenosol [25] of granitic origin and sandy texture (74% of sand). Its pH value is 6.3, it has 1.2% of oxidizable carbon, and 3.22% of the oxidizable carbon corresponds to microbial biomass carbon. This sample has the characteristics required by the Organisation for Economic Co-operation and Development (OECD) method to test for carbon mineralization processes [26].

Terrestrial plants test

The seedling emergence and seedling growth tests were performed with the seeds of two monocotyledon plants, onion (*Allium cepa*) and grass (*Lolium perenne*), and one dicotyledon, radish (*Raphanus sativus*), in 20-ml plastic pots, with four replicates of five seeds, in 15 g of soil for each application rate. Aqueous solutions of the three protic ionic liquids were added to the dry soil at nominal concentrations of 1, 10, 100, 1,000, and 5,000 mg/kg, including the control samples in which no PILs were added to dry soil. The final water content of all samples and controls was equivalent to 60% of the soil's water-holding capacity; water lost during the assay was restored daily. The plant germination and growth assay lasted until 14 to 21 d after the emergence of 50% of the seedlings in the control group, and was performed according to OECD guideline 208 [27]. At the end of this assay, shoot length was measured.

Soil microorganisms: Carbon transformation test

In the carbon transformation test, 50 g soil sample was used, adjusting the water content to 60% of the soil's water-holding capacity, as determined by using International Organization for Standardization (ISO) method 11274 [28]. All experiments were done at least in triplicate. The soil was treated with the following nominal concentrations of PILs: 10, 100, 1,000, 5,000, and 10,000 mg/kg, including the control samples, in which no PILs were added to the soil. These samples were incubated in manometric respirometers, which allow the determination of the samples' oxygen consumption (Oxitop OC 110, WTW). The samples were kept in the dark at 25°C, in an incubator equipped with a thermostat for 28 d. Oxygen consumption was periodically registered. Cumulative respiration (CR) was determined by the cumulative oxygen consumption at the end of the incubation period. Once incubation was completed, substrate-induced respiration (SIR) was determined according to OECD method 217 for testing carbon transformation [26]. This test was performed by adding an aqueous solution equivalent to 4,000 mg glucose per kilogram soil to

the incubated samples and determining the oxygen consumed during the 12 h following glucose addition. Basal respiration rate (BR) was estimated as the average hourly respiration rate over the last 5 d of incubation when respiration was stable. The respiratory activation quotient (Q_R) was calculated by dividing BR by SIR [29]. The carbon transformation test also was performed with amines or acids separately to evaluate the behavior of the anionic and cationic moiety. For amines and acids, the concentration used corresponded to 1000 mg/kg of the analyzed PIL; these compounds are not quantitatively dissociated in aqueous solutions because of the hydrogen-bonded networks that are formed [30]; thus, in the soil solution, dissociated and undissociated forms can be found. The ammonium moiety was found to be the part of the molecule most correlated to toxicity (Peric et al., unpublished data). The anionic parts (organic acids) usually are found in natural media and are known biodegradable compounds. The incubated samples in the carbon transformation test were analyzed to determine possible decreases in their concentrations in soil as a consequence of eventual degradation processes that were expected through the respirometric results. After 0, 7, and 28 d of contact with the soil (1,000 mg/kg of the PILs), 1:5 0.1N KCl extracts were obtained and analyzed by ion chromatography in a Dionex DX300 (Sunnyvale), with a Hamilton PRP-X200 column (Reno, internal diameter 4.1 mm, length 250 mm) and electrical conductivity detector without ion suppression. The eluent used was 2 mM nitric acid at 1 ml/min.

Soil microorganisms: Nitrogen transformation test

The nitrogen transformation test was performed according to OECD guideline 216 [31]. The soil was treated with the following nominal concentrations of PILs: 10, 100, 1,000, 10,000, and 20,000 mg/kg, including the control samples, in which no PILs were added to the soil. The test was performed with quadruplicates of each concentration and control. The concentration of the nitrates after the incubation period of 28 d was determined by means of the brucine method ([32]; http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/2007_07_10_methods_method_352_1.pdf).

The ionic liquids were added to the soil as aqueous solutions in all tests. These compounds are soluble in water. The estimated solubilities, according to the preliminary test of OECD guideline 105 [33], were greater than 100 g/L for 2-HEAF, greater than 50 g/L for 2-HDEAPr, and greater than 10 g/L for 2-HTEAPe.

Statistical analysis

To determine the statistical significance of the differences between treated soil samples and controls, an analysis of variance test was done followed by a post hoc Duncan test ($p < 0.05$). Based on the results obtained by the analysis of variance test, a lowest-observable-adverse-effect concentration (LOAEC) of the ionic liquids was established under the experimental test conditions. A dose–response relationship was assessed, and the effective concentrations 10, 20, and 50 (EC10, EC20, and EC50, respectively) with their 95% confidence intervals were calculated for each compound from suitable regression models (linear, Gompertz, hormetic, or logistic) using Statistica 6.0 (StatSoft). The choice of the model was based on the best fit to the data [34,35]. Some studies have concluded that no-observed-effect concentration values have high variability and that estimated EC*x* values are more consistent [36]. Other authors indicate that most models agree with the estimation of EC*x* between EC10 and EC90 [37]. The parameter EC50 was selected because it is used in the Spanish regulations and most of the literature. A 20% level of negative effect is a significant alteration, considering that this work was completed in controlled laboratory experimental conditions [38]. For EC10, the lowest bound of its confidence interval was found to be close to a no-observed-effect concentration [39]; therefore, it can be considered a safe concentration.

RESULTS AND DISCUSSION

Terrestrial plants seedling emergence and growth test

The results of the terrestrial plant test are shown in Table 1 and are presented as the values of stem length percentage with respect to the control for plants tested in the presence of various concentrations of PILs. After the analysis of variance and subsequent Duncan test of all data obtained after the plant germination and growth test, the LOAEC values were higher than 1,000 mg/kg in all cases, except in one instance of *R. sativus* in which the inhibitory effect of 2-HTEAPe was observed at 1,000 mg/kg. After applying an analysis of variance test on the values of stem length in the presence of 2-HEAF, significant differences were seen at 5,000 mg/kg for *R. sativus* ($p < 0.05$). For *L. perenne* and *A. cepa*, the LOAEC value was higher than 5,000 mg/kg. The LOAEC value for 2-HDEAPr was 5,000 mg/kg for all three plant species. The LOAEC values for 2-HTEAPe were 5,000 mg/kg for *A. cepa* and *L. perenne*, and 1,000 mg/kg for *R. sativus*.

Table 1. Effects of the three protic ionic liquids on stem length expressed as percentage of stem length compared to the control[a]

			Ionic liquid concentration				
	Plant	Control	1	10	100	1000	5000
	Allium cepa	100.00AB	112.14B	99.53AB	91.04AB	98.04AB	80.87A
2-HEAF	*Lolium perenne*	100.00A	122.83A	114.74A	108.45A	112.84A	96.29A
	Raphanus sativus	100.00B	94.79B	115.81B	97.10B	94.49B	27.90A
	A. cepa	100.00B	75.37AB	73.99AB	76.74AB	81.14B	13.28A
2-HDEAPr	*L. perenne*	100.00B	86.82B	91.37B	118.22B	102.14B	0.00A
	R. sativus	100.00BC	106.63C	102.83C	72.90BC	84.31B	11.59A
	A. cepa	100.00B	102.63B	108.56B	92.71B	99.40B	0.00A
2-HTEAPe	*L. perenne*	100.00B	87.45B	88.66B	93.29B	95.59B	0.00A
	R. sativus	100.00C	98.86C	106.45C	97.88C	44.74B	2.27A

2-HEAF = 2-hydroxyethanolamine formate; 2-HDEAPr = 2-hydroxydiethanolamine propionate; 2-HTEAPe = 2-hydroxytriethanolamine pentanoate.

[a] The nominal concentrations of the ionic liquids are 1, 10, 100, 1,000, and 5,000 mg/kg dry soil. The capital letters A, B, and C indicate the homogeneous groups within rows, determined by post hoc Duncan test, $p < 0.05$.

Table 2. Results of dose–response curves in mg/kg dry soil for the seedling emergence and growth and nitrogen transformation tests, $p < 0.05$[a]

		EC10	CI 95%	EC20	CI 95%	EC50	CI 95%
2-HEAF	*Allium cepa*	1,814	740–4,443	2,627	1,564–4,412	6,887	3,805–12,459
	Lolium perenne	3,580	810–6,276	4,374	2,662–7,185	7,166	6,878–14,257
	Raphanus sativus	1,184	481–2,915	1,544	678–3,516	3,383	2,464–4,645
	N min.	3,347	1148–6,456	5,014	1,982–7,361	10,014	4,865–14,157
2-HDEAPr	*A. cepa*	1,981	1278–3,660	3,483	2,162–5,890	3,891	2,468–6,025
	L. perenne	1,156	857–3,480	2,820	1,161–4,964	3,163	1,951–5,158
	R. sativus	951	709–1,277	1,232	977–1,553	2,128	1,714–2,640
	N min.	1,787	1,233–3,459	3,454	1,433–4,045	8,454	3,973–9,704
2-HTEAPe	*A. cepa*	579	150–2,192	818	240–2,785	1,428	422–3,655
	L. perenne	1,381	1,082–2,210	1,698	1,235–1,989	2,326	1,930–2,568
	R. sativus	155	59–350	285	140–582	826	605–1,128
	N min.	1,240	1,050–2,975	2,480	1,285–4,098	6,201	3,763–8,184

2-HEAF = 2-hydroxyethanolamine formate; 2-HDEAPr = 2-hydroxydiethanolamine propionate; 2-HTEAPe = 2-hydroxytriethanolamine pentanoate; N min. = nitrogen mineralization test.
[a] Refer to Table 1 for plant abbreviations. EC = effective concentration; CI = confidence interval.

The values of EC10, EC20, and EC50 (mg/kg) and the confidence interval of dose–response curves for the three PILs are shown in Table 2. The observed toxicity profile, in descending order and based on EC50 values, for testing germination of seeds was that 2-HTEAPe was the most toxic for the three plants tested, followed by 2-HDEAPr; 2-HEAF was the least toxic. According to the results, all plants used in this test gave results of the same order of magnitude, except *Raphanus sativus,* which has been shown to be somewhat more sensitive to the presence of certain ionic liquids. As far as phytotoxicity is concerned, according to the Globally Harmonized System of Classification and Labeling of Chemicals [40], these PILs cannot be classified as toxic for the terrestrial environment (EC50 $\geq$ 1,000 mg/kg), except for 2-HTEAPe, which has the most complex molecule structure. It is the most toxic of the three PILs analyzed and could be included in the acute 3 category of this classification. Comparing the results from this test to those obtained by other authors for aprotic ionic liquids [41–43], the PILs analyzed were less toxic than aprotic ionic liquids. Even though the plant species used in the test were not the same as those used by other authors, EC50 values for aprotic ionic liquids are generally one order of magnitude higher than EC50s for the PILs tested in the present study.

Soil microorganisms: Carbon transformation test

The graphs in Figure 1 show the cumulative respiration (CR) curves corresponding to control and the nominal concentrations 1, 10, 100, 1,000, 5,000, and 10,000 mg/kg of the three PILs. For 2-HEAF, none of the tested concentrations produced a value of accumulated oxygen below the control. This indicates that this ionic liquid has no toxicity to soil microbiota, reflected by the respiratory activity of the soil. The cases of 2-HDEAPr and 2-HTEAPe are very similar. The curves for the lowest concentrations (1, 10, and 100 mg/kg) showed no difference when compared with the control, whereas at higher concentrations (1,000 and 5,000 mg/kg) an initial inhibitory effect on the soil microbiota managed to recuperate and began to respirate. However, at the highest concentration (10,000 mg/kg) the inhibition of the respiratory activity occurred throughout the whole assay. With regard to temporary inhibition and posterior recovery of respiratory activity, the levels of accumulated oxygen were higher than in the control sample, which could be attributed to the degradation of labile organic matter coming from the microorganisms affected by the initial toxicity or to the biodegradation of the substance itself [44].

The amine and acid parts of the ionic liquid molecule were assayed separately to establish whether they have any effect on soil microbiota or are easily biodegraded in soil. This respirometric assay was performed at a concentration equivalent to 1,000 mg/kg of the corresponding protic ionic liquid. In Figure 2, cumulative respiration curves are shown for treatments with acids, amines, and also control soil samples. No inhibition of respiratory activity occurred because of these compounds. Soil respiration increased significantly ($p < 0.05$) in all soil samples treated with organic acids and amines, which may indicate that both anionic and cationic parts of the ionic liquid molecule were probably nontoxic for soil microorganisms. Thus, the possibility of biodegradation of these compounds by soil microorganisms cannot be discarded. According to the ion chromatography quantification results, after 7 d of incubation in soil, 2-HEAF could not be detected in the soil extracts (the cationic moiety), whereas for 2-HDEAPr and 2-HTEAPe the corresponding cationic moiety remained at available concentrations of 70% (standard deviation of 14) and 68% (standard deviation of 20), respectively. At 28 d of incubation, none of the compounds was detectable in the extracts.

Table 3 shows the results for the CR, expressed as the percentage relative to the control soil, BR, SIR, and Q_R for the three PILs, acids, and amines. The CR shows the overall soil state (microbiota, nutrient availability), and the results showed that at the lower concentrations of ionic liquids (1, 10, and 100 mg/kg) no significant difference is seen from the control samples ($p < 0.05$). A significant increase of CR can be observed at 1,000 and 5,000 mg/kg ($p < 0.05$). Inhibition of respiration occurred only at the highest concentration of 2-HTEAPe and 2-HDEAPr (10,000 mg/kg). For acids and amines, a significant increase of CR occurred at the concentration equivalent to 1,000 mg/kg of the corresponding PIL ($p < 0.05$). This might indicate again that the cationic and anionic moieties of the tested ionic liquids are degradable in soil. Basal respiration rate indicates actual biological activity remaining after soil respiration is stabilized after the labile carbon source has been exhausted and the microbiota have adapted to the conditions of the incubation. This depends on the microbiota and nutritional state of the soil. According to the results obtained in the present study, only the highest concentration of 2-HTEAPe (10,000 mg/kg) yielded a substantial decrease in BR values ($p < 0.05$). At concentrations of 5,000 and 10,000 mg/kg for 2-HEAF, a significant increase of the BR was noted, as with concentrations of 5,000 mg/kg for

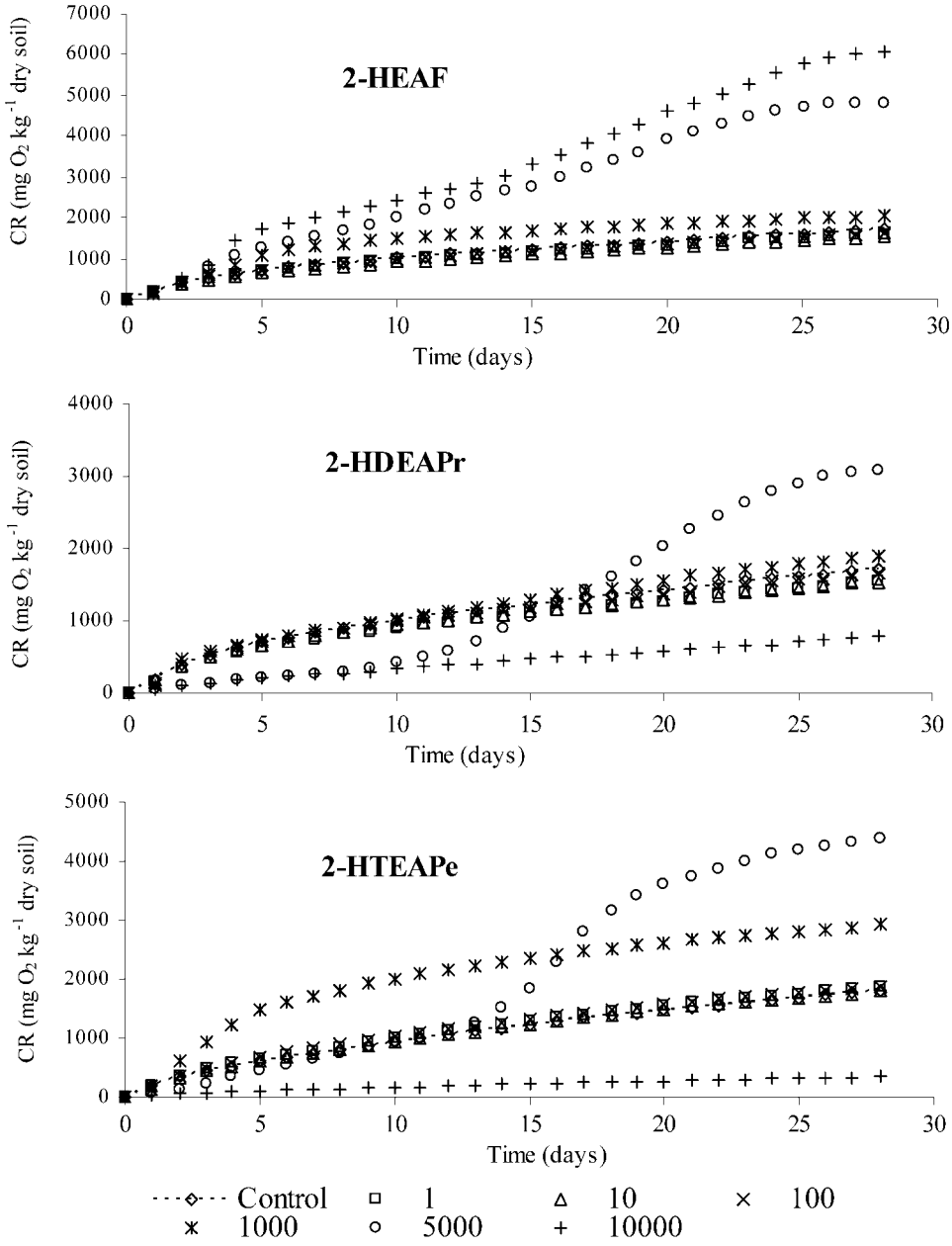

Fig. 1. Cumulative respiration (CR) data expressed as the cumulative oxygen consumed during the 28 d of the respirometric assay for the control and different nominal concentrations (in mg/kg) of the protic ionic liquids treatment groups: 2-hydroxyethanolamine formate (2-HEAF, top panel), 2-hydroxydiethanolamine propionate (2-HDEAPr, middle panel), 2-hydroxytriethanolamine pentanoate (2-HTEAPe, bottom panel). The values correspond to the averages of at least three replicates. The standard deviation is between 20 and 186 mg/kg for 2-HEAF, between 29 and 101 mg/kg for 2-HDEAPr, and between 10 and 100 mg/kg for 2-HTEAPe.

2-HDEAPr and 5,000 mg/kg for 2-HTEAPe ($p < 0.05$). For acids and amines, the level of activity was somewhat higher than in the control sample. The SIR is proportional to the active microbial biomass and is often used as an indicator of this parameter. Only the highest concentration of 2-HDEAPr or 2-HTEAPe (10,000 mg/kg) showed a substantial decrease in SIR values ($p < 0.05$). The higher concentrations of 2-HEAF (5,000 and 10,000 mg/kg) produced an increase in SIR values, as did concentrations of 1,000 and 5,000 mg/kg for 2-HDEAPr and 2-HTEAPe.

According to ISO standards for determining abundance and activity of soil microflora using respiration curves, values of Q_R higher than 0.30 indicate the toxic effect of the contaminants [29]. This effect could be noted only for the highest concentrations of 2-HDEAPr and 2-HTEAPe.

The LOAEC values for the three PILs tested were high (10,000 mg/kg for 2-HDEAPr and 2-HTEAPe and above 10,000 mg/kg for 2-HEAF), indicating no toxic effect of these PILs on carbon mineralization processes according to the Globally Harmonized System of classification and labeling of chemicals [40].

Soil microorganisms: Nitrogen transformation test

Table 4 shows concentrations of nitrate obtained as percentages of control content at different nominal concentrations of PILs (10, 100, 1,000, 10,000, and 20,000 mg/kg) at the end of the test (after 28 d). The values of EC10, EC20, and EC50 (in mg/kg) for dose–response curves are provided in Table 2. With 2-HEAF, concentrations lower than 1,000 mg/kg had a slight increase of nitrate presence compared with the control

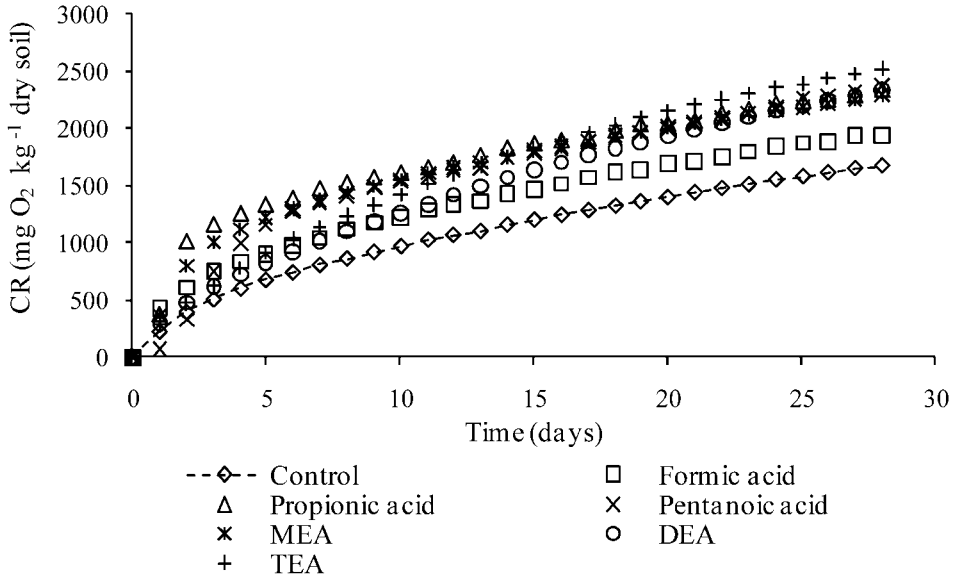

Fig. 2. Cumulative respiration (CR) data expressed as the cumulative oxygen consumed during the 28 d of the respirometric assay for the control and nominal concentrations of formic, propionic, and pentanoic acids and amines monoethanolamine (MEA), diethanolamine (DEA), and triethanolamine (TEA), equivalent to 1,000 mg/kg of the ionic liquid. The standard deviation is between 21 and 99 mg/kg.

sample ($p < 0.05$), whereas at the highest concentrations (10,000 and 20,000 mg/kg), a clear inhibition of nitrification could be observed. For 2-HDEAPr, a decrease of the nitrate concentration (85% of the control) occurred at the concentration of 1,000 mg/kg. Starting from the concentration of 10,000 mg/kg, a significant decrease of the nitrate presence ($p < 0.05$) occurred, reaching only 5% in the highest concentration (20,000 mg/kg). The third analyzed compound, 2-HTEAPe, showed a tendency similar to the other two PILs: a slight increase of nitrate at lower concentrations (10 and 100 mg/kg) and a strong inhibition at concentrations of 10,000 and 20,000 mg/kg.

The LOAEC values were high (10,000 mg/kg for the three ionic liquids tested), indicating no apparent toxicity of these PILs to the nitrifying microbiota according to the test used. Inhibition of the nitrification process can be observed only at the highest concentrations. A significant increase ($p < 0.05$) of nitrogen occurred, possibly because of at least two different factors, the hormetic effect and the possible degradation and mineralization of the ionic liquid molecule. The ECx values were all above 1,000 mg/kg and increased inversely to molecule size.

Based on all results obtained in different tests, with 2-HEAF, soil microorganisms showed greater sensitivity in the nitrogen

Table 3. Average rate of cumulative respiration (CR) during the incubation period expressed as the % relative to the control soil; BR (mg O_2/h/kg dry soil), SIR (mg O_2/h/kg dry soil), and Q_R[a]

			Ionic liquid concentration					
		Control	1	10	100	1,000	5,000	10,000
	%CR	100.00A	93.21A	89.40A	95.38A	120.07B	280.87C	354.62C
2-HEAF	BR	1.35A	1.05A	1.24A	1.40A	1.14A	2.78B	6.92C
	SIR	11.43A	10.00A	9.11A	11.79A	10.71A	15.00B	28.57C
	Q_R	0.12	0.11	0.14	0.12	0.11	0.19	0.24
	%CR	100.00B	90.22B	89.40B	96.74B	110.60B	179.35C	46.47A
2-HDEAPr	BR	1.40A	1.32A	1.09A	1.20A	1.63A	3.77B	1.22A
	SIR	12.57B	10.84B	11.27B	12.70B	20.29C	25.86C	2.48A
	Q_R	0.11	0.12	0.1	0.09	0.08	0.15	0.49
	%CR	100.00B	103.61B	99.74B	103.87B	161.27C	242.06D	18.88A
2-HTEAPe	BR	1.71B	1.55B	1.59B	1.48B	1.42B	3.18C	0.36A
	SIR	5.89B	5.71B	6.07B	6.07B	13.57C	19.28D	0.63A
	Q_R	0.29	0.27	0.26	0.24	0.11	0.17	0.58
		Control	Formic acid	Propionic acid	Pentanoic acid	MEA	DEA	TEA
	%CR	100.00A	124.68AB	139.75BC	153.45C	137.15BC	139.94BC	150.84C
	BR	1.20A	1.06A	1.42AB	1.94B	1.24AB	1.75AB	1.57AB
	SIR	8.57A	8.72A	14.75B	15.74B	9.29A	14.29B	13.49B
	Q_R	0.14	0.12	0.1	0.12	0.13	0.12	0.12

2-HEAF = 2-hydroxyethanolamine formate; 2-HDEAPr = 2-hydroxydiethanolamine propionate; 2-HTEAPe = 2-hydroxytriethanolamine pentanoate; MEA = monoethanolamine; DEA = diethanolamine; TEA = triethanolamine; %CR = percentage of cumulative respiration; BR = basal respiration rate; SIR = substrate-induced respiration; Q_R = respiratory activation quotient.

[a] The nominal concentrations of the ionic liquids are between 1 and 10,000 mg/kg dry soil. The capital letters indicate different homogeneous groups determined by Duncan test ($p < 0.05$) within rows.

Table 4. The percentage of nitrates formed compared with the control for the three ionic liquids analyzed[a]

		Ionic liquid concentration				
	Control	10	100	1,000	10,000	20,000
2-HEAF	100.00B	104.33B	120.64B	111.73B	30.45A	8.87A
2-HDEAPr	100.00B	114.55B	111.10B	84.65A,B	34.74A	4.96A
2-HTEAPe	100.00B	101.83B	99.50B	114.59B	0.77A	0.00A

2-HEAF = 2-hydroxyethanolamine formate; 2-HDEAPr = 2-hydroxydiethanolamine propionate; 2-HTEAPe = 2-hydroxytriethanolamine pentanoate.

[a] Values followed by the same capital letter are not significantly different (Duncan test, $p < 0.05$).

transformation test than in the carbon transformation test. The group of microorganisms involved in the process of nitrification is small and is only a part of the soil aerobic population that is evaluated in the respirometric assay. The effect on the nitrifying bacteria is of greater relevance because it is a process that only a few groups of microorganisms can perform, whereas the mineralization of organic carbon is a less selective process. In the nitrogen transformation test, 2-HTEAPe proved to be the most toxic of the three ionic liquids analyzed, although at very high concentrations.

Considering the EC50 obtained for different ionic liquids and including all plants, the toxicity increased with the complexity of the PIL molecule. These results agree with those obtained by other authors [45], indicating that aprotic ionic liquids with long alkyl chains have higher toxicity than ionic liquids with short chains. Other authors have concluded that the cationic part of the ionic liquid molecule is responsible for the toxicity of the molecule [46]. These effects have not been observed in the three tests described here, because the three compounds that were analyzed have linear hydroxylamines in the cationic part of the molecule, whereas ionic liquids analyzed by other authors contained heterocycles. Few references exist on ecotoxicity of protic ionic liquids, because this group is still in development [47]. The findings in this work are consistent with the fact that the three analyzed protic ionic liquids have a short and lineal molecular structure with few functional groups, whereas for most classical ionic liquids the structure is more complex, with heterocycles and long alkyl side chains, which can produce higher toxicity.

CONCLUSIONS

The PILs analyzed in the present study showed no toxicity, with EC50s above 1,000 mg/kg in all assays except for the *R. sativus* plant test with 2-HTEAPe (EC50 = 826 mg/kg). Within the group of terrestrial organisms, higher plants (that is, the three plant species tested) were more sensitive to the presence of PILs than soil microbiota, with *R. sativus* being the most sensitive to the presence of PILs. In general, compounds with more complex molecular structures had a greater tendency to cause inhibition in the organisms tested than compounds with smaller molecules and simpler structures. The three analyzed PILs seemed to be nontoxic in terms of chronic toxicity for plants and C and N cycles. Also, they could be biodegradable in the soil matrix as deduced from the respirometric test and the subsequent quantification of pollutants in the soil matrix. These compounds could be safer alternatives to other, more toxic substances. This, together with their low production cost, simple synthesis, and functional profile in various industrial applications, suggests great potential for the future. Further analyses will be conducted on other test organisms and trophic levels to confirm this hypothesis.

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REFERENCES

1. Stolte S, Stepnowski P. 2011. Ionic liquids: Analytical and environmental issues. *Curr Org Chem* 12:1871–1872.
2. Ranke J, Mölter K, Stock F, Bottin-Weber U, Poczobutt J, Hoffmann J, Ondruschka B, Filser J, Jastorff B. 2004. Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays. *Ecotoxicol Environ Saf* 58:396–404.
3. Sheldon RA. 2005. Green solvents for sustainable organic synthesis: State of the art. *Green Chem* 7:267–278.
4. Zhu S, Chen R, Wu Y, Chen Q, Zhang X, Yu Z. 2009. A mini-review on greenness of ionic liquids. *Chem Biochem Eng Q* 23:207–211.
5. Brennecke JF, Maginn EJ. 2001. Ionic liquids: Innovative fluids for chemical processing. *AIChE J* 47:2384–2388.
6. Seddon KR, Plechkova NV. 2008. Applications of ionic liquids in the chemical industry. *Chem Soc Rev* 37:123–150.
7. Bernot RJ, Brueseke MA, Evans-White MA, Lamberti GA. 2005. Acute and chronic toxicity of imidazolium-based ionic liquids on *Daphnia magna*. *Environ Toxicol Chem* 24:87–92.
8. Latala A, Stepnowski P, Nedzi M, Mrozik W. 2005. Marine toxicity assessment of imidazolium ionic liquids: Acute effects on the Baltic algae *Oocystis submarina* and *Cyclotella meneghiniana*. *Aquat Toxicol* 73:91–98.
9. Samorì C, Pasteris A, Galletti P, Tagliavini E. 2007. Acute toxicity of oxygenated and nonoxygenated imidazolium-based ionic liquids to *Daphnia magna* and *Vibrio fischeri*. *Environ Toxicol Chem* 26:2379–2382.
10. Stolte S, Marianne M, Arning J, Böschen A, Pitner W-R, Welz-Biermann U, Jastroff B, Ranke J. 2007. Effects of different head groups and functionalised side chains on the aquatic toxicity of ionic liquids. *Green Chem* 9:1170–1179.
11. Pham TPT, Cho C-W, Vijayaraghavan K, Min J, Yun Y-S. 2008. Effect of imidazolium-based ionic liquids on the photosynthetic activity and growth rate of *Selenastrum capricornutum*. *Environ Toxicol Chem* 27:1583–1589.
12. Latała A, Nedzi M, Stepnowski P. 2009. Toxicity of imidazolium and pyridinium based ionic liquids towards algae: *Bacillaria paxillifer* (a microphytobenthic diatom) and *Geitlerinema amphibium* (a microphytobenthic blue green alga). *Green Chem* 11:580–588.
13. Zhao DB, Liao YC, Zhang ZD. 2007. Toxicity of ionic liquids. *Clean Soil Air Water* 35:42–48.
14. Iglesias M, Gonzalez-Olmos R, Cota I, Medina F. 2010. Brønsted ionic liquids: Study of physico-chemical properties and catalytic activity in aldol condensations. *Chem Eng J* 162:802–808.
15. Álvarez VH, Iglesias M, Dosil N, Gonzalez-Cabaleiro R, Martin-Pastor M, Mattedi S, Navaza JM. 2010. Brønsted ionic liquids for sustainable processes: synthesis and physical properties. *J Chem Eng Data* 55:625–632.
16. European Union. 2006. Regulation (EC) No. 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). *Off J Eur Union* 396:1–856.
17. Bicak N. 2005. A new ionic liquid: 2-hydroxy ethylammonium formate. *J Mol Liq* 116:15–18.
18. Bicak N, Filiz Senkal B, Sezer E. 2005. Preparation of organo-soluble polyanilines in ionic liquid. *Synthetic Met* 155:105–109.
19. Cota I, Gonzalez-Olmos R, Iglesias M, Medina F. 2007. New short aliphatic chain ionic liquids: synthesis, physical properties, and catalytic activity in aldol condensations. *J Phys Chem B* 111:12468–12477.
20. Khodadadi-Moghaddam M, Habibi-Yangjeh A, Gholami MR. 2008. Kinetic study of heterogeneous catalytic hydrogenation of cyclohexene to cyclohexane in ionic liquid-alcohols mixtures. *Appl Catal A* 341: 58–64.
21. Khodadadi-Moghaddam M, Habibi-Yangjeh A, Gholami MR. 2009. Solvent effects on the reaction rate and selectivity of synchronous

heterogeneous hydrogenation of cyclohexene and acetone in ionic liquid/alcohols mixtures. *J Mol Catal A: Chem* 306:11–16.
22. Roth M. 2009. Partitioning behaviour of organic compounds between ionic liquids and supercritical fluids. *J Chromatogr A* 1216:1861–1880.
23. Iglesias M, Torres A, Gonzalez-Olmos R, Salvatierra D. 2008. Effect of temperature on mixing thermodynamics of a new ionic liquid: {2-Hydroxyethylammonium formate (2-HEAF)+short hydroxylic solvents}. *J Chem Thermodyn* 40:119–133.
24. Yuan X, Zhang S, Liu J, Lu X. 2007. Solubilities of CO_2 in hydroxyl ammonium ionic liquids at elevated pressures. *Fluid Phase Equilib* 257:195–200.
25. Food and Agriculture Organization-United Nations Educational, Scientific and Cultural Organization. 1998. World Reference Base for Soil Resources. Rome, Italy.
26. Organisation for Economic Co-operation and Development. 2000. Soil microorganisms: Carbon transformation test. Guideline 217. Paris, France.
27. Organisation for Economic Co-operation and Development. 2006. Terrestial plants test: Seedling emergence and seedling growth test. Guideline 208. Paris, France.
28. International Organization for Standardization. 1998. Soil quality. Determination of the water-retention characteristic. Guideline 11274. Geneva, Switzerland.
29. International Organization for Standardization. 2002. Soil quality. Determination of abundance and activity of soil microflora using respiration curves. Guideline 17155. Geneva, Switzerland.
30. Greaves TL, Drummond CJ. 2008. Protic ionic liquids: Properties and applications. *Chem Rev* 108:206–237.
31. Organisation for Economic Co-operation and Development. 2000. Soil microorganisms: Nitrogen transformation test. Guideline 216. Paris, France.
32. U.S. Environmental Protection Agency. 1971. Brucine method for determination of nitrates, Method 352.1. Washington, DC.
33. Organisation for Economic Co-operation and Development. 1995. Water solubility. Guideline 105. Paris, France.
34. Stephenson GL, Koper N, Atkinson GF, Solomon KR, Scroggins RP. 2000. Use of nonlinear regression techniques for describing concentration-response relationships for plant species exposed to contaminated site soils. *Environ Toxicol Chem* 19:2968–2981.
35. Domene X, Alcañiz JM, Andrés P. 2007. Ecotoxicological assessment of organic wastes using the soil collembolan *Folsomia candida*. *Appl Soil Ecol* 35:461–472.
36. Chapman PM, Chapman PF, Caldwell RS. 1996. A warning: NOECs are inappropriate for regulatory use. *Environ Toxicol Chem* 15:77–79.
37. Moore DRJ, Caux P-Y. 1997. Estimating low toxic effects. *Environ Toxicol Chem* 16:794–801.
38. Isnard P, Flammarion P, Roman G, Babut M, Bastien P, Bintein S, Esserméant L, Férard JF, Schmitt S, Saouter E, Saroli M, Thiébaud H, Tomassone R, Vindimian E. 2001. Statistical analysis of regulatory ecotoxicity tests. *Chemosphere* 45:659–669.
39. Fulladosa E, Murat JC, Villaescusa I. 2005. Study on the toxicity of binary equitoxic mixtures of metals using the luminescent bacteria *Vibrio fischeri* as a biological target. *Chemosphere* 58:551–557.
40. United Nations. 2006. Globally harmonized system of classification and labeling of chemicals. UN/SCEGHS/12/INF5. New York, NY, USA.
41. Matzke M, Stolte S, Böschen A, Filser J. 2008. Mixture effects and predictability of combination effects of imidazolium based ionic liquids as well as imidazolium based ionic liquids and cadmium on terrestrial plants (*Triticum aestivum*) and limnic green algae (*Scenedesmus vacuolatus*). *Green Chem* 10:784–792.
42. Matzke M, Stolte S, Thiele K, Juffernholz T, Arning J, Ranke J, Welz-Biermann U, Jastorff B. 2007. The influence of anion species on the toxicity of ionic liquids observed in an (eco)toxicological test battery. *Green Chem* 9:1198–1207.
43. Wang L-S, Wang L, Wang L, Wang G, Li Z-H, Wang J-J. 2009. Effect of 1-butyl-3-methylimidazolium tetrafluoroborate on the wheat (*Triticum aestivum* L.) seedlings. *Environ Toxicol* 24:296–303.
44. Martí E, Sierra J, Cáliz J, Montserrat G, Vila X, Garau MA, Cruañas R. 2011. Ecotoxicity of chlorophenolic compounds depending on soil characteristics. *Sci Total Environ* 409:2707–2716.
45. Pham TPT, Cho C-W, Yun Y-S. 2010. Environmental fate and toxicity of ionic liquids: A review. *Water Res* 44:252–272.
46. Matzke M, Stolte S, Arning J, Uebers U, Filser J. 2009. Ionic liquids in soils: Effects of different anion species of imidazolium based ionic liquids on wheat (*Triticum aestivum*) as affected by different clay minerals and clay concentrations. *Ecotoxicology* 18:197–203.
47. Pernak J, Goc I, Mirska I. 2004. Anti-microbial activities of protic ionic liquids with lactate anion. *Green Chem* 6:323–329.