Evaluation of (eco)toxicity and biodegradability of short aliphatic protic ionic liquids

Avaluació de l'(eco)toxicitat i la biodegradabilitat de líquids iònics pròtics alifàtics de cadena curta

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EVALUATION OF (ECO)TOXICITY AND BIODEGRADABILITY OF SHORT ALIPHATIC PROTIC IONIC LIQUIDS

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2014

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

PROGRAMA DE DOCTORAT
Ciències i tecnologies del medi ambient

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AVALUACIÓ DE L'(ECO)TOXICITAT I LA BIODEGRADABILITAT DE LÍQUIDS IÒNICS PRÒTICS ALIFÀTICS DE CADENA CURTA

Memòria presentada per Brezana Peric per optar al títol de doctor per la Universitat de Barcelona

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2014

ACKNOWLEDGMENTS

ACKNOWLEDGMENTS

First and foremost, I would like to thank the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development for awarding me the Grant MAEC-AECID, without which this thesis project would not be possible in the first place.

A very special thank you to Teresa Maria de Manuel Ochoa, who was not only the scholarship supervisor, but also a great friend, giving me support and encouraging me during years, and also providing me with a shoulder to cry on when needed!

Than I would like to thank my thesis directors Dra. Mary Garau and Dr. Jordi Sierra for years of marvelous collaboration and for always being there when I needed help or advice in and outside of the laboratory.

I would also like to thank the other members of my team: Dr. Robert Cruañas, for giving me the opportunity to do a thesis project in the Laboratori d'Edafologia, for his endless patience in listening to all of my ideas, doubts and problems, and Dra. Esther Martí for providing me valuable suggestions and moral support. I thank Màrius Mumbrú for his help with the ionic chromatography analysis.

Thanks to all of the staff of Unitat d'Edafologia, you've been wonderful! These six years have been like a dream thanks to you, folks! A special thank you to Angelito "de mi alma". Miquel, thank you for always being there for me and helping me with every problem or doubt that I have had during the years, and also for making me feel less lonely and more at home, especially while sharing the "castle" during festive days and holidays. Cèlia, Silvana and Miquelet, thank you for the laughs and chitchats.

Girls&guys from Fisiologia Vegetal, thank you for all of the great moments spent together: Xavi, Jean, Laura, Míriam, Strahil, Kosta, Nati and many others whom I came to know during my PhD.

During my stay in the Center for Environmental Research and Sustainable Technology (UFT) of the University of Bremen I had some top collaborators and I came across young people from around the world. To mention but a few members of the UFT staff: Stefan, Jürgen, Ulrike, Jen, Andrea, Hà, Cho, Jan, the two Antjes, Ruth... Thank you all for turning my stay in your work group into a unique and unforgettable experience, despite the polar cold. Magda and Alke, thank you for making me feel like at home. Albert, Brittany and Catherine thank you for helping me survive the northern coldness.

I would also like to thank my colleagues from the Environmental Analysis and Management Group of the Rovira i Virgili University, especially Neus, for the great time during scientific meetings and for letting me perform the Microtox® test in their laboratory.

José Luis, thank you for being the best boss in the whole wide world. Thank you for your understanding, advices and chats. I have learned a lot from you. Glòria and Francisco, thank you for being there for me every time I needed help or someone to talk to. Many thanks to the rest of the informatics team as well.

M. Àngels, Pepita and Ana, thank you for your patience and help, and for guiding me through the labyrinths of administration.

I thank Jihad and Roy for being so "pura vida" and wonderful persons and friends. I also thank Laura, who has been a good friend beyond the walls of our Unit. A special thank you to my marvelous Mexican friend Vanessa, I enjoyed so much our year in Barcelona! A big and "chachi piruli" thanks to my "inspiración profunda" Isabel, who has not only enriched my Spanish vocabulary, but also three years of my life in Barcelona. Many thanks to Ziortza, I will always admire her implacable Basque character. To Anita-pharmacist-singer-actress-Libra I owe many thanks, for being such a good friend since the day one and for always having some sensible advice and point of view. Señor G., thank you for all of those last-minute-decision tickets and for nearly 25 years of friendship. Abuelit@, thank you for endless chats, talks and mails, and for always believing in me. Thanks to Jeca^2, Caca, Milena, Danijela, Dušica and the rest of my BJ&BG crew. I also express my gratitude to Marija, Rajka i Gocita for their support and care. A very special thank you to Ljubiša, for always being there for me for nearly two decades.

All of my Catalan and other teachers I thank for everything they have thought me during. various courses that I have assisted throughout these years. And all of my classmates I thank for their friendships that have lasted a looot longer than the classes.

A big and very special thanks to the Professorat team. Guys, you are the best! Thank you for making me feel like one of the team and for all of the great talks during Saturday post-match coffee&drinks sessions.

Neus and Paco, my "adoptive" family, thank you for being there 24/7. Neus, thank you for your patience and interest, for many hours of talking, and for always having something nice and comforting to say. Paco, you are the most precious cat in the whole universe!

Micifú&Kitties, thank you for your unconditional affection and support, you rock!!!

And last but not least, I thank my family for their understanding and support throughout all of these PhD years, and especially my mother, to whom I dedicate this thesis.

It is impossible to mention each and every one here, but I do thank every person who has passed through my life during the duration of my PhD studies.

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INTRODUCTION

1. INTRODUCTION

1.1. Ionic liquids

Ionic liquids (ILs) are a fairly new and promising group of compounds that has generated a lot of interest as potential environmentally benign substitutes of organic solvents. An ionic liquid (IL) is generally defined as a salt composed entirely of ions (cation and anion) with a melting point below 100°C (Rogers and Seddon, 2002). The ILs are liquid in a wide range of temperatures, and some ILs can be liquid even at room temperature (Ranke et al., 2007a) and then they are called room temperature ionic liquids (RTILs). Most of the ILs used up to date have a bulky organic cation (imidazolium[a], pyridinium[b], pyrrolidinium[c], piperidinium[d], ammonium[e], phosphonium[f], etc.), substituted with alkyl chains of different length (from C_1 to C_{22}) and inorganic anions such as $[Cl]^-$, $[Br]^-$, $[I]^-$, $[N(CN)_2]^-$, $[BF_4]^-$, $[PF_6]^-$, etc. (Figure 1). New ILs are constantly being developed and new applications of ILs are being discovered.

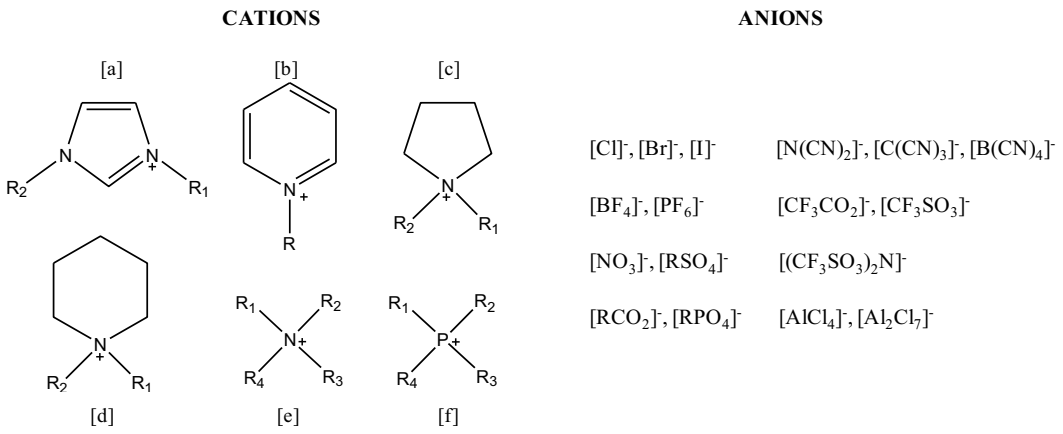


Figure 1. Principal ions present in most ILs.

Ionic liquids (ILs) are not newly discovered compounds, they have been known for almost a century. The first documented observation of ionic liquids by chemists was the so-called "red oil" formed during Friedel–Crafts reactions in the mid-nineteenth century. The structure of the red oil was later identified by NMR as a stable intermediate composed of a carbocation and a tetrachloroaluminate anion

(Forsyth et al., 2004). The earliest example of a room temperature ionic liquid was ethylammonium nitrate $[EtNH_3^+(NO_3)]^-$ with a melting point of 12ºC. It was described by Paul Walden in 1914 and obtained by the neutralization of ethylamine with concentrated nitric acid. One of the first 1,3-dialkylimidazolium RTILs was reported in the early 1980s by Wilkes and co-workers. It was obtained through the mixing of 1-ethyl-3-methylimidazolium chloride with aluminum trichloride (Wilkes, 2002). Organoaluminate ILs have a limited range of applications due to the high reactivity of the chloroaluminate anion towards water (Kruger, 2008). In the early 1990s, Wilkes reported two new ILs such as 1-butyl-3-methylimidazolium tetrafluoroborate and 1-butyl-3-methylimidazolium hexafluorophosphate (Wilkes, 2002). The cation 1-ethyl-3-methylimidazolium has been the most widely studied until 2001, and nowadays, 1-3-dialkylimidazolium salts are one of the most popularly used and investigated class of ILs (Keskin et al., 2007).

The great number of potential ionic liquids and the possible applications makes their classification a very difficult task because different criteria can be used (physical, chemical or structural characteristics, industrial applications, etc.). So, according to the chemical properties, ILs can be divided into protic (PILs) and aprotic (AILs). The distinguishing feature between both is that all PILs have a proton available for hydrogen bonding (Greaves and Drummond, 2008), whereas AILs haven't.

However, for some authors, the most useful way of grouping them is based on the properties that have conditioned the evolution of their use. According to Hough et al. (2007a) the first generation includes ILs that have unique physical properties such as decreased vapor pressure and high thermal stability. Second generation ILs have potential use as functional materials (energetic materials, lubricants, metal ion complexing agents, etc.) which utilize novel tunable physical and chemical property sets. The third and most recent generation of ILs involves biological properties combined with chosen physical and chemical properties. This third generation of ILs with biological properties may be a breakthrough for the pharmaceutical industry because it opens up many possibilities to generate active

pharmaceutical ingredients (APIs) in the form of ionic liquids (IL-APIs). Most of APIs are crystalline salts that present some problems related to dissolution, transport, bioavailability and polymorphism control, which can reduce the pharmacological activity (Schuster et al., 2005 and Hough et al., 2007b). A possible way to overcome the drawbacks of a drug with an ionic active group is to change the complementary ion for another able to bring IL physico-chemical properties to the new substance and thus modify its initial pharmacokinetic properties.

A new family of PILs with different cations and anions than those used up to now has been designed and it has been the subject of this study. These PILs are composed of substituted amines (mono-, di- and triethanolamine) as cations and aliphatic organic acids (formic, acetic, propionic, butyric, isobutyric and pentanoic acid) as anions (Cota et al., 2007). The AILs analyzed in this study are one of the most frequently used and are derived from imidazolium and pyridinium (Masten, 2004, Werner et al., 2010, Domínguez de María, 2012 and Mohammad Ali, 2012). The PILs analyzed in the present study are: 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxyethanolamine butanoate (2-HEAB), 2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine propionate (2-HDEAPr), 2-hydroxydiethanolamine butanoate (2-HDEAB), 2-hydroxydiethanolamine isobutanoate (2-HDEAiB), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), 2-hydroxytriethanolamine butanoate (2-HTEAB) and 2-hydroxytriethanolamine pentanoate (2-HTEAPe). The analyzed AILs are: 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-methyl-3-octylimidazolium chloride ([OMIM]Cl) and N-butylpyridinium chloride ([BPy]Cl). The structures of analyzed PILs are shown in Figure 2 and the structures of AILs in Figure 3.

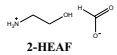
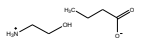
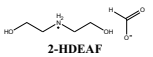
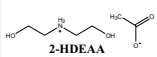
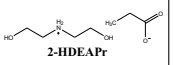
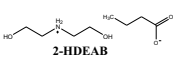
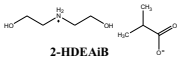
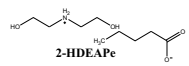
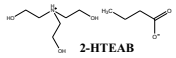
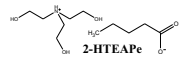
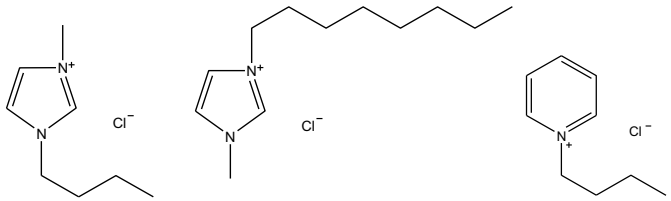
	Formic acid	Acetic acid	Propionic acid	Butiric acid	Isobutiric acid	Pentanoic acid
Monoethanolamine	 2-HEAF			 2-HEAB		
Diethanolamine	 2-HDEAF	 2-HDEAA	 2-HDEAPr	 2-HDEAB	 2-HDEAiB	 2-HDEAPe
Triethanolamine				 2-HTEAB		 2-HTEAPe

Figure 2. Structures and abbreviations of the analyzed PILs.



[BMIM]Cl **[OMIM]Cl** **[PBy]Cl**

Figure 3. Structures and abbreviations of the analyzed AILs.

The remarkably fast rising interest in ILs is manifested in a still increasing number of publications every year. More than 95% of all studies have been published during the last twenty years, with the number of publications dramatically rising in the last decade. The 20 papers published in 1994 increased to nearly 50 in 1997, as they were considered as sort of exotic compounds at that time. But just 5 years later, in 2002, there were already more than 500 papers published, and they were to become more than 2500 in 2008. In 2011 more than 3000 papers dealt with this class of compounds, increasing to nearly 4000 in 2013 and this number is still growing worldwide, due to constant research into synthesis and possible applications of existent and new ILs (Figure 4). This number has already been exceeded during 2014. There has been a remarkable development of the Chinese research groups in this field, with more than 1200 papers published in 2011 by these groups alone (Barrosse-Antle et al., 2010, Endres, 2012, Rogers et al., 2012, and Web of Science®).

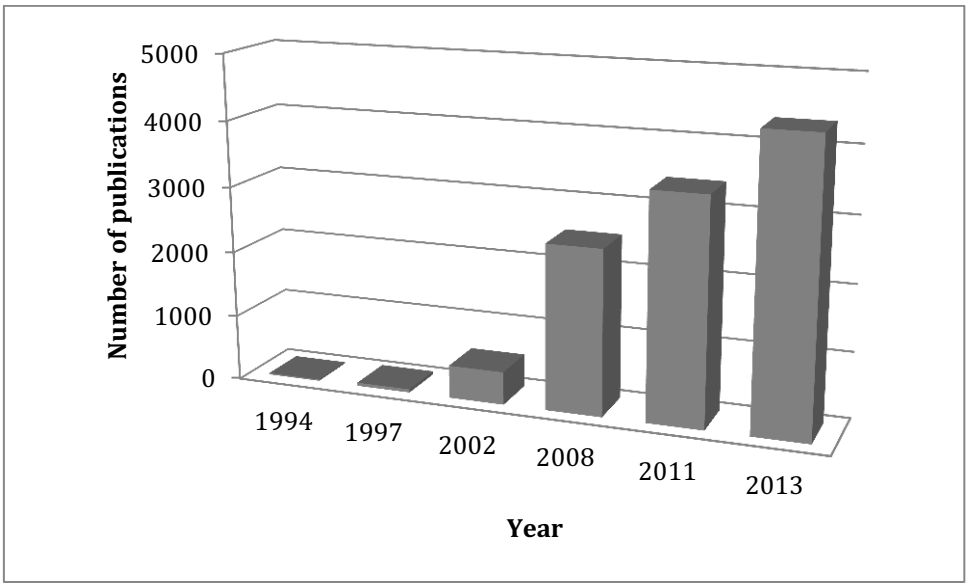


Figure 4. The number of publications on ionic liquids per year, between 1994 and 2013

1.2. Properties and applications of ionic liquids

The physicochemical properties of the ILs, like all other materials, depend upon the intermolecular and intramolecular forces and, subsequently, upon the structure of the cation and the anion. A significant number of investigations have been conducted for ILs on the relationship between their physicochemical properties and the structure of their cation and anion (Tokuda et al., 2004, 2005, 2006 and Greaves et al., 2006). In general, ILs have negligible vapor pressure so they are not volatile and no atmospheric pollution can be expected due to their use. The ILs are also thermally stable so they can be used in chemical processes that require heat input. These and other characteristics (Table 1) such as nonflammability make them useful for many applications, and make that they meet up to the criteria of green chemistry.

Table 1. Some physico-chemical characteristics of ionic liquids (IL Thermo NIST Standard Reference Database).

Decomposition point	150 – 500°C
Electrical conductivity at 25°C	< 0.6 S m^{-1} (maximum value 11.9)
Flammability	Non-flammables
Melting point	< 100°C (maximum value 239°C)
Temperature range liquid phase	-96 – 300°C
Thermal conductivity	0.117 – 0.199 W/m/K
Vapor pressure	Negligible
Viscosity	0.013 – 0.22 Pa·s (maximum value 1.02)

There are literally millions of different structures that may be formed by combining different cations and anions and the number of possible combinations is estimated to be as high as 10^{18} (Visser et al., 2002 and Chiappe and Pieraccini, 2005). This enormous quantity of possible ILs would permit, based on physicochemical characteristics, to select the most appropriate for a particular purpose. In particular, the room temperature ionic liquids (RTILs) are often called "designer solvents" because it is possible to create an IL with a required property. The RTILs have been used in numerous applications, and their development continues at a considerable rate owing to their peculiar physical and chemical properties such as high thermal and chemical stability, lack of inflammability, low volatility, and tunable solubility in several organic compounds (Earle and Seddon, 2000). Due to their low volatility, many of the RTILs have been used as greener alternatives to conventional toxic and volatile organic solvents by taking advantage of their unique properties (Wasserscheid and Welton, 2007). This set of properties allows the design of very attractive reaction systems that can solve some of the main drawbacks of currently used methods of synthesis or to obtain new procedures for making various products (Welton, 1999 and Wasserscheid and Welton, 2007). Polarity, hydrophilicity/hydrophobicity and other properties of ILs can be adjusted by an appropriate combination of cations and anions (Sheldon, 2001).

As the unique properties of the ILs were being discovered, there was a rising interest in applying them as a reaction medium in a wide variety of chemical transformations that until recently could only be carried out in organic solvents. The literature describes numerous uses of ILs, some of them being: as reaction media for many organic transformations (Sheldon, 2001), in separations and extractions (Han and Armstrong, 2007), as electrolytes for electrochemistry (Hapiot and Lagrost, 2008), in nanotechnology (Ichikawa et al., 2007), in biotechnology (Van Rantwijk and Sheldon, 2007), in engineering processes (Greaves and Drummond, 2008), in whole-cell biotechnological applications (Quijano et al., 2011), absorption of gases (CO_2) (Rogers and Seddon, 2002), as catalysts in organic synthesis (Olivier-Bourbigou et al., 2010), and organometallic and radical polymerization (Zhu et al., 2009). Other authors have described ILs specific applications for extraction of active ingredients from medicinal plants (Du et al., 2009, Jin et al., 2011 and Liu et al., 2011). The ILs have a largely unexplored potential to be used in the pharmaceutical industry as well. They can be used as drug reservoirs for the controlled release (Jaitely et al., 2008), solvents of pharmaceuticals (Azevedo et al., 2013) or ingredients in topical drug delivery systems (Dobler et al., 2013). In order to overcome the problems associated with low water solubility, and consequently low bioavailability of active pharmaceutical ingredients (APIs), ionic liquids containing APIs are being investigated, as in case of ampicillin-based ionic liquids (Florindo et al., 2013). Some of the ILs applications are presented in the Figure 5.

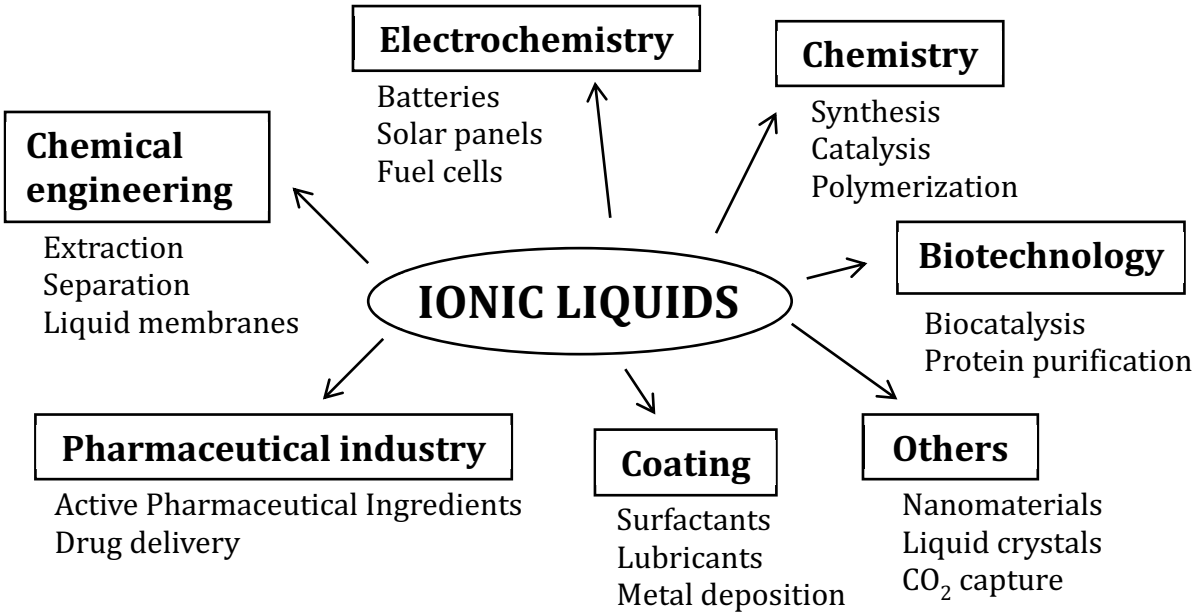


Figure 5. Some of the possible applications of ionic liquids (adapted from Pham et al., 2010).

The possible applications for the studied new family of PILs are numerous. The 2-hydroxyethanolamine formate (2-HEAF) has been found to be able to dissolve many inorganic salts, hydroxylated compounds and some insoluble polymers such as polyaniline and polypyrrole (Bicak, 2005). Also, 2-HEAF has been analyzed as a potential solvent in the preparation of organo-soluble polyanilines with reasonable molecular weights (Bicak et al., 2005 and Cota et al., 2007). Application and prospects of a two-phase, tunable solvent system composed of ionic liquids and supercritical fluids with an emphasis on supercritical carbon dioxide have been reviewed (Roth, 2009). The representatives of this family like e.g. 2-HDEAF, 2-HDEAA and 2-HDEAPe, have been analyzed for their potential catalytic activity in the aldol condensation reactions (Iglesias et al., 2010 and Cota et al., 2013) and for the heterogeneous catalytic hydrogenation of cyclohexene and acetone catalyzed by Pt/Al_2O_3 with methanol, ethanol and propan-1-ol (Khodadadi-Moghaddam et al., 2009). Other PILs, also derived from 2-hydroxyetanolamine and organic acids, have been studied in order to develop a new process for absorption of CO_2 (Yuan et al., 2007). A potential corrosion effect of PILs on the metal frame of the systems of absorption of CO_2 has been studied recently and very low corrosion index have been found (especially for 2-HEAA) in case of carbon steel plates A285, which is a

widely used material in absorption operations (Dos Santos et al., 2014). The immobilization of the lipase from *Burkholderia cepacia* by the sol–gel technique using monoethanolamine derived PILs as additives to protect against inactivation of the lipase due to release of alcohol and shrinkage of the gel was successfully performed by De Souza et al. (2013). The same set of PILs was immobilized on alanine as a support in another study, obtaining active heterogeneous catalysts. They proved to have an excellent catalytic potential in citral-acetone and benzaldehyde-acetone condensations (two reactions of interest for fine chemistry industry) and they can be recycled and reused for three consecutive cycles without significant loss of activity (Cota et al., 2014). The newest potential application of the PILs (2-HEAA, 2-HDEAA, 2-HEAPr and 2-HDEAPr) is still in development, involving a search for an alternative method for dyeing cotton fiber using PILs as water substitutes (Iglesias et al., 2014).

1.3. Ecotoxicity of ionic liquids

Even though ILs have a potentially "green" profile, they are basically chemical products, and as such, have to fulfill the criteria of the current legislation on chemicals in the European Union called Registration, Evaluation, Authorization, and Restriction of Chemical Substances (REACH) (European Union, 2006). Taking into account their possible commercial use, a very important aspect that has to be studied in sufficient depth is the potential negative impact of ILs, both on the environment and humans. The REACH regulates the safety of chemical products, their manufacturing, toxicity, biodegradability, transport and use in the industrial sectors. This regulation applies to substances manufactured or imported into the European Union in quantities of 1 ton or more per year. REACH came into force on the 1st of June 2007. The REACH demands all manufacturers and importers of substances to register them with the European Chemicals Agency (ECHA). Registration means that a manufacturer or importer has to provide a registration dossier to the ECHA. The third registration deadline is the 31st of May 2018 for all substances produced, imported or used in the quantity of 1-100 tons per year, so it could affect the ILs, if produced and used in a larger scale.

Manufacturers and importers need to submit two documents: a technical dossier, for substances in quantities of 1 ton or more per year, and, in addition, a chemical safety report, for substances manufactured or imported in quantities of 10 tons or more per year. The technical dossier contains information on the properties and classification of a substance as well as on uses and guidance on safe use. The information required to determine the properties of the substances varies according to the tonnage in which the substance is manufactured or imported. The higher the tonnage the more information on the intrinsic properties of the chemical is required. The chemical safety report documents the hazards and classification of a substance and the assessment as to whether the substance is PBT (Persistent, Bioaccumulative and Toxic) or vPvB (very Persistent very Bioaccumulative). The chemical safety report also includes exposure scenarios (European Union, 2006).

Requirements for environmental information can be divided in two main information types:

- Ecotoxicity information: long term aquatic toxicity (invertebrates), short-term or long-term toxicity on terrestrial organism, long-term toxicity on sediment organisms, long-term or reproductive toxicity to birds.
- Environmental fate and pathways: biotic degradation (simulation testing), information on degradation products, bioaccumulation and adsorption-desorption.

Despite the fact that the ILs are considered as non-volatile and thus cannot contribute to the air pollution, the water solubility of many ionic liquids is not negligible. The potential release of ionic liquids into aquatic and terrestrial environment may lead to water and soil pollution, and related risks. So, it is important to evaluate ecotoxicity, biodegradability, bioaccumulation and environmental fate of these chemicals. Taking into account the possible use and commercialization of ILs, they should pass the REACH evaluation, which recommends the tests for the assessment of the potential toxicity of chemicals to be done in accordance with OECD guidelines. These tests can be divided into three groups: effects on biotic systems, health effects, and degradation and accumulation.

1.3.1. Aquatic ecotoxicity

Several properties of ionic liquids and their effects on aquatic organisms have been investigated in different works. Toxicological research studies concerning ionic liquids have been undertaken in the past decade and indicate that the ILs might not be as "green" as they were considered initially.

Green algae are ecologically relevant organisms that are at the base of the food chain. The algal test has an advantage over tests done with e. g. fish or invertebrates because it measures a population-level response. Algae have short life cycle which makes them ideal for toxicological studies, as they can respond quickly to environmental change (Blaise and Férard, 2005). Cho and co-workers (2008) found that the toxicity of 1-butyl-3-methylimidazolium, 1-butyl-3-methylpyridinium, 1-butyl-1-methylpyrrolidinium, tetrabutylammonium and tetrabutylphosphonium bromides on *Pseudokirchneriella subcapitata* was between two and four orders of magnitude greater than those of the organic solvents examined (methanol, dimethylformamide and 2-propanol). This group was also investigating other series of imidazolium based ILs and the conclusion was that the toxicity of ILs increased with the increase in side chain length (Cho et al., 2007). Latała et al. (2005) examined the effect of imidazolium based ILs on other species of Baltic algae (*Oocystis submarina* and *Cyclotella meneghiniana*). They found that the response of the two species differed dramatically being *Oocystis submarina* more resistant to the toxicity of ILs than the other one, and that the toxicity was reduced in more saline waters. Matzke et al. (2007) investigated the effect of imidazolium based ILs with C_4 and C_8 side chains on *Scenedesmus vacuolatus* especie. Their findings confirmed other authors conclusions, i.e. that the toxicity strongly depends on the side chain effect.

The results of the studies of toxicity of ILs towards *Daphnia magna* (a cladoceran freshwater water flea) showed the link between toxicity and alkyl chain length of the tested ILs containing imidazolium, pyridinium or quaternary ammonium as cations (Couling et al., 2006, Wells and Coombe, 2006, Samorì et al., 2007 and Pretti et al., 2009). Other authors (Bernot et al., 2005a) indicate that ILs

derivated of imidazolium present an acute toxicity to *Daphnia magna* higher than benzene and some organochlorine solvents (tri- and tetra-chloromethane).

Similar results were obtained in other studies on the snail *Physa acuta* with imidazolium and pyridinium based ILs (Bernot et al., 2005b) and also on *Dreissena polymorpha* (zebra mussel) (Costello et al., 2009). In these tests a positive relationship between alkyl chain length and toxicity of ILs was also demonstrated, as well as the lower sensitiveness of *Physa acuta* than *Daphnia magna* to ILs.

Concerning toxicity of ILs to the zebrafish (*Danio rerio*), Pretti et al. (2006) evaluated the toxicity of commonly used (imidazolium and pyridinium ILs) and new AMMOENG® ILs (quaternary ammonium derivates). The results of this study revealed that ILs may cause a completely different effect on fish than expected according to their chemical structures. Imidazolium, pyridinium and pyrrolidinium could be regarded as non-highly lethal towards zebrafish. On the other hand, some ammonium salts showed EC_{50} remarkably lower than that organic solvents and tertiary amines. They also demonstrated that fish species are less sensitive to ILs toxicity compared to other species belonging to lower trophic levels.

The duckweed, *Lemna minor*, is a common aquatic plant that has been frequently a focus of the investigation of phytotoxicity of ILs (Jastorff et al., 2005, Matzke et al., 2007, Stolte et al., 2007a and Larson et al., 2008). In general, 1-alkyl-3-methylimidazolium compounds with longer alkyl chains were more toxic to *Lemna minor* than those with short alkyl chain lengths. Imidazolium and pyridinium cations with butyl groups had similar EC_{50} (the concentration that produces a 50% reduction in plant growth) while the equivalent ammonium cation had a much higher EC_{50} and thus proved to be less toxic.

Microtox® is a standardised toxicity test system which is based on the reduction of the bioluminescence of the marine bacteria *Vibrio fischeri* when exposed to a range of concentrations of the tested chemical. This is a rapid, sensitive, reproducible, ecologically relevant and cost effective test. It is recognised and used throughout the world as a standard test for aquatic toxicity testing.

Docherty and Kulpa (2005) investigated the toxicity of imidazolium and pyridinium ILs and found that the hydrophobicity, which corresponds to the increasing alkyl chain length of the IL cation, induces rising toxicity. These findings are consistent with those from other authors (Ranke et al., 2004, Matzke et al., 2007). Couling and co-workers (2006) have expanded the range of investigated ILs and noted that the quaternary ammonium compounds were less toxic than the imidazolium and pyridinium analogues. In comparison to some commonly used industrial solvents such as phenol, toluene and benzene, long chain (> C_4) ILs are more toxic. The values of EC_{50} for the most frequently used organic solvents for the Microtox® test are presented in the Table 2.

Table 2. Acute toxicity of organic solvents to *Vibrio fischeri* (Kaiser and Palabrica, 1991).

Solvent	**log EC_{50} (μmol L^{-1})**
Methanol	3.5
Acetonitrile	2.77
Acetone	2.52
Benzene	2.03
Phenol	1.49

Generally speaking, all of the aquatic toxicity tests showed that the head group (cationic part of the molecule) was responsible for the toxicity of the ILs. In most cases there was no influence of the anionic part of the ILs molecule on the toxicity. Only the anion bis[(trifluoromethyl)sulfonyl]imide $[(CF_3SO_2)_2N]^-$ showed higher toxicity than others (Ranke et al., 2004, Matzke et al., 2007 and Stolte et al., 2007a). The side chains on the head groups were proven to have a very strong influence on the toxicity. The longer and more branched the side chain is, the more toxic is the ionic liquid. The most toxic ionic liquids have an alkyl chain with more than eight carbons (Wells and Coombe, 2006, Pham et al., 2010 and Radošević et al., 2013). These results point to the fact that some ILs may be more toxic than the commonly used organic solvents, so they could to be not as green as expected.

In the Table 3 is presented the toxicity of ILs to different levels of biological complexity.

Table 3. Toxicity of different ILs expressed as $logEC_{50}$ (μmol L^{-1}).

Ionic liquid	*Vibrio fischeri*	*P. subcapitata*	*Lemna minor*	*Daphnia magna*
$[C_4MIM]Cl$	2.95 [1]	2.34 [6]	2.82 [6]	1.93 [4]
$[C_4MIM]Br$	3.07 [2]	3.46 [7]	N.D.	1.57 [4]
$[C_4MIM]BF_4$	3.55 [2]	N.D.	2.49 [10]	1.68 [4]
$[C_4MIM]PF_6$	3.07 [3]	2.20 [8]	N.D.	1.85 [4]
$[C_6MIM]Br$	1.42 [4]	2.57 [9]	N.D.	0.78 [4]
$[C_6MIM]BF_4$	3.18 [2]	N.D.	N.D.	N.D.
$[C_8MIM]Cl$	1.19 [3]	1.46 [8]	N.D.	N.D.
$[C_8MIM]BF_4$	1.41 [2]	N.D.	N.D.	N.D.
$[C_4Py]Cl$	3.18 [5]	N.D.	2.32 [5]	N.D.
$[C_4MPy]Br$	2.12 [1]	3.46 [7]	N.D.	1.76 [4]
$[C_4MPyr]Cl$	>4.30 [5]	3.67 [7]	N.D.	N.D.
$[C_4Pip]Br$	4.27 [5]	3.27 [5]	0.47 [5]	N.D.

Abbreviations: 1-butyl-3-methylimidazolium (C_4MIM), 1-hexyl-3-methylimidazolium (C_6MIM), 1-octyl-3-methylimidazolium (C_8MIM), 1-butylpyridinium (C_4Py), 1-butyl-3-methylpyridinium (C_4MPy), 1-butyl-3-methylpyrrolidinium (C_4MPyr), 1-butyl-3-methylpiperidinium (C_4Pip). N.A. non available.

The results are taken from: [1] Docherty and Kulpa, 2005; [2] Ranke et al., 2004; [3] Garcia et al., 2005; [4] Couling et al., 2006; [5] Stolte et al., 2007a; [6] Matzke et al., 2007; [7] Cho et al., 2008; [8] Wells and Coombe, 2006; [9] Cho et al., 2007; [10] Jastorff et al., 2005.

1.3.2. Terrestrial ecotoxicity

Studies on the effects of the ILs on soil and sediment organisms are very limited or still missing so far. Terrestrial organism such as the spring tail *Folsomia candida*, a soil invertebrate (Matzke et al., 2007), the earthworm *Eisenia foetida* (Luo et al.,

2010) or a roundworm *Caenorhabditis elegans* (Swatloski et al., 2004) have been tested. In this last case, the authors suggest the use of *C. elegans* as a model organism for inexpensively and quickly exploring toxicological effects of 1-alkyl-3-methylimidazolium chloride.

Concerning higher plants, Matzke et al. (2008) investigated the toxicity of ILs derived from imidazolium with different anions to wheat (*Triticum aestivum*) and cress (*Lepidium sativum*). The side chain effect was once again confirmed, with slightly diverse patterns of toxicity depending on the anion used. Matzke et al. (2009) investigated the influence of different clay minerals and clay concentrations on the toxicity of the anionic moieties of imidazolium based ILs towards wheat plants. The obtained data showed that 1-butyl-3-methyl-imidazolium bis[(trifluoromethyl)sulfonyl] imide appeared to be the most toxic, independently of the type of clay added to a reference soil. The toxicity of different ILs with the same cationic moiety (1-butyl-3-methylimidazolium chloride, tetrafluoroborate and hydrogen sulfate) was mainly dependent on the cation and the observed effects varied according to the added clay type and clay concentration to the reference soil. An increase of smectite clay content resulted in less inhibitory effects of these ILs. Studzinska and Buszewski (2009) proved that hazardous effects of imidazolium ILs are closely connected with organic matter content in soil. Soil with more organic carbon was observed to sorb IL cations more extensively than soil with little or no organic matter; hence, the more fertile the soil, the lower probability of hazardous effect of ILs to plants.

Wang and co-workers (2009) conducted a study on the effect of 1-butyl-3-methylimidazolium tetrafluoroborate on wheat seedlings. The increase of this IL concentration in soil showed a significant negative effect both on germination and roots and shoot length of the wheat plants. At low concentrations, 1-butyl-3-methylimidazolium tetrafluoroborate did not inhibit, and even promoted wheat seedling growth. At high concentrations, this IL inhibited wheat seedling growth significantly and decreased chlorophyll content, thereby reducing photosynthesis and plant growth. In another research, the phytotoxicity tests of chiral ILs containing (-)-nopyl derivatives were carried out in a greenhouse using spring

barley (*Hordeum vulgare*) which is a monocotyledonous plant, and a common radish (*Raphanus sativus* L. subvar. *radicula* Pers.) which is a dicotyledonous plant (Bałczewski et al., 2007). According to the obtained results, increasing the concentration of ILs resulted in a systematic decrease in the crop fresh weight of total sprouts and the crop fresh weight per plant, both for spring barley and for common radish. It could also be noted that barley was more resistant than the radish and tolerated higher concentrations of ILs in soil.

In a recent study of the multilevel toxic effects of imidazolium based ILs on barley seedlings (Cvjetko Bubalo et al., 2014), the investigated ILs had different anions and length of alkyl chains linked to imidazolium ring. Once again, the ILs with longer alkyl side chains have proven to be more toxic, in terms of germination and early growth of barley. The novel information on the alternation in the extent of oxidative stress and antioxidant enzymes activities that were found in barley plants due to the ILs treatment can be useful for assessing the potential risk for humans and for better understanding of the ILs toxicity mechanisms on the enzymatic level.

Although the investigation of the phytotoxicity of ILs and their effect on soil has not been intensive, the available data can give a boost for the environmental scientists to start dealing more with the potential impact of ILs towards plants and soil.

1.4. Biodegradability of ionic liquids

1.4.1. Biodegrability in water

The biodegradation potential of ILs in aqueous media has been investigated in many studies. Wells and Coombe (2006) investigated the biodegradability of ammonium, imidazolium, phosphonium and pyridinium compounds by measuring the biological oxygen demand (BOD). They observed that the cations with short side chains (C_4) were not biodegradable. A strong inhibitory potential to the inoculum used in the test was observed for series with longer side chains (C_{12}, C_{16} and C_{18}), indicating the toxicity of these ILs towards the microorganisms used. Docherty and co-workers (2007) examined the biodegradability of N-methylimidazolium and 3-methylpyridinium compounds substituted with butyl, hexyl and octyl side chains and bromide as the anion. A dependency between biodegradability and the side chain length was found in the dissolved organic carbon (DOC) die-away tests and in tests monitoring the changes in the total dissolved nitrogen concentration. Another IL, 1-octylpyridinium bromide, met the OECD criterion for being classified as readily biodegradable (reaching the biodegradation level higher than 60% of Theoretical Oxygen Demand within 28 days of testing), whereas 1-hexylpyridinium bromide exhibited a decreased degradation rate. Compared to the pyridinium ILs the mineralisation of the imidazolium ILs was lower. The 1-methyl-3-octylimidazolium cation showed significant degradation rates, but those were not high enough for a classification as readily biodegradable. For the pyridinium and imidazolium head groups carrying a butyl side chain no significant biodegradation was observable. In another study, Gathergood et al. (2006) found that the influence of anion was important only in a case of the octyl sulfate anion, which proved to be considerably more biodegradable than the other commonly used anions. The introduction of an ester group in the side chain of the 1,3-dialkylimidazolium cation lead to biodegradation values very close to the pass level of the Closed Bottle test.

Stolte et al. (2008) investigated the biodegradation of different imidazolium, pyridinium and 4-dimethylaminopyridinium compounds substituted with various

alkyl side chains. They found a significant biodegradation for ecotoxicologically unfavourable compounds carrying long alkyl side chains (C_6 and C_8). In contrast for ecotoxicologically more recommendable imidazolium ionic liquids with short alkyl ($\leq C_6$) and short functionalised side chains, no biological degradation could be found. The introduction of different functional groups into the side chain moiety, thus offering a higher chemical reactivity, did not lead to the expected improvement of the biological degradation potential. After an incubation period of 24 days for the 1-methyl-3-octylimidazolium cation different biological transformation products carrying hydroxyl, carbonyl and carboxyl groups were identified. Furthermore, shortened side chain moieties were identified indicating the degradation of the octyl side chain via β-oxidation.

Gathergood and co-workers (2004) intended to design, synthesize and evaluate biodegradable ILs containing ester or amide groups in the alkyl side chain. The introduction of a group susceptible to enzymatic hydrolysis improved greatly the biodegradation, compared with the commonly used dialkylimidazolium ionic liquids, 1-butyl-3-methylimidazolium tetrafluoroborate and hexafluorophosphate. For the 3-methyl-1-alkyloxycarbonylmethylimidazolium bromide series, the greatest biodegradation was observed when alkyl side chain had four or more carbons. The corresponding amide analogues proved to be poorly biodegradable. They also tried to establish the influence of the anionic moiety on the biodegradability of ILs. Different ILs with 1-butyl-3-methylimidazolium cation combined with Br^-, $[BF_4]^-$, $[PF_6]^-$, $[N(CN)_2]^-$, $[(CF_3SO_2)_2N]^-$ and octylsulfate as the counter ion were analyzed using the Sturm and Closed Bottle test protocols. No compound showed significant degree of biodegradation with the exception of the IL containing octylsulfate anion which had higher levels of biodegradability. The same group did a further research toward the discovery of biodegradable ILs (Gathergood et al., 2006). The aims of the study were to incorporate additional structural modifications in order to improve biodegradability. They incorporated 2-methyl group into the molecule of imidazolium ILs to provide an additional site for metabolism, parting from a fact that 2-methylimidazole is significantly more biodegradable than imidazole. The incorporation of a methyl group in the 2-position of imidazolium cation had no significant effect on biodegradability. The

commonly used 1-butyl-3-methylimidazolium core showed negligible levels of degradation. Part of this team continued their research by trying to design, synthesize and evaluate biodegradable pyridinium ILs. Harjani et al. (2008) prepared ILs bearing an ester side chain moiety, using either pyridine or nicotinic acid. These ILs showed high levels of biodegradation under aerobic conditions and can be classified as "readily biodegradable". In contrast, pyridinium ILs with alkyl side chains showed significantly lower levels of biodegradability in the same test. In a recent comprehensive study of the biodegradability of 27 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium ILs cations under aerobic conditions, the authors' findings indicate that all of the five head groups are readily or inherently biodegradable (Neumann et al., 2014).

The fact that only the long side chains in ionic liquids improved biodegradability creates a conflict of aims between minimizing the toxicity and maximizing the biodegradability. The issue of biodegradability seems to be a problem in the development of environmentally safer ionic liquids.

1.4.2. Biodegradability in soil

Like in the case of the terrestrial ecotoxicity, the data on the biodegradability in soil is scarce. Kumar et al. (2006) investigated the fate of 1-butyl-3-methylimidazolium tetrafluoroborate when in contact with soil-microorganisms, wastewater microorganisms, *Pseudomonas putida* and *Escherichia coli*. Although 1-butyl-3-methylimidazolium tetrafluoroborate was indicated to be recalcitrant in Sturm and Closed Bottle test assays as mentioned above, it was observed in this study that *P. putida* was able to break down 1-butyl-3-methylimidazolium tetrafluoroborate after 15 days of incubation.

The aerobic biodegradation processes of ionic liquids in soil were monitored for the first time by Modelli et al. (2008), working with four ionic liquids obtained from the 1-butyl-3-methylimidazolium and 1-methoxyethyl-3-methylimidazolium cations combined with the tetrafluoroborate and dicyanamide counter anions, by measuring the total production of CO_2 over six months, according to test method

ASTM D 5988-96. The results indicate that the biodegradability rate ranges between 17 and 52% for 1-butyl-3-methylimidazolium and between 0.1 and 3.6 % for 1-methoxyethyl-3-methylimidazolium, with dicyanamide and tetrafluoroborate anions respectively. In both cases the biodegradation rate did not exceed 10 % in 28 days of the test duration.

In the first report showing complete biodegradation by *Corynebacterium* sp. of an N-substituted pyridinium to low molecular organic acid degradation products, Zhang et al. (2010) also found that the 1-butyl-3-methylimidazolium-hexaflurophosphate was not metabolized, proving once again that the imidazolium based ILs are not only more toxic, but also more resistant to biodegradation, both in water and in soil.

The study of Deive et al. (2011) focused on identification of the microbial strains with higher survival rates towards ILs and which could be potentially used in soil remediation. The surviving microbial strains (containing both fungi and bacteria) were isolated and taxonomically identified, and the ionic liquid degradation was analyzed during their cultivation. The degradation of the imidazolium cation was observed to be null. But amongst the isolated microbial strains there were both fungi and bacteria which were able to degrade, even if only partially, the short chain anions (ethanoate, lactate, ethylsulfonate and ethylsulfate).

But there is no complete information or study done in order to evaluate the toxic effect of ILs on the soil microbiota in terms of carbon and nitrogen transformation.

1.5. Enzymatic and cellular toxicity

Several enzymatic and cellular assays have been used in order to predict the impact of ionic liquids on humans. Acetylcholinesterase (AChE) is an enzyme that can be found in nearly all higher organisms. Arning et al. (2008) found that the positively charged nitrogen atom, a widely delocalized aromatic system, and the

lipophilicity of the side chains of the cationic head groups are the key elements for the potential enzyme inhibition. The influence of the cationic head groups, anions and different side chains of the cation on the inhibitory potential of the ILs was analyzed. The authors concluded that aromatic cation head groups exhibit a very strong inhibitory potential to the enzyme, in contrast to the polar and nonaromatic head groups that exhibited only a weak inhibition of the enzyme activity. The introduction of polar hydroxy, ether or nitrile functions into the alkyl side chain lowers the inhibitory potential. Practically none of the anions exhibited negative effect on AChE. These findings are in concordance with the conclusions of ecotoxicity studies. Składanowski et al. (2005) studied the *in vitro* inhibition of the enzyme adenosine monophosphate (AMP) deaminase. The 1-butyl-3-methylimidazolium ILs were seen to inhibit AMP deaminase activity. Antioxidant enzymes of the mouse liver were investigated by Yu et al. (2008) and it was discovered that 1-octyl-3-methylimidazolium bromide causes damage on mouse liver and thus significantly decreased the catalase activity.

In vitro studies with different cell lines have also been conducted, namely with cancerous cell lines from rat (promyelocytic leukemia – IPC-81 and glioma – C6 cell lines) and human cells (colonic – HT-29 and CaCo-2, cervical – HeLa and breast – MCF-7 carcinoma cell lines). The effects of ILs on IPC-81 cell line has been frequently studied, and the imidazolium based ILs with long side chains have proved to be more toxic than the non-aromatic ILs (Ranke et al., 2004, Ranke et al., 2007b, Stolte et al., 2007b and Stasiewicz et al., 2008). The toxicity of 1-butyl-3-methylimidazolium increased when the halogen was substituted by BF_4 or NTf_2, showing an anion dependence effect. The 24-hour EC_{50} for MCF-7 cell line was lower for the imidazolium than for the piperidinium based ILs. The anion also affected MCF-7 viability like in the IPC-81 studies (Kumar et al., 2009). In the case of the HeLa cell line, there was a clear influence of the side chain on toxicity. Quaternary ammonium ILs were seen to be not very harmful for this cell line (Wang et al., 2007). Concerning the CaCo-2 cell line, 1-butyl-3-methylimidazolium ILs did not affect significantly the cell viability, independently on the chosen anion, but the toxicity increased with the alkyl chain length. The introduction of a carboxylic group in the end of a C_{10} alkyl chain demonstrated a considerable

reduction of 1-decyl-3-methylimidazolium toxicity, which did not occur when the carboxylic group was replaced by an ester (Frade et al., 2007 and 2009). Similar results were obtained for the HT-29 cell line (Frade et al., 2007).

1.6. *In silico* methods for the prediction of toxicity of ionic liquids

The main goal of the REACH Regulation is to ensure a high level of protection of human health and environment. One of the main reasons for developing and adopting the REACH Regulation was to fill the data gaps for the large number of substances already in use. For many of these substances there is inadequate information on the hazards they pose to human health and the environment. Filling the data gaps would allow the industry to assess hazards and risks, and also to identify and implement any risk management measures that are necessary to protect human health and the environment. CAS REGISTRY^{SM} contains around 87 million unique organic and inorganic chemical substances and it is updated daily with about 15000 substances (last accessed on 10th of September 2014), while European Chemicals Agency's (ECHA) database contains 12636 unique substances and contains information from 48801 Dossiers, done in compliance with the requirements of REACH (last updated 14th of August 2014, ECHA website). It is currently estimated that there are around 30000 chemicals in use in the EU that have never been subject to approved testing. The REACH legislation requires that existing chemicals are formally evaluated and registered. Regulatory concern to protect human health and the environment means that the situation is now changing. The REACH places the responsibility on industry to generate the necessary evidence of safety/toxicity, to explain how the risks are managed in practice and to submit the documentation to regulators. To evaluate so many chemicals using traditional animal tests would take decades, require millions of laboratory animals and cost billions to the industry. The REACH requires that companies which use the same chemical share their existing data, collaborate on further evaluation and registration and use alternative methods where possible. Some of the proposed alternative methods are *in silico* methods (ECHA, 2008).

In silico methods can be used for the prediction of toxicity and help to avoid the costly and time consuming testing, either on animals or in laboratory. Many different *in silico* methods have been developed since the late 70s, mainly being used in the area of pharmacology and drug formulation. The term *in silico* comes from the computer component silicium; *in silico* methods, meaning that these methods of prediction are based on computational approaches, like in the case of Quantitative Structure-Activity Relationship (QSAR). *In silico* methods have the advantage over the laboratory ones that they can make fast predictions for a large set of compounds using data processing and specific softwares (Amberg, 2013). Another advantage of the *in silico* methods is that they can make predictions based on the structure of a compound even before it has been synthesized or used in a wider scale, which is precisely the case with ILs. So, *in silico* methods can be used at a very early stage in the ILs development process, helping to discard the potentially toxic ILs.

The QSAR methods are based on the quantitative relationship between a chemical structure of a compound and its biological/toxicological activity, with the use of the descriptors that are generated from the molecular structure. QSAR methods can make predictions of EC_{50} values and thus significantly reduce laboratory costs and testing time needed by the industry in order to fulfill the demands that REACH legislation imposes on the chemical industry. The REACH recognizes that properties determined with alternative methods are equal to tests and testing methods performed *in vivo* and *in vitro*. And for the ECHA, it is very important to avoid unnecessary tests on animals. In this context, the usage of QSAR is also strongly recommended and supported by ECHA. In the last six years, ECHA has published several documents on QSAR (ECHA, 2008, 2010 and 2012a).

The QSAR studies on toxicity of ILs have not been as frequent as compared to other chemicals (Das and Roy, 2013). One of the first studies emphasizing the importance of relating the structure and toxicity of the ILs was published by Jastorff et al. (2003), where a new strategy was presented, which aimed to provide the environmental risk assessment of ILs using a combination of structure–activity relationships (SAR), toxicological and ecotoxicological tests and modelling. The

authors considered that by using the QSAR and SAR models, the costs of a sustainable ILs design would be reduced substantially. This idea was further developed by the same team (Stock et al. 2004, Jastorff et al., 2007, Ranke et al., 2007a, Arning et al., 2008), reaching a fully developed *in silico* model by Cho et al. (2013) that allowed the prediction of cationic hydrophobicity and cytotoxicity of ionic liquids towards the leukemia rat cell line, *Vibrio fischeri* and *Scenedesmus vacuolatus* based on molecular interaction potentials of ions. Couling et al. (2006) developed a model predicting ILs toxicity to *V. fischeri* and *D. magna* based on electronic, spatial, structural, thermodynamic, and topological descriptors. The group contribution QSAR model based on multiple linear regression proposed by Luis et al. (2007) was successfully applied to a larger set of ILs by the same group three years later (Luis et al., 2010), but including only the toxicity data for Microtox® test.

AIMS

2. AIMS

The objective of the thesis is to evaluate the (eco)toxicity and biodegradability of selected short aliphatic protic ionic liquids belonging to a new family of protic ionic liquids (PILs) derived from substituted amines and organic acids.

(Eco)toxicity and biodegradability of the representatives of the family of the most frequently used aprotic ionic liquids (AILs), derived from imidazolium and pyridinium, is also to be evaluated, in order to make a comparative study and see if the ionic liquids of the new family of PILs are less toxic and have less environmental impact than the AILs.

In order to determine the impact of the ionic liquids on the aquatic environment, ecotoxicity tests involving the following test organisms will be performed: marine bacteria *Vibrio fischeri*, green algae *Pseudokirchneriella subcapitata* and higher aquatic plant *Lemna minor*. The impact of the ionic liquids on terrestrial environment will be determined by performing terrestrial ecotoxicity tests that evaluate the toxic effects on terrestrial plants (onion *Allium cepa*, grass *Lolium perenne* and radish *Raphanus sativus*) and soil microorganisms involved in carbon and nitrogen transformation. Two additional test systems with an enzyme (acetylcholinesterase inhibition) and isolated leukemia IPC-81 cells from rats (cytotoxicity) will be performed in order to provide more in-depth evaluation of toxicity. Studies of biodegradability in soil and water will also be conducted.

The (eco)toxicity data and the chemical structure of ionic liquids will be related by means of group contribution quantitative structure-activity relationship (QSAR) modelling, with the goal of predicting the (eco)toxicity of previously untested PILs. This kind of modelling can also provide useful information for the design and synthesis of ionic liquids with lower environmental impact.

RESULTS

3. RESULTS

The results of the thesis have been published in form of research papers in three different scientific journals and the forth one has been submitted and is currently under review.

PAPER 1

Peric B., Sierra J., Martí E., Garau M.A., Cruañas R., Iglesias M., 2011. Terrestrial ecotoxicity of short aliphatic protic ionic liquids. *Environmental Toxicology and Chemistry Journal*, 30, 2802-2809.

PAPER 2

Peric B., Sierra J., Martí E., Cruañas R., Garau M.A., Arning J., Bottin-Weber U., Stolte S., 2013. (Eco)toxicity and biodegradability of short aliphatic protic ionic liquids. *Journal of Hazardous Materials*, 261, 99-105.

PAPER 3

Peric B., Sierra J., Martí E., Cruañas R., Garau M.A., 2014. A comparative study of the terrestrial ecotoxicity of selected protic and aprotic ionic liquids. *Chemosphere*, 108, 418-425.

PAPER 4

Peric B., Sierra J., Martí E., Cruañas R., Garau M.A., 2014. Quantitative structure-activity relationship (QSAR) prediction of (eco)toxicity of short aliphatic protic ionic liquids. *Ecotoxicology and Environmental Safety* – submitted, manuscript number assigned, under review.

3.1. Paper 1

TERRESTRIAL ECOTOXICITY OF SHORT ALIPHATIC PROTIC IONIC LIQUIDS

Summary

Ionic liquids (ILs) are a fairly new and promising group of compounds with a large variety of possible structures and uses. The new structures of ILs are being constantly synthesized, but the information of their ecotoxicity is often neglected or not studied enough, especially when it comes to terrestrial toxicity, where the available information is scarce. In this article, the first results on terrestrial ecotoxicity of the new family of short aliphatic protic ionic liquids (PILs) are presented. Three representatives of this family were analyzed: 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxydiethanolamine propionate (2-HDEAPr), and 2-hydroxytriethanolamine pentanoate (2-HTEAPe). These three compounds were chosen as they are derived from different amines and organic acid, being the 2-HEAF the most simple of all of the representatives of the new family, the 2-HDEAPr being the "middle" one, and the 2-HTEAPe being the most complex in structure. By applying this criterion of selection, the initial structure-toxic effect relationship was established and thus gave a preview of the ecotoxicity of the new family of PILs. Tests performed within the study included the assessment of the effects of PILs on terrestrial plants (*Allium cepa*, *Lolium perenne* and *Raphanus sativus*), and on soil microbiota involved in carbon and nitrogen mineralization. The results showed that the investigated PILs were not toxic for the three assayed plant species, as all but one of the EC_{50} values were a lot higher than 1000 mg kg^{-1} (dry soil), which is a threshold for terrestrial plants toxicity established by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Only the 2-HTEAPe in case of *R. sativus* had an EC_{50} of 826 mg kg^{-1} (dry soil). They were not toxic for the soil nitrifying microbiota either, as all of the EC_{50} values were several orders of magnitude higher than 100 mg kg^{-1} (dry soil), the threshold for soil microorganisms toxicity according to the GHS. The cumulative respiration curves obtained show an inhibition of the microbiota activity only in case of the highest tested concentration for 2-HDEAPr and

2-HTEAPe (10000 mg kg^{-1} of dry soil), thus indicating a potential biodegradability. So, these compounds are non-toxic for soil microbiota and analyzed plants, and are potentially biodegradable in soils. The toxicity increases with the complexity of PILs molecule (longer aliphatic chains), and the 2-HTEAPe proved to be the most toxic one in all of the performed tests. This finding corroborates the previous literature findings on ILs toxicity.

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 30, No. 12, pp. 2802–2809, 2011

Printed in the USA
DOI: 10.1002/etc.683

TERRESTRIAL ECOTOXICITY OF SHORT ALIPHATIC PROTIC IONIC LIQUIDS

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(*Submitted* 2 *February* 2011; *Returned for Revision* 14 *March* 2011; *Accepted* 22 *August* 2011)

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

Keywords—Protic ionic liquids Terrestrial ecotoxicity Biodegradability

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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Published online 20 September 2011 in Wiley Online Library (wileyonlinelibrary.com).

[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
2-HEAF	*Allium cepa*	100.00AB	112.14B	99.53AB	91.04AB	98.04AB	80.87A
	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
2-HDEAPr	*A. cepa*	100.00B	75.37AB	73.99AB	76.74AB	81.14B	13.28A
	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
2-HTEAPe	*A. cepa*	100.00B	102.63B	108.56B	92.71B	99.40B	0.00A
	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

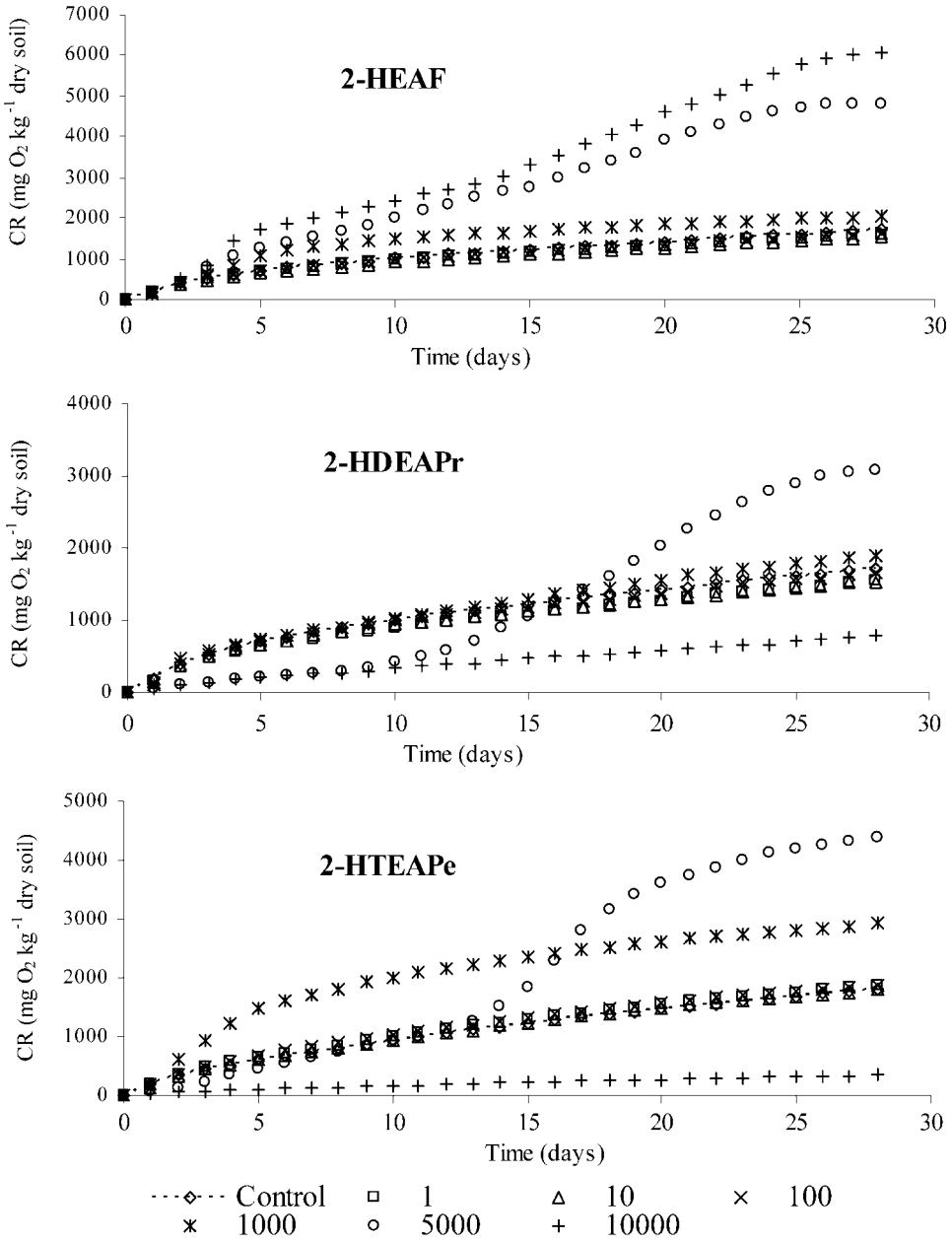

Fig. 1. Cumulative respiration (CR) data expressed as the cumulative oxygen consumed during the 28 d of the respirometric assay for the control and different nominal concentrations (in mg/kg) of the protic ionic liquids treatment groups: 2-hydroxyethanolamine formate (2-HEAF, top panel), 2-hydroxydiethanolamine 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

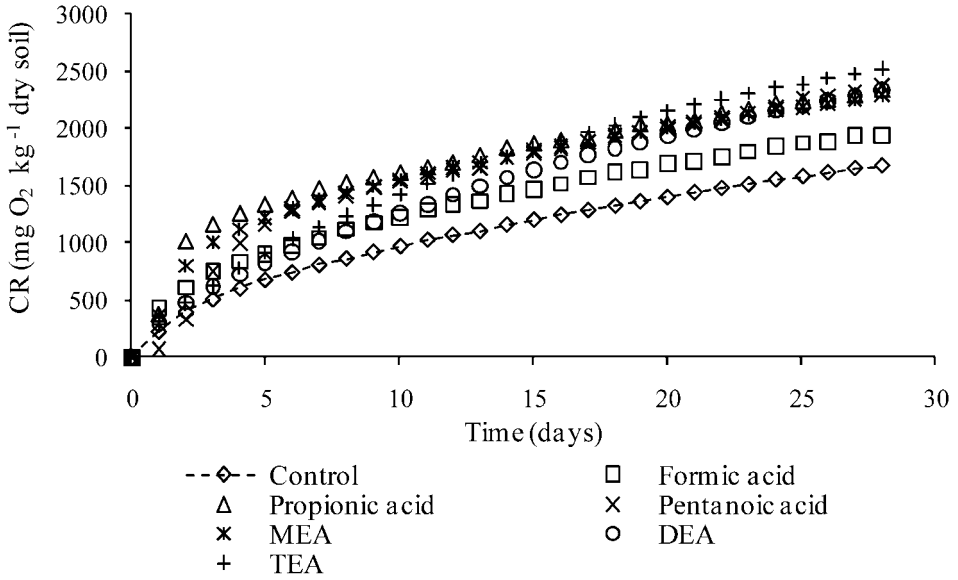

Fig. 2. Cumulative respiration (CR) data expressed as the cumulative oxygen consumed during the 28 d of the respirometric assay for the control and nominal concentrations of formic, propionic, and pentanoic acids and amines monoethanolamine (MEA), diethanolamine (DEA), and triethanolamine (TEA), equivalent 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
2-HEAF	%CR	100.00A	93.21A	89.40A	95.38A	120.07B	280.87C	354.62C
	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
2-HDEAPr	%CR	100.00B	90.22B	89.40B	96.74B	110.60B	179.35C	46.47A
	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
2-HTEAPe	%CR	100.00B	103.61B	99.74B	103.87B	161.27C	242.06D	18.88A
	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.

Acknowledgement—B. Peric thanks the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development (Grant MAEC-AECID II.A). M. Iglesias acknowledges the Fundacion Ibercaja (Programa de Excelencia en Investigacion 2009) for support in developing this research.

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.

3.2. Paper 2

(ECO)TOXICITY AND BIODEGRADABILITY OF SHORT ALIPHATIC PROTIC IONIC LIQUIDS

Summary

Ionic liquids (ILs) are a promising group of compounds with a large variety of possible structures and uses. They are often considered as a potential "green" replacement for traditional volatile organic solvents, due to their negligible vapor pressure and other attractive properties and physicochemical characteristics. But, it is necessary that they exhibit low ecological footprint, and not only to be suitable for a desired application. In the present study, selected representatives of two ILs groups were analyzed: a new family of PILs (Protic Ionic Liquids) and some frequently used AILs (Aprotic Ionic Liquids) namely substituted imidazolium and piridinium chlorides. Aquatic toxicity (test organisms *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Lemna minor*) and biodegradability tests were performed. Additional tests with enzyme (acetylcholinesterase AChE) and leukaemia rat cells (IPC-81) provided more in-depth evaluation of toxicity. In this comparative hazard assessment the PILs showed EC_{50} values >100 mg L^{-1}, the threshold for aquatic ecotoxicity established by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) in all of the tests performed, except in the case of three representatives towards *Lemna minor*. They also show good biodegradability rates. The EC_{50} values for AILs are various orders of magnitude lower than the ones for PILs in most of the aquatic ecotoxicity tests and they have low biodegradability potential. None of the analyzed PILs showed inhibitory potential towards AchE, and they exhibited no o very low cytotoxicity. In contrast, the AILs proved to be inhibitors of AchE and more cytotoxic than the PILs. The toxicity increases with the increase of the number of carbons in the anionic part of the molecule of PILs and the length of the side alkyl chain in the molecule of AILs, thus leading to a higher toxicity of the more complex structures. These findings indicate that the PILs can be considered as environmentally safer alternatives for more toxic AILs, especially if found in the aquatic environment.

Journal of Hazardous Materials 261 (2013) 99–105

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

(Eco)toxicity and biodegradability of selected protic and aprotic ionic liquids

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HIGHLIGHTS

- We analyzed (eco)toxicity of selected protic (PILs) and aprotic (AILs) ionic liquids.
- The PILs proved to have by far lower aquatic toxicity than the AILs.
- Complementary toxicity tests also showed the PILs as less toxic.
- The analyzed PILs are potentially biodegradable in water, unlike the AILs.
- The new family of PILs could be an environmentally safer alternative to AILs.

ARTICLE INFO

Article history:
Received 2 May 2013
Received in revised form 25 June 2013
Accepted 28 June 2013
Available online 6 July 2013

Keywords:
Ionic liquids
Toxicity
Aquatic ecosystems
Biodegradability

ABSTRACT

Ionic liquids (ILs) are a promising group of compounds with a large variety of possible structures and uses. They are considered as a potential "green" replacement for traditional volatile organic solvents, but their impact on the environment is often neglected or not studied enough. In the present study, selected representatives of two ILs groups were analyzed: a new family of protic ILs (derived from aliphatic amines and organic acids) and some frequently used aprotic ILs (substituted imidazolium and piridinium chlorides). The aquatic toxicity (test organisms *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Lemna minor*) and biodegradability tests were carried out. The additional tests with enzyme (acetylcholinesterase) and leukemia rat cells (IPC-81) provided more in-depth evaluation of toxicity. In our comparative hazard assessment protic ILs have EC_{50} values >100 mg L^{-1} in all of the tests performed, except in the case of three representatives toward *Lemna minor*. They also show good biodegradability rates. The EC_{50} values for aprotic ILs are various orders of magnitude lower than the ones for protic ILs in most of the tests and they show a lower biodegradability potential. These findings indicate that protic ILs can be considered as environmentally safer alternatives for more toxic ILs and organic solvents.

1. Introduction

In recent years, ionic liquids (ILs) have generated attention for their potential use as environmentally friendly chemicals and as a potential replacement for traditional volatile organic solvents. They are defined as salts consisting only of ions with melting point below 100 °C [1]. The interest in using ILs as solvents in chemical processes is that these compounds are thermally stable, have a high polarity, remain unchanged when mixed with different organic compounds and catalysts, have a very small vapor pressure and are liquid at a wide temperature range [2]. Thanks to their properties, ILs have been utilized in a variety of applications, such as solvents in catalysis [3], extractions [4], polymerization [5], nanotechnology [6], composite molecule dissolution and preparation [7] and renewable resource utilization [8].

Based on their chemical behavior, ILs can be divided in two groups: aprotic (AILs) and protic (PILs) ionic liquids. The structure of AILs is mainly based on bulky organic cations such as imidazolium or pyridinium with long alkyl chain substituents and large variety of anions such as Cl^-, Br^-, BF_4^-, PF_6^-, $N(CN)_2^-$, etc. The aprotic group can be considered as "classic" ILs, whilst the representatives of the protic group are in recent development. The here selected PILs belong to a newly designed family of short aliphatic PILs, with a different structure than the "classic" ILs, that includes compounds

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http://dx.doi.org/10.1016/j.jhazmat.2013.06.070

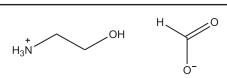
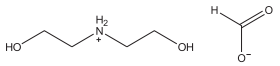
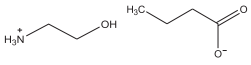
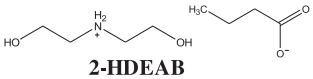
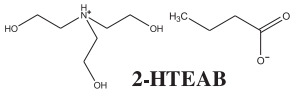
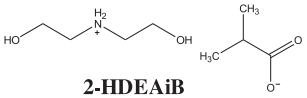
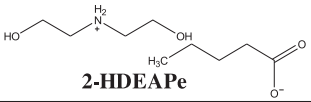
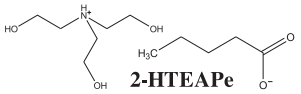
	Monoethanolamine	Diethanolamine	Triethanolamine
Formic acid	 2-HEAF	 2-HDEAF	
Acetic acid		 2-HDEAA	
Propionic acid		 2-HDEAPr	
Butiric acid	 2-HEAB	 2-HDEAB	 2-HTEAB
Isobutiric acid		 2-HDEAiB	
Pentanoic acid		 2-HDEAPe	 2-HTEAPe

Fig. 1. Structures and abbreviations of the analyzed PILs.

based on substituted amines (monoethanolamine, diethanolamine or triethanolamine) as cations and organic acids with different numbers of carbon atoms (formic, propionic, butanoic, isobutanoic and pentanoic acid) as anions. Both cationic and anionic part of the molecule are organic and have a relatively low molecular weight [9]. The potential environmental impact of these new PILs is expected to be smaller than the impact of AILs, due to their simpler structure.

There are numerous possible applications for this new family of PILs. To name but a few: the 2-hydroxyethanolamine formate (2-HEAF) has been found to be able to dissolve many inorganic salts, hydroxylated compounds and some insoluble polymers such as polyaniline and polypyrrole [10]. Also, 2-HEAF together with some other representatives of this family (2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), etc.) have been analyzed for their potential catalytic activity in the aldol condensation reactions [9], for the heterogeneous catalytic hydrogenation of cyclohexene and acetone catalyzed by Pt/Al_2O_3 with methanol, ethanol and propan-1-ol [11]. Other PILs, also derived from 2-hydroxyethylammonium and organic acids have been studied in order to develop a new process for absorption of CO_2 [12]. Also, the catalytic activity in the aldol condensation processes of the PILs derived from mono, di and triethanolamine and penthanoic acid has been studied, with successful results [13].

However, apart from good technical performance, current European Union environmental legislation including REACH (Regulation concerning registration, evaluation, authorization and restriction of chemicals) [14] makes insistent demands for safety of chemicals and, among others, ecotoxicological as well as biodegradation data are required. As the group of PILs is fairly new in their use and still in development, it is very important to assess their hazard potential before they can be safely used.

The aim of the present study is therefore an initial hazard assessment of the new PILs and a comparative analysis with classical AILs. For our investigations we applied tests to determine aquatic toxicity (with bacteria *Vibrio fischeri*, green algae *Pseudokirchneriella subcapitata* and higher aquatic plant *Lemna minor*) and the ready biodegradability in water. We used additional test systems with an enzyme (acetylcholinesterase inhibition) and isolated leukemia IPC-81 cells from rats (cytotoxicity), which have been proven to be useful for determining the acute toxicological hazards of ILs [15,16].

2. Experimental

2.1. Ionic liquids

The PILs analyzed in the present study are: 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxyethanolamine butanoate (2-HEAB), 2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine propionate (2-HDEAPr), 2-hydroxydiethanolamine butanoate (2-HDEAB), 2-hydroxydiethanolamine isobutanoate (2-HDEAiB), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), 2-hydroxytriethanolamine butanoat (2-HTEAB) and 2-hydroxytriethanolamine pentanoate (2-HTEAPe) and some of the most frequently used AILs [17–19]: 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-methyl-3-octylimidazolium chloride ([OMIM]Cl) and N-butylpyridinium chloride ([BPy]Cl). The structures of analyzed PILs are shown in the Fig. 1 and the AILs in the Fig. 2.

The PILs were obtained through synthesis starting from the amine compounds (monoethanolamine, diethanolamine and triethanolamine) and the corresponding acids (formic, acetic, n-propionic, n-butyric, isobutyric and n-pentanoic acid) as described in detail by Iglesias et al. [20]. To confirm the products

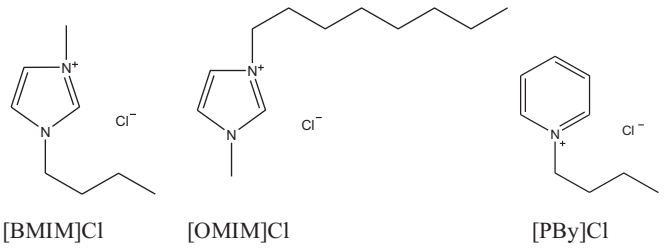


Fig. 2. Structures and abbreviations of the analyzed AILs.

structures ^{1}H NMR were performed [21,22]. The obtained NMR spectrums confirmed the synthesized structure.

The AILs were obtained commercially from Sigma–Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

2.2. Inhibition of bacterial luminescence (Microtox®) test

The toxicity of ionic liquids was determined by a bioassay according to the ISO 11348-3 procedure for Microtox® Basic test [23] based on the decrease of luminescence of the marine bacteria *V. fischeri* (strain NRRL B-11177) after exposure to a toxic substance. Isotonic solutions of the ionic liquids were prepared using 2% NaCl as solvent. The prepared concentrations of ILs were 0.5% or 1%. Starting from these concentrations, four serial dilutions in 1:2 ratio were prepared in triplicate, along with the control samples that contained no ILs. The reduction of light emission was measured at least two times after 15 min of contact between bacteria previously reconstituted with 2% NaCl and serial dilutions of ILs, at 15 °C. Bacterial luminescence is directly linked with cellular respiration, so a decrease in luminescence corresponds to bacterial toxicity. The equipment used was the Microtox® M500 Analyzer (SDIX, Newark, DE, USA, 2008).

2.3. Algal growth inhibition test

The conventional algal chronic toxicity assay was done according to the procedures set out in Organization for Economic Cooperation and Development (OECD) guidelines 201 [24]. For this assay, the unicellular limnic green algae *P. subcapitata* (strain 61.81, SAG, Culture Collection of Algae, Universität Göttingen, Germany) was used. The stock culture was grown under conditions recommended in OECD guideline 201 and diluted to a cell density of 5×10^4 cells mL^{-1}. The nominal concentrations of ILs used in the test were: 0.001, 0.01, 0.1, 1, 10, 100, 1000 and 5000 mg L^{-1}. The test was done with quadruplicates of each concentration of PILs and six control samples, two of which were stored in the refrigerator (4 °C) to prevent growth and have a referral from the initial concentration of cells and the other four were incubated under the same conditions as the treated samples. The incubation was carried out in a chamber for 72 h at 22 °C temperature and white light with intensity of 1000 lux. During the three days, regular agitation was done to promote gas exchange but preventing cell lysis. The cell count was done using the Coulter cell counter (Beckman, Nürnberg, Germany).

2.4. Growth inhibition test with duckweed

The growth inhibition assay with *Lemna minor* (duckweed) was performed as described in detail by Drost et al. [25]. The *L. minor* plants (Department of Agricultural Science, The Royal Veterinary and Agricultural University, Taastrup, Denmark) were grown in open Erlenmeyer flasks in sterilized Steinberg medium, in an illuminated climate chamber with a constant temperature of 25 ± 2 °C. The assays were conducted in 6-well titer plates. The nominal concentrations of ILs used in the test were between 0.1 and 5000 mg L^{-1}. All ILs were tested at least twice, with a minimum of 6 controls, containing only pure Steinberg medium, for each test. The test started with 18 fronds, 3 in each well. The resulting growth inhibition was determined after 7 days of incubation, basing on different end points like the number of fronds or frond area calculated in relation to the controls. The growth rate was determined on the basis of the counted fronds; for the detection of the frond area (mm^2) a Scanalyzer from Lemnatec GmbH (Würselen, Germany) was used.

2.5. Acetylcholinesterase inhibition assay

The assay was performed according to Ellman et al. [26] as modified for use with microtiter plates [27]. The inhibition of acetylcholinesterase (AChE) was measured using a colorimetric assay based on the reduction of the dye 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) by the enzymatically formed thiocholine moiety from the AChE substrate acetylthiocholine iodide. A dilution series (1:1) of the ILs in phosphate buffer (0.02 M, pH 8.0) containing maximum 1% methanol was prepared directly in the wells of a 96-well microtiter plate. The start concentration was 5000 mg L^{-1}. DTNB (2 mM, 0.185 mg mL^{-1} $NaHCO_3$ in phosphate buffer pH 8.0) and the enzyme (0.2 U mL^{-1}, 0.25 mg mL^{-1} bovine serum albumin in phosphate buffer pH 8.0) were added to each well. The reaction was started by the addition of acetylthiocholine iodide (2 mM in phosphate buffer). The final test concentrations were 0.5 mM of DTNB and acetylthiocholine iodide and 0.05 U mL^{-1} acetylcholinesterase, respectively. Each plate contained blanks (no enzyme) and controls (no toxicant). Enzyme kinetics were measured at 405 nm at 30 s intervals in a microplate-reader (MRX Dynatech) for 5 min with on at least two different 96-well plates. The enzyme activity was expressed as the slope of optical density (in OD min^{-1}) from a linear regression.

2.6. Cell viability assay with IPC-81 cells

The cytotoxicity of ILs was determined using the WST-1 reagent. The assay was described in detail in Ranke et al. [16]. Briefly, promyelocytic leukemia cells from the rat IPC-81 cell line were incubated for 4 h in 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent. Each plate contained blanks (no cells) and controls (no toxicant). The cell viability assays were carried out in a 1:1 dilution series, starting from 5000 mg L^{-1} of aqueous solution of ILs. Each dose-response curve was recorded for at least 9 parallel dilution series on three different 96-well plates.

2.7. Biodegradability in water

The determination of potential biodegradability of ILs in water was done according to OECD guideline 301 [28] in manometric respirometers (Velp® Scientifica, Usmate, Italy) which allow the determination of the sample oxygen consumption. The ILs were added at concentration of 100 mg L^{-1} in 250 mL of the mineral nutrient solution and inoculated with a mixture of microorganisms from the sludge of an urban biological treatment plant. All the experiments were done at least in triplicate, including the control sample where no ILs were added. During 28 days, at a constant temperature of 22 ± 2 °C, the amount of oxygen consumed (Biochemical oxygen demand – BOD) was determined. The percentage of biodegradation was obtained by dividing BOD values with ThOD (Theoretical Oxygen Demand) and multiplying by 100 for each ionic liquid. Theoretical oxygen demand is the total amount of oxygen required to oxidize a chemical completely, it is a calculated value, expressed as mg oxygen required per mg of the test compound. After 0, 5 and 28 days of the incubation during the biodegradation test, the samples containing PILs were analyzed by ion chromatography in a Dionex DX300 (Sunnyvale, CA, USA), with a Hamilton PRP-X200 cationic column (Reno, NV, USA, I.D. 4.1 mm, 250 mm long) and electrical conductivity detector without ion suppression. The eluent used was 2 mM nitric acid at 1 mL min^{-1}. In the case of the AILs the samples were analyzed via HPLC [29]. The chromatography analysis was done in order to determine possible decreases in cationic moiety concentration of ILs during the course of the test, as a consequence of eventual primary degradation process.

OECD guideline 301 defines primary biodegradation as the alteration of the chemical structure of a substance, brought about by biological action, resulting in the loss of a specific property of that substance. The percentage of primary degradation of each sample was calculated referring to the initial concentration.

2.8. Statistical analysis

Dose-response relationship was assessed, and the effective concentrations 50 (EC_{50}) with their 95% confidence intervals were calculated for each compound from suitable regression models (Gompertz, hormetic, or logistic) using Statistica 6.0 (Stat Soft Inc., Tulsa, OK, USA) and the drfit package (version 0.05-92) for the R language and environment for statistical computing (R Development Core Team, 2005). The choice of the model was based on the best fit to the data. In the case of Microtox® test the log-lineal model was used to obtain the dose-response curve and the corresponding EC_{50} value (MicrotoxOmni™ Software for Windows, Azur Environmental, Carlsbad, CA, USA, 1999). The parameter EC_{50} was selected because it is used in the environmental regulation and most of the literature. Spearman's coefficient of correlation was calculated using PASW Statistics 17.0.1 program (IBM Corporation, NY, USA).

3. Results and discussion

3.1. Toxicity tests

3.1.1. Aquatic ecotoxicity tests

For all ILs tested dose-response curves were obtained and EC_{50} values were calculated in mg L^{-1} and mmol L^{-1} (Table 1).

In the case of the test with marine bacteria *V. fischeri* the EC_{50} values for the analyzed PILs were between 1.69 and 15.13 mmol L^{-1} (350–2239 mg L^{-1}), with 2-HDEAPe being the most toxic and 2-HEAB the least toxic compound. The two compounds with the lowest EC_{50}s (2-HDEAPe and 2-HTEAPe) have the same pentanoic anionic part, which is indicating the influence of the anion on toxicity. The EC_{50}s corresponding to AILs were up to several orders of magnitude lower, being between 0.002 and 1.72 mmol L^{-1} (0.5–295 mg L^{-1}), with [OMIM]Cl being the most toxic one. The obtained EC_{50} values for the PILs in test of inhibition of the growth of *P. subcapitata* range between 0.70 and 22.93 mmol L^{-1} (104–2453 mg L^{-1}). The highest as well as the lowest toxicity was found for the 2-HEA cation showing again the strong influence of the anionic moiety formiate vs. butyrate. In the case of AILs, the obtained EC_{50} are several orders of magnitude lower, between 0.005 and 0.17 mmol L^{-1} (1.21–30 mg L^{-1}), with [OMIM]Cl once again being the most toxic one. As far as the growth inhibition test of duckweed (*L. minor*) is concerned, three of the analyzed PILs showed some toxic effect on duckweed. The 2-HDEAB, 2-HEAB and 2-HDEAiB have EC_{50} values of 0.17 (33 mg L^{-1}), 0.40 (59 mg L^{-1}), and 0.41 (79 mg L^{-1}) mmol L^{-1} respectively. The AILs showed to be more toxic toward *L. minor* than the most of the PILs, with [OMIM]Cl having the EC_{50} of 0.005 mmol L^{-1} (1.15 mg L^{-1}), the lowest of all analyzed ILs.

L. minor has proved to be more sensitive to the presence of PILs than *P. subcapitata* or *V. fischeri*. In the case of algae and bacteria the cell membrane is a target site and entry route for the contaminants, so the increased lipid solubility can be related to the higher toxicity of a lipophilic contaminant [30] as in the case of the investigated AILs. The nutrient uptake from soluble phase in the case of *L. minor* is via its fronds (mainly) and roots [31], and being an aquatic plant there is an increased exposure area during a prolonged period of time that can also lead to higher sensitivity. The analyzed PILs tend to be more soluble and more hydrophilic than the analyzed AILs. Other authors have also proven that duckweed was more sensitive

Table 1
Results of dose-response curves in mg L^{-1} and mmol L^{-1} for the aquatic ecotoxicity tests.

Ionic liquid	MW (mg mol^{-1})	*Vibrio fischeri*				*Pseudokirchneriella subcapitata*				*Lemina minor*			
		EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval	EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval	EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval
2-HEAF	107	700	225–920	6.54	2.10–8.60	2453	1584–3801	22.93	14.80–35.52	288	234–380	2.70	2.19–3.55
2-HEAB	148	2239	1400–3500	15.13	9.46–23.65	104	23–482	0.70	0.16–3.26	59	44–81	0.40	0.29–0.55
2-HDEAF	151	800	455–1950	5.30	3.01–12.91	976	615–1548	6.46	4.07–10.25	525	398–631	3.48	2.64–4.18
2-HDEAA	165	1750	1330–2600	10.61	8.06–15.76	870	630–1201	5.27	3.82–7.28	631	550–708	3.82	3.33–4.29
2-HDEAPr	179	650	250–1650	3.63	1.40–9.22	2569	1478–4365	14.35	8.26–24.38	209	229–363	1.17	1.28–2.03
2-HDEAB	193	800	300–1100	4.15	1.55–5.70	294	151–574	1.52	0.78–2.97	33	13–60	0.17	0.07–0.31
2-HDEAiB	193	850	450–1540	4.40	2.33–7.98	1275	709–2290	6.61	3.67–11.86	79	60–68	0.41	0.31–0.35
2-HDEAPe	207	350	75–525	1.69	0.36–2.54	574	129–1965	2.77	0.62–9.49	155	148–158	0.75	0.71–0.77
2-HTEAB	237	501	195–1500	2.11	0.82–6.32	1287	630–2690	5.43	2.66–11.35	178	145–219	0.75	0.61–0.92
2-HTEAPe	251	461	257–828	1.84	1.02–3.30	757	138–1952	3.02	0.55–7.78	525	490–562	2.09	1.95–2.24
[BMIM]Cl	175	287	178–559	1.64	1.02–3.19	14	7.00–27	0.08	0.04–0.15	48.98	42–59	0.28	0.24–0.34
[OMIM]Cl	231	0.50	0.29–0.88	0.002	0.001–0.004	1.21	1.10–1.35	0.005	0.004–0.006	1.15	0.95–1.35	0.005	0.004–0.006
[BPy]Cl	172	295	255–336	1.72	1.48–1.95	30	22–47	0.17	0.13–0.27	51.29	44–60	0.30	0.25–0.36

$p < 0.05$; MW, molecular weight.

than algae and bacteria [32,33] and that the compounds with a higher lipophilicity affect more the algae than the duckweed [34].

According to the Globally Harmonized System of Classification and Labelling of Chemicals [35,36], most of the PILs can be classified as non-toxic for aquatic organisms, because their EC_{50}s are higher than 100 mg L^{-1}. Only 2-HDEAB, 2-HEAB, 2-HDEAiB fit into the category of harmful for the aquatic environment (category Acute 3) with their EC_{50} between 10 and 100 mg L^{-1} in the case of *L. minor*. All of the AILs can be placed into some of the acute toxicity categories according to their EC_{50} values. In the case of the Microtox test [OMIM]Cl can be classified as very toxic for aquatic organisms, belonging to the category Acute 1 (EC_{50} < 1 mg L^{-1}). The rest of the analyzed AILs cannot be placed in none of the categories of substances considered as dangerous for the aquatic environment, because their EC_{50} are all higher than 100 mg L^{-1}. In the test of inhibition of the growth of *P. subcapitata* [OMIM]Cl can be classified as toxic (category Acute 2, EC_{50} between 1 and 10 mg L^{-1}) and [BMIM]Cl and [BPy]Cl as harmful for aquatic organisms (category Acute 3). According to the results of the inhibition of growth of *L. minor*, [OMIM]Cl can be placed into the category of very toxic (category Acute 1) and [BMIM]Cl and [BPy]Cl into harmful for the aquatic environment (category Acute 3).

The higher toxicity of [OMIM]Cl compared to the other two representatives of AILs is supported by other authors' findings, where the increase of toxicity is provoked by the elongation of the alkyl side chain [37,38]. The higher toxicity of the AILs compared with the PILs analyzed in this study is also related to the structural differences, the AILs have a voluminous heterocyclic cation with alkyl side chains in their structure, whilst the PILs have simpler structure with smaller, highly hydrophilic molecule. The differences in toxicity among the PILs can also be seen in case of the more complex structure, when the anionic part has 4 or 5 carbons (butyric, isobutyric and pentanoic acid). The ionic liquids with short alkyl chains should be always be preferred if technically possible [39] and the introduction of the hydrophilic functional groups in the side chain reduces the toxicity of the ionic liquid [40]. These facts also lead to a decrease of toxicity of the PILs compared to the AILs.

3.1.2. Cytotoxicity and inhibition of acetylcholinesterase

The PILs and AILs were also assayed with respect to their inhibitory activity toward enzyme acetylcholinesterase (AChE) and their cellular toxicity toward IPC-81 rat promyelocytic leukemia cell line. These test systems yielded reproducible measurements of the acute toxicity of ILs, and many data are available for comparison [41]. The obtained EC_{50} values are shown in Table 2.

EC_{50} data for [BMIM]Cl, [OMIM]Cl and [BPy]Cl are taken from The UFT/Merck Ionic Liquids Biological Effects Database [41]

In the test of inhibition of acetylcholinesterase, the PILs showed a very low inhibitory potential with EC_{50}s ranging from 1.27 to 59.02 mmol L^{-1}. The AILs show a moderate inhibition potential [41], with EC_{50} values between 0.04 and 0.08 mmol L^{-1}. The EC_{50} of AILs were once again several orders of magnitude lower than the ones found for PILs. The [OMIM]Cl is the most toxic one of the three AILs.

Regarding cytotoxicity toward IPC-81, the EC_{50} values for the analyzed PILs ranged between 1.76 and 30.95 mmol L^{-1}, while the AILs had EC_{50} between 0.1 and 5 mmol L^{-1}. One of the PILs, 2-hydroxydiethanolamine formate, showed no inhibitory activity. The AILs proved to be more toxic than the PILs, with [OMIM]Cl being the most toxic one, as in the previously described aquatic toxicity tests. Their EC_{50} indicate moderate cytotoxicity [41,42].

3.2. Biodegradability

The biodegradation results are shown in Table 3. There are two types of data: the percentage of biodegradation after 5, 14 and 28 days according to OECD 301 manometric respirometry test procedure and the primary biodegradation of the ILs molecule determinated by means of ionic chromatography (IC).

According to OECD 301 guidelines, the compounds which reach a biodegradation level higher than 60% of ThOD during a 10-day window within 28 days of testing can be considered as "readily biodegradable". PILs show much better biodegradation rates than AIL in these tests. After 28 days of the test, the biodegradation rates of most of the PILs are higher than 60%. Three of the tested PILs do not fit into "readily biodegradable" criteria: 2-HDEAF, 2-HTEAB and 2-HTEAPe. The results of 59 and 57% for the 2-HTEAB and 2-HTEAPe respectively are very close to the 60% limit, so they can be considered as potentially readily biodegradable. But the results of 13% for the 2-HDEAF indicates that this PIL has a low biodegradation rate. The levels of biodegradation are usually dependent on compound's stability and toxicity. The analyzed AILs have proven to be resistant to water biodegradation, with the % of biodegradability being around 1%.

According to the chromatographic quantification results, after 5 days of incubation in water during the biodegradability test, the cationic moiety of the 2-HEAF, 2-HEAB, 2-HDEAF and 2-HDEAPr cannot be detected in the water samples, whereas for the rest of the PILs the corresponding cationic moiety remains at available concentrations between 30 (for 2-HDEAiB) and 84% (for 2-HDEAPe). After 28 days of incubation, only the cationic moiety of 2-HDEAPe was detectable in the extracts, which indicates that all but one of the PILs were completely degraded after 28 days. The 2-HDEAPe was not completely metabolized by the activated sludge microbial community, remaining at available concentration of 38%. The primary degradation of 2-HEAF, 2-HEAB, 2-HDEAF and 2-HDEAPr was complete within 5 days, while the other PILs molecules (except in the case of 2-HDEAPe) are completely mineralized during the period between the fifth and the twenty-eighth day. In a study performed by Stolte et al. [29], imidazolium and pyridinium ILs with short alkyl side chains ([BMIM]Cl and [BPy]Cl) were highly resistant to mineralization during the incubation period. A complete primary biodegradation could be detected for the [OMIM]Cl after 28 days.

3.3. Correlation analysis of the toxicity and biodegradability data

The Spearman's correlation coefficients were determined in order to get more information on potential toxic modes of action of the analyzed PILs. The correlation between the structure and the toxic effects and potential biodegradability of the PILs was investigated. For this analysis the molecular weight of PILs, the number of C-atoms in the cationic (amine) and anionic (acid) part of the molecule and EC_{50} values were used. All statistically relevant results of the correlation analysis are presented in the Table 4.

In the case of the Microtox test, there is an increase of toxicity with the increase of the molecular weight and elongation of alkyl chains of the anionic part of the molecule, and with the complexity of the cationic part. These three findings indicate that the complexity of PILs structure has a positive influence on the potential toxicity in the case of the Microtox test and also in the test of the inhibition of acetylcholinestherase, where the same correlations were found. A positive influence of the elongation of the carboxyl chain in the anionic part of the PILs molecule on their toxicity has also been found in the case of the tests performed on green algae, duckweed and IPC-81 cells. In these three tests, the acid part of the PILs molecule seems to have more influence on the toxicity then the amine part. These findings confirmed other authors conclusions,

Table 2
Results of dose-response curves in mg L^{-1} and mmol L^{-1}.

Ionic liquid	AChE				IPC-81			
	EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval	EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval
2-HEAF	3467	3090–3890	32.41	29.88–36.36	3311	2089–7943	30.95	19.53–74.24
2-HEAB	7079	6166–8128	47.83	41.66–54.92	269	232–295	1.82	1.57–1.99
2-HDEAF	8912	7413–10965	59.02	49.09–72.61	>5000	nd	>5000	nd
2-HDEAA	2089	1950–2291	12.66	11.82–13.88	1778	1349–2344	10.77	8.18–14.21
2-HDEAPr	2399	2239–2570	13.40	12.51–14.36	1047	912–1202	5.85	5.10–6.72
2-HDEAB	2344	2188–2570	12.15	11.34–13.32	339	302–372	1.76	1.56–1.93
2-HDEAiB	1995	1820–2138	10.34	9.43–11.08	575	468–708	2.98	2.42–3.67
2-HDEAPe	1862	1738–1995	9.00	8.40–9.64	1096	977–1259	5.30	4.72–6.08
2-HTEAB	302	288–324	1.27	1.22–1.37	589	468–741	2.48	1.97–3.13
2-HTEAPe	427	398–447	1.70	1.59–1.78	851	741–977	3.39	2.95–3.89
[BMIM]Cl	14.40	13.10–15.70	0.08	0.07–0.09	626	530–751	3.58	3.03–4.29
[OMIM]Cl	9.10	8.47–9.27	0.04	0.03–0.04	24	21–27	0.10	0.09–0.12
[BPy]Cl	8.59	8.30–8.89	0.05	0.04–0.05	862	781–960	5.01	4.54–5.58

$p < 0.05$.

Table 3
Percentage of biodegradation of the ILs after 5, 14 and 28 days and the percentage of primary biodegradation after 5 and 28 days analyzed by ionic chromatography and HPLC (results for AILs are taken from Stolte at al. [29]).

Ionic liquid	Ready biodegradability (% biodegradation)			Chromatography -IC and HPLC (% primary biodegradation)	
	5 days	14 days	28 days	5 days	28 days
2-HEAF	11	61	86	100	100
2-HEAB	64	69	95	100	100
2-HDEAF	4	4	13	100	100
2-HDEAA	21	46	69	65	100
2-HDEAPr	45	62	68	100	100
2-HDEAB	47	66	78	21	100
2-HDEAiB	46	71	79	70	100
2-HDEAPe	38	57	69	16	62
2-HTEAB	28	32	59	34	100
2-HTEAPe	23	38	57	33	100
[BMIM]Cl	0.23	0.66	1.17	0	0
[OMIM]Cl	0.29	0.35	1.33	31	100
[BPy]Cl	0.31	0.46	0.61	0	0

Table 4
Spearman's correlation coefficient for different correlation pairs.

	EC_{50} (mmol L^{-1})					% biodegradation	
	Microtox	Green algae	*L. minor*	AChE	IPC-81	5 days	28 days
MW	−0.863**	ns	ns	−0.924**	ns	ns	ns
Amine	−0.716**	ns	ns	−0.771**	ns	ns	−0.773**
Acid	−0.661*	−0.617*	−0.574*	−0.736**	−0.661*	0.567*	ns

ns, not significant; MW, molecular weight.
* Level of significance $p < 0.05$.
** Level of significance $p < 0.01$.

i.e. that the toxicity strongly depends on the side chain elongation in the case of AILs [33,43,44].

The correlation analysis for the biodegradation results shows that the complexity of an anionic part of the molecule (acid) is directly correlated with BOD_5, whilst there is an inverse correlation between the increase of the complexity of the cationic part of the molecule (amine) and BOD_{28} values. This means that during the first 5 days of biodegradation in water, it is the increase of the number of carbons in the anionic part of the molecule that causes higher biodegradation rate, but in the later stages of the biodegradation process the complexity of the cationic part of the molecule causes lower rates of biodegradability, which is in concordance with a previously established inverse correlation in the case of Microtox, meaning that the PILs are more toxic to microbial communities if they have a more complex structure.

The correlation analysis performed by other authors [33] showed that there is correlation between lipophilicity and toxicity for bacteria and algae in the case of AILs. In addition, a correlation between an increasing chain length of the side chains connected to the cationic head groups and an enhanced inhibitory potential on the enzyme acetylcholinestherase was found [39]. In the case of the rat leukemia cell line, the same group found a correlation between a HPLC derived lipophilicity parameter and the observed cytotoxicity [42].

4. Conclusions

The PILs analyzed in the present study have shown no toxicity in the performed aquatic toxicity tests, with EC_{50} values above 100 mg L^{-1}, with the exception of three PILs in the case of duckweed growth inhibition test. *L. minor* was found to be the most sensitive species in the case of the aquatic toxicity tests for the PILs, but not for the AILs, probably due to the hydrophilicity of the PILs. The [OMIM]Cl proved to be the most toxic in all of the aquatic

toxicity tests, and the other two representatives of AILs proved to be harmful for the aquatic organisms. In the case of cytotoxicity and inhibition of acetylcholinesterase tests, none of the analyzed ionic liquids showed very high toxicity. There is a clear difference in EC_{50} values between the two groups of ILs, with the EC_{50} for AILs being various orders of magnitude lower than the EC_{50} for PILs in most of the tests performed. From the results we can deduce that, in general, the analyzed AILs with more complex molecular structure have a greater tendency to cause toxic effect in the aquatic organisms tested than the PILs with the smaller molecule and simpler structure. Based on the biodegradation test results and ulterior chromatographic quantification, the analyzed PILs are potentially biodegradable in water, whilst the AILs have shown practically no biodegradation. The complexity of the ILs molecules has a positive impact on the toxicity. The initial comparative hazard assessment showed that PILs are, in terms of toxicity as well as in terms of biodegradability, much favorable than the here tested AILs. This fact, together with their low production cost, simple synthesis and possible applications, suggests that they have a potential "greener" profile among other ionic liquids and a good prospect for a wider use.

Acknowledgments

B. Peric would like to thank the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development (Grant MAEC-AECID). The authors would like to thank Màrius Mumbrú for his technical help with the ionic chromatography and Andrea Böschen for her advices and positive spirit during the Acetylcholinesterase inhibition assay.

References

[1] R.D. Rogers, K.R. Seddon, Ionic Liquids as Green Solvents: Progress and Prospects, ACS Symposium Series, American Chemical Society, Washington D.C, 2003, 856.
[2] J.O. Valderrama, R.E. Rojas, Critical properties of ionic liquids, Ind. Eng. Chem. Res. 48 (2009) 6890–6900, Revisited.
[3] Z.C. Zhang, Catalysis in ionic liquids, Adv. Catal. 49 (2006) 153–237.
[4] M.L. Dietz, Ionic liquids as extraction solvents: where do we stand? Sep. Sci. Technol. 41 (2006) 2047–2063.
[5] P. Kubisa, Application of ionic liquids as solvents for polymerization processes, Prog. Polym. Sci. 29 (2004) 3–12.
[6] M. Antonietti, D. Kuang, B. Smarsly, Y. Zhou, Ionic liquids for the convenient synthesis of functional nanoparticles and other inorganic nanostructures, Angew. Chem. Int. Ed. 43 (2004) 4988–4992.
[7] M.B. Turner, S.K. Spear, J.D. Holbrey, R.D. Rogers, Production of bioactive cellulose films reconstituted from ionic liquids, Biomacromolecules 5 (2004) 1379–1384.
[8] S. Zhu, Y. Wu, Q. Chen, Z. Yu, C. Wang, S. Jin, Y. Ding, G. Wu, Dissolution of cellulose with ionic liquids and its application: a mini-review, Green Chem. 8 (2006) 325–327.
[9] I. Cota, R. Gonzalez-Olmos, M. Iglesias, F. Medina, New short aliphatic chain ionic liquids: synthesis, physical properties, and catalytic activity in aldol condensations, J. Phys. Chem. B 111 (2007) 12468–12477.
[10] N. Bicak, A new ionic liquid: 2-hydroxy ethylammonium formate, J. Mol. Liq. 116 (2005) 15–18.
[11] M. Khodadadi-Moghaddam, A. Habibi-Yangjeh, M.R. Gholami, Kinetic study of heterogeneous catalytic hydrogenation of cyclohexene to cyclohexane in ionic liquid–alcohols mixtures, Appl. Catal. A 341 (2008) 58–64.
[12] X. Yuan, S. Zhang, J. Liu, X. Lu, Solubilities of CO_2 in hydroxyl ammonium ionic liquids at elevated pressures, Fluid Phase Equilib. 257 (2007) 195–200.
[13] M. Khodadadi-Moghaddam, A. Habibi-Yangjeh, M.R. Gholami, 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 (2009) 11–16.
[14] European Union, 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 (2006) 1–856.
[15] J. Arning, S. Stolte, A. Boeschen, F. Stock, W.R. Pitner, U. Welz-Biermann, B. Jastorff, J. Ranke, Qualitative and quantitative structure activity relationships for the inhibitory effects of cationic head groups, functionalised side chains and anions of ionic liquids on acetylcholinesterase, Green Chem. 10 (2008) 47–58.
[16] J. Ranke, K. Mölter, F. Stock, U. Bottin-Weber, J. Poczobutt, J. Hoffmann, B. Ondruschka, J. Filser, B. Jastorff, Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays, Ecotoxicol. Environ. Saf. 58 (2004) 396–404.
[17] National Toxicology Program (NTP) and National Institute of Environmental Health Sciences (NIEHS), Review of Toxicological Literature for Ionic Liquids, prepared by Integrated Laboratory Systems Inc., Research Triangle Park, NC, 2004.
[18] A. Mohammad, D. Inamuddin (Eds.), Green Solvents II. Properties and Applications of Ionic Liquids, 2012. Springer.
[19] P. Domínguez de María (Ed.), Ionic Liquids in Biotransformations and Organocatalysis: Solvents and Beyond, John Wiley & Sons, Inc., 2012.
[20] M. Iglesias, R. Gonzalez-Olmos, I. Cota, F. Medina, Brønsted ionic liquids: study of physico-chemical properties and catalytic activity in aldol condensations, Chem. Eng. J. 16 (2010) 2802–2808.
[21] V.H. Álvarez, N. Dosil, R. Gonzalez-Cabaleiro, S. Mattedi, M. Martin-Pastor, M. Iglesias, J.M. Navaza, Brønsted ionic liquids for sustainable processes: synthesis and physical properties, J. Chem. Eng. Data 55 (2010) 625–632.
[22] B. Peric, E. Martí, J. Sierra, R. Cruañas, M. Iglesias, M.A. Garau, Terrestrial ecotoxicity of short aliphatic ionic liquids, Environ. Toxicol. Chem. 30 (2011) 2802–2809.
[23] ISO guideline 11348-1, Water quality. Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test), Part 1: Method using freshly prepared bacteria, Geneva, 2007.
[24] OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems Test No. 201: Freshwater Alga and *Cyanobacteria*, Growth Inhibition Test, Paris, France, 2011.
[25] W. Drost, M. Matzke, T. Backhaus, Heavy metal toxicity to *Lemna minor*: studies on the time dependence of growth inhibition and the recovery after exposure, Chemosphere 67 (2007) 36–43.
[26] G.L. Ellman, K.D. Courtney, V.A. Andres Jr., R.M. Featherstone, A new coloromet-ric determination of acetylcholinesterase activity, Biochem. Pharm. 7 (1961) 88–95.
[27] T.C. Fisher, M. Crane, A. Callaghan, An optimized microtiter plate assay to detect acetylcholinesterase activity in individual *Chironomus riparius* Meigen, Environ. Toxicol. Chem. 19 (2000) 1749–1752.
[28] OECD Guidelines for the Testing of Chemicals, Guideline 301: Ready biodegradability, Paris, France, 1992.
[29] S. Stolte, S. Abdulkarim, J. Arning, A.K. Blomeyer-Nienstedt, U. Bottin-Weber, M. Matzke, J. Ranke, B. Jastorff, J. Thöming, Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazolium chloride and electrochemical wastewater treatment of poorly biodegradable compounds, Green Chem. 10 (2008) 214–224.
[30] N. Cedergreen, J.C. Streibig, The toxicity of herbicides to non-target aquatic plants and algae: Assessment of predictive factors and hazard, Pest Manage. Sci. 61 (2005) 1152–1160.
[31] N. Cedergreen, T.V. Madsen, Light regulation of root and leaf NO_3^- uptake and reduction in the floating macrophyte *Lemna minor*, New Phytol. 161 (2003) 449–457.
[32] M. Mohammad, T. Kishimoto, K. Itoh, K. Suyama, H. Yamamoto, Comparative sensitivity of *Pseudokirchneriella subcapitata* vs. *Lemna* sp. to eight sulfonylurea herbicides, Bull. Environ. Contam. Toxicol. 75 (2005) 866–872.
[33] S. Stolte, M. Matzke, J. Arning, A. Böschen, W.R. Pitner, U. Welz-Biermann, B. Jastorff, J. Ranke, Effects of different head groups and functionalised side chains on the aquatic toxicity of ionic liquids, Green Chem. 9 (2007) 1170–1179.
[34] J.F. Fairchild, D.S. Ruessler, P.S. Haverland, A.R. Carlson, Comparative sensitivity of *Selenastrum capricornutum* and *Lemna minor* to sixteen herbicides, Arch. Environ. Contam. Toxicol. 32 (1997) 353–357.
[35] UN/SCEGHS/12/IN.F.5, Sub-Committee of Experts on the Globally Harmonized System of classification and labelling of chemicals (GHS), United Nations, New York and Geneva, 2007.
[36] Globally Harmonized System of classification and labelling of chemicals (GHS), 4th revised edn, United Nations, New York and Geneva, 2011.
[37] M. Stasiewicz, E. Mulkiewicz, R. Tomczak-Wandzel, J. Kumirska, E. Siedlecka, M. Gołębiowski, J. Gajdus, P. Stepnowski, Assessing toxicity and biodegradation of novel, environmentally benign ionic liquids (1-alkoxymethyl-3-hydroxypyridinium chloride, saccharinate and acesulfamates) on cellular and molecular level, Ecotoxicol. Environ. Saf. 71 (2008) 157–165.
[38] S.P.M. Ventura, C.S. Marques, A.A. Rosatella, C.A.M. Afonso, F. Gonçalves, J.A.P. Coutinho, Toxicity assessment of various ionic liquid families towards *Vibrio fischeri* marine bacteria, Ecotoxicol. Environ. Saf. 76 (2012) 162–168.
[39] F. Stock, J. Hoffmann, J. Ranke, R. Störmann, B. Ondruschka, B. Jastorff, Effects of ionic liquids on the acetylcholinesterase – a structure-activity relationship consideration, Green Chem. 6 (2004) 286–290.
[40] C. Samorì, Introduction of oxygenated side chain into imidazolium ionic liquids: evaluation of the effects at different biological organization levels, Ecotoxicol. Environ. Saf. 73 (2010) 1456–1464.
[41] The UFT/Merck Ionic Liquids Biological Effects Database: http://www.ileco.uft.uni-bremen.de/ (last accessed on 30.11.12).
[42] S. Stolte, J. Arning, U.Bottin-Weber, A. Müller, W.R. Pitner, U. Welz-Biermann, B. Jastorff, J. Ranke, Effects of different head groups and functionalised side chains on the cytotoxicity of ionic liquids, Green Chem. 9 (2007) 760–767.
[43] P. Luis, I. Ortiz, R. Aldaco, A. Irabien, A novel group contribution method in a developnment of a QSAR for predicting the toxicity (*Vibrio fischeri* EC_{50}) of ionic liquids, Ecotoxicol. Environ. Saf. 67 (2006) 423–429.
[44] T.P.T. Pham, C.W. Cho, Y.S. Yun, Environmental fate and toxicity of ionic liquids: a review, Water Res. 44 (2010) 252–272.

3.3. Paper 3

A COMPARATIVE STUDY OF THE TERRESTRIAL ECOTOXICITY OF SELECTED PROTIC AND APROTIC IONIC LIQUIDS

Summary

Ionic liquids (ILs) have been usually considered as potentially "green" and the determination of their environmental impact tends to be overshadowed by the ever rising interest in obtaining new ILs with interesting applications. This is particularly obvious in the area of terrestrial ecotoxicity, as there are only few studied published, especially when it comes to the toxic effect towards carbon and nitrogen transforming microbiota, both in case of aprotic (AILs) and protic ionic liquids (PILs). In this article a comparative study of the terrestrial ecotoxicity of selected representatives of two ILs groups is presented: ten representatives of a new family of PILs (derived from aliphatic amines and organic acids) and some frequently used AILs (substituted imidazolium and piridinium chlorides). Toxicity of these ILs towards three terrestrial plant species (*Allium cepa*, *Lolium perenne* and *Raphanus sativus*) and soil microorganisms involved in carbon and nitrogen transformation was analyzed. The tested AILs were more toxic than the PILs, with EC_{50} values being various orders of magnitude lower than the ones for PILs. All of the AILs were toxic for *A. cepa*, with one of them being toxic for the three assayed plants. In the case of PILs, only one representative had the EC_{50} lower than 1000 mg kg^{-1} (the GHS threshold for terrestrial plants toxicity) for all of the analyzed plants, while other three other PILs were classified as harmful for only one plant species. The analyzed ionic liquids showed EC_{50} values over the GHS threshold of toxicity for nitrifying microbiota, especially in case of PILs. The shape of soil respiration curves for PILs indicates that they stimulate the activity of the soil carbon transforming microbiota and thus that they are potentially biodegradable in soil. On the other hand, the AILs proved to be resistant to biodegradation, having the respiratory curves lower than the one for the control sample. The most toxic ILs are the most complex ones in both of the analyzed groups. The findings of this study indicate that the PILs can be considered as less toxic and safer for the terrestrial environment than the AILs.

Chemosphere 108 (2014) 418–425

Contents lists available at ScienceDirect

Chemosphere

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

A comparative study of the terrestrial ecotoxicity of selected protic and aprotic ionic liquids

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HIGHLIGHTS

- Toxicity of selected ionic liquids (ILs) towards terrestrial organisms was analyzed.
- The aprotic ILs have lower EC_{50} than the protic ILs in all of the performed tests.
- The analyzed protic ILs are potentially biodegradable in soil, unlike the aprotic ILs.
- The new family of protic ILs is environmentally more favourable than the aprotic ILs.

ARTICLE INFO

Article history:
Received 10 November 2013
Received in revised form 1 February 2014
Accepted 6 February 2014
Available online 13 March 2014

Keywords:
Protic ionic liquids
Aprotic ionic liquids
Terrestrial ecotoxicity
Phytotoxicity
Carbon and nitrogen transformation

ABSTRACT

Ionic liquids (ILs) are a fairly new and very promising group of compounds with a vast variety of possible structures and uses. They are considered to be potentially "green", but their impact on the environment tends to be neglected or not studied enough, especially when it comes to terrestrial ecotoxicity, where there are very few studies performed to date. This work presents a comparative study of the terrestrial ecotoxicity of selected representatives of two ILs groups: a new family of protic ILs (derived from aliphatic amines and organic acids) and some frequently used aprotic ILs (substituted imidazolium and piridinium chlorides). Toxicity of the ILs towards three terrestrial plant species (*Allium cepa*, *Lolium perenne* and *Raphanus sativus*) and soil microorganisms involved in carbon and nitrogen transformation was analyzed. Protic ILs have shown no toxic effect in most of the tests performed. The EC_{50} values for aprotic ILs are various orders of magnitude lower than the ones for protic ILs in all of the tests. The most toxic ILs are the most complex ones in both of the analyzed groups. Protic ILs seem to have a potential for biodegradation in soil, while aprotic ILs exhibit inhibitory effects towards the carbon transforming microbiota. These findings indicate that protic ILs can be considered as less toxic and safer for the terrestrial environment than the aprotic ILs.

1. Introduction

During the last decades ionic liquids (ILs) have generated a lot of interest as new promising group of compounds. They are composed entirely of ions, liquid over a wide range of temperatures and practically non-volatile. The number of combinations of ions that form ILs is estimated to be as high as 10^{18}, so they have the potential to be tailored to suite the desired application. Their use spreads from chemical laboratories to various industrial processes, mostly being used as a reaction medium in a wide variety of chemical transformations, separations and extractions that until recently could only be carried out in organic solvents (Han and Row, 2010; Olivier-Bourbigou et al., 2010; Mohammad Ali, 2012). New possible applications of ILs are being constantly discovered (Kokorin, 2011).

One of many possible classifications of ILs is dividing them into two groups: aprotic (AILs) and protic (PILs) ionic liquids. The first group has been in use for more than a decade and the second one is still in development. A new family of PILs with simple and lineal cations and anions has been designed and they could have a potentially smaller environmental impact than the bulky and heterocyclic "classic" ILs which have been mainly in use up to date. The new PILs are composed of polysubstituted amines (mono-, di- and triethanolamine) as cations, and aliphatic organic acids (formic, acetic, propionic, butyric, isobutyric and pentanoic acid) as anions (Cota et al., 2007). The AILs analyzed in this study are one of the most frequently used and are derived from imidazolium and pyridinium (Masten, 2004; Werner et al., 2010; Domínguez de María, 2012; Mohammad Ali, 2012).

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http://dx.doi.org/10.1016/j.chemosphere.2014.02.043
0045-6535/

There are many possible applications for this new family of PILs. The 2-hydroxyethanolamine formate (2-HEAF) has been found to be able to dissolve many inorganic salts, hydroxylated compounds and some insoluble polymers such as polyaniline and polypyrrole (Bicak, 2005). The representatives of this family have been analyzed for their potential catalytic activity in the aldol condensation reactions (Iglesias et al., 2010) and for the heterogeneous catalytic hydrogenation of cyclohexene and acetone catalyzed by Pt/Al_2O_3 with methanol, ethanol and propan-1-ol (Khodadadi-Moghaddam et al., 2009). Other PILs, also derived from 2-hydroxyethylammonium and organic acids, have been studied in order to develop a new process for absorption of CO_2 (Yuan et al., 2007).

Ionic liquids are usually described as "green", but they are chemical products, and as such have to fulfill the current European chemical legislation for Registration, Evaluation, Authorisation and Restriction of Chemical Substances (REACH) (European Union, 2006). A very important aspect that has to be studied in sufficient depth is the potential negative environmental impact of ILs. This feature is often neglected, because the usual focus is to obtain new compounds with desired characteristics, sometimes without taking into account the environmental hazards. It is true that due to their extremely low vapour pressure ILs cannot be found in the atmosphere, but they can be soluble in water, like in case of the ILs presented in this study, so they could be found in water and soil as part of industrial and laboratory effluents. And as the new family of PILs is still under development and fairly new in their use, it is very important to assess their hazard potential, both in terms of aquatic (Peric et al., 2013) and terrestrial ecotoxicity.

The information on terrestrial ecotoxicity and biodegradability of ILs in literature is scarce. There are some articles published on the sorption and transport of ILs in soil (Studzińska et al., 2008, 2009; Matzke et al., 2008a; Mrozik et al., 2012) where the authors showed that the ILs cations can adsorb onto different types of soils and sediments and that hydrophobic ILs with long side chains adsorb much more strongly than the ILs with short and hydroxylated side chains, so their minimal retention could result in contamination of soils and consequently ground waters. The study of Deive et al. (2011) focused on identification of the microbial strains with higher survival rates towards ILs which could be potentially used in soil remediation. The potential biodegradability of ILs in water has been frequently studied (Stolte et al., 2008; Harjani et al., 2009; Coleman and Gathergood, 2010; Pham et al., 2010) with different conclusions, from zero biodegradation to potential biodegradability, depending on the hydrophylicity and hydrophobicity of the ILs molecule. But, there is a very limited number of publications on their biodegradation in soil (Modelli et al., 2008; Zhang et al., 2010), where the authors analyzed four ILs, three of which are derived from imidazolium and one from pyridinium, and found limited biodegradation after two months or complete lack of biodegradation. The studies published on the phytotoxicity of ILs have been mainly focused on the aquatic plants (Zhu et al., 2009; Pham et al., 2010) with certain degree of toxicity of ILs for *Lemna minor*, which depends on the cation present in the molecule, and on the length of the side chains. But there is few data available on the ecotoxicological effects of ionic liquids on terrestrial plants, with data published on toxicity of ILs (mainly derived from imidazolium) to wheat (Matzke et al., 2007; Wang et al., 2009), garden cress (Jastorff et al., 2005; Matzke et al., 2007), barley and radish (Bałczewski et al., 2007) and watercress (Studzińska and Buszewski, 2009) where the toxicity was found to be increasing with the decrease of the hydrophobicity of the ILs molecule. In their two studies Matzke et al. (2008b, 2009) analyzed the relation between the toxic effects of imidazolium based ionic liquids (depending on their anions and side chains) on wheat and cress and the content of different clay minerals and clay concentrations in soil, and found that the toxicity of the ILs was mainly dominated by the cation and that higher clay content lowered the toxicity, whilst the addition of clay minerals caused higher toxic effects in comparison to the control soil sample. To our knowledge, there is no complete information or study done in order to evaluate the toxic effect of ILs on the soil microbiota in terms of carbon and nitrogen transformation.

The present study aimed to evaluate and compare the terrestrial ecotoxicity of the representatives of a new family of PILs and frequently used AILs 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).

2. Materials and methods

2.1. Ionic liquids

The PILs analyzed in the present study are: 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxyethanolamine butanoate (2-HEAB), 2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine propionate (2-HDEAPr), 2-hydroxydiethanolamine butanoate (2-HDEAB), 2-hydroxydiethanolamine isobutanoate (2-HDEAiB), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), 2-hydroxytriethanolamine butanoate (2-HTEAB) and 2-hydroxytriethanolamine pentanoate (2-HTEAPe). The analyzed AILs are: 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-methyl-3-octylimidazolium chloride ([OMIM]Cl) and N-butylpyridinium chloride ([BPy]Cl).

The PILs were synthesized and characterized by Dr. Miguel Iglesias's group at the University of Santiago de Compostela (Spain) as described in detail by Cota et al., 2007; Álvarez et al., 2010; Iglesias et al., 2010; Peric et al., 2011. The AILs were obtained commercially from Sigma–Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

The structures of analyzed PILs are shown in Fig. S1 and the structures of AILs in Fig. S2 of Supplementary material.

2.2. Soil samples

Soil samples (air dried and 2 mm-sieved) were obtained from the superficial layer (A horizon) of natural pine wood forest soil sampled in Vilassar de Mar, Barcelona (UTM: 31T 444376E 4596459N). The soil corresponds to a Haplic arenosol (FAO, 1998) granitic origin and sandy texture (74% of sand). Its pH value is 6.3 and it has 1.2% of oxidizable carbon and around 3% 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) guideline for testing soil microorganisms involved in carbon transformation process (OECD, 2000b).

2.3. Terrestrial plants test: Seedling emergence and seedling growth test

The seedling emergence and seedling growth test was performed with the seeds of two monocotyledonae plants: *Allium cepa* (onion) and *Lolium perenne* (grass) and one dicotyledonae *Raphanus sativus* (radish). These plant species were chosen based on the criteria for selection of test species and the list of species historically used in plant testing, included in the OECD 208 Terrestrial plant test guideline (OECD, 2006). The seeds were put in 20 mL plastic pots, with four replicates of 5 seeds, in 15 g of soil for each application rate and control sample. Aqueous solutions of the ILs were added to the soil at concentrations of 1, 10, 100, 1000, and 5000 mg kg^{-1}, including the control samples where no ILs were added to the soil. The final water content of all samples was the

equivalent to 60% of the soil water holding capacity, and the water lost during the assay was periodically restored. The plant germination and growth assay lasted 14–21 d after the emergence of 50% of the seedlings in the control group, and was performed according to OECD 208 guideline (OECD, 2006). Once the assay was concluded the shoot length was measured. The morphological changes of the plants were also noted, such as chlorosis.

2.4. Soil microorganisms: Carbon transformation test

In the carbon transformation test, 50 g of soil sample was used, adjusting the water content to the 60% of the soil water holding capacity. All the experiments were done at least in triplicate. The soil was treated with the following concentrations of ILs: 1, 10, 100, 1000, 5000, and 10000 mg kg^{-1}, including the control samples where no ILs were added to the soil. These samples were incubated in manometric respirometers, which allow the determination of the sample oxygen consumption (Oxitop® OC 110, WTW GmbH, Weilheim, Germany). The samples were kept at 25 °C in the darkness, in an incubator equipped with a thermostat for 28 d. The oxygen consumption was periodically registered. Cumulative respiration (CR) was determined by the cumulative oxygen consumption at the end of the incubation period. Once the incubation was completed, substrate induced respiration (SIR) was determined according to OECD 217 carbon transformation test guideline (OECD, 2000b). This former test was done by adding an aqueous solution equivalent to 4000 mg glucose per kg of soil to the incubated samples and the determination of 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 the respiration was stable. The respiratory activation quotient (Q_R) was calculated dividing BR by SIR (ISO, 2002).

2.5. Soil microorganisms: Nitrogen transformation test

The nitrogen transformation test was done according to the OECD 216 guideline (OECD, 2000a). The soil was treated with the following concentrations of ILs: 10, 100, 1000, 10000, and 20000 mg kg^{-1}, including the control samples where no ILs were added to the soil. The test was done with the 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 (US EPA, 1971).

2.6. Statistical analysis

In order to know the statistical significance of the differences between treated soil samples and controls an analysis of variance (ANOVA) test was done followed by post hoc Duncan test ($p < 0.05$). Based on the results obtained by the ANOVA test, a Lowest Observable Adverse Effect Concentration (LOAEC) for the ionic liquids has been determined after the carbon transformation test. Dose–response relationship was assessed, and the effective concentrations 10, 20 and 50 (EC_{10}, EC_{20} and EC_{50}) with their 95% confidence intervals were calculated for each compound from suitable regression models (Gompertz, hormetic, or logistic) using Statistica 6.0 (Stat Soft Inc., 1984–2001). The choice of the model was based on the best fit to the data. The EC_{50} was selected because it is the parameter used in the environmental regulations and most of the available literature. It can be considered that EC_{20}, which represents 20% level of negative effect, is a significant alteration, taking into account that this work was done in controlled laboratory experimental conditions (Isnard et al., 2001). In the case of EC_{10}, the lowest bound of its confidence interval was found to be close to No Observed Effect Concentration (NOEC) (Fulladosa et al., 2005), so it can be considered as a safe concentration. Spearman and Pearson's coefficient of correlation were calculated using IBM® SPSS® Statistics 20 program (IBM Corporation, NY, USA).

3. Results and discussion

3.1. Terrestrial plants test: Seedling emergence and seedling growth test

The values of EC_{50} (mg kg^{-1}) and the confidence interval of dose–response curves for the analyzed ILs are shown in Table 1.

In the case of *A. cepa* EC_{50} values for the analyzed PILs are between 655 and 7793 mg kg^{-1}, with 2-HDEAPe being the most toxic and 2-HDEAF the least toxic compound. The AILs showed to be more toxic towards *A. cepa* than the PILs, with considerably lower EC_{50} values, ranging between 150 and 930 mg kg^{-1}. The [OMIM]Cl is the most toxic one, with the lowest EC_{50} of all analyzed ILs for the three tested plants. The EC_{50} values obtained for the PILs in the test with *L. perenne* range between 503 and 7166 mg kg^{-1}, with 2-HDEAPe and 2-HEAB being the most toxic ones, and 2-HEAF and 2-HDEAF the least toxic ones. In the case of AILs, the obtained EC_{50} are in general lower, being between 561 and 2890 mg kg^{-1}, with [OMIM]Cl once again being the most toxic one. As far as the inhibition of growth of *R. sativus* is concerned, the EC_{50} values vary between 422 and 6430 mg kg^{-1}, with 2-HDEAPe being the most toxic one and 2-HDEAF the least toxic compound, as in the case of *A. cepa*. The EC_{50} values corresponding to AILs are lower, being between 371 and 3742 mg kg^{-1}, with [OMIM]Cl as the most toxic one. *L. perenne* and *R. sativus* have proven to be some more sensitive to the presence of PILs than *A. cepa*. In contrast, for the AILs the most sensitive species is *A. cepa*.

It can be observed that the most toxic of all the analyzed ILs is the [OMIM]Cl, with EC_{50} values far below 1000 mg kg^{-1} in case of all of the three plants. According to the Classification criteria for substances hazardous to plants (soil exposure) of the Globally Harmonized System of Classification and Labelling of Chemicals (United Nations, 2006) this AIL can be classified as toxic for the terrestrial environment in the Acute 3 category of this classification ($100 < EC_{50} \leqslant 1000$ mg kg^{-1}). Two of the PILs show some toxic effect and belong to the Acute 3 category, 2-HDEAPe for the *A. cepa*, 2-HEAB and 2-HDEAPe for *L. perenne* and 2-HDEAPe and 2-HTEAPe for *R. sativus*. The most toxic of the analyzed PILs is 2-HDEAPe, with EC_{50} values below 1000 mg kg^{-1} for the three plants. It can also be classified into the Acute 3 category. The other two representatives of AILs can also be classified into the Acute 3 category in the case of *A. cepa*, but with a significant difference in EC_{50} values from the [OMIM]Cl, being 930 and 588 mg kg^{-1} for [BMIM]Cl and [BPy]Cl respectively. The analyzed PILs are less toxic than the AILs. The comparison of the results from this study with those obtained by other authors for ILs (Jastorff et al., 2005; Bałczewski et al., 2007; Matzke et al., 2007, 2008b, 2009; Wang et al., 2009; Pham et al., 2010) shows that the analyzed PILs are less toxic than the AILs in general, including the ones analyzed in this study. Even though the plant species used in the test were not the same as those used by other authors, the values of EC_{50} for the AILs generally have lower EC_{50} values than the ones obtained for the PILs tested during the present study. Increased toxicity of [OMIM]Cl in comparison with the other two representatives of AILs is consistent with the findings of other authors that indicate that the elongation of the alkyl side chains leads to increased toxicity (Ranke et al., 2004; Matzke et al., 2008b; Pham et al., 2010).

When it comes to EC_{20} values, in the case of PILs most of them are above 1000 mg kg^{-1}, with the values ranging between 272 and 4374 mg kg^{-1}, whilst the EC_{10} values range between 87 and 3580 mg kg^{-1}. If we consider EC_{20} as the concentration where

Table 1
Results of dose–response curves in mg kg^{-1} for the seedling emergence and growth and nitrogen transformation tests, $p < 0.05$.

	Allium cepa		*Lolium perenne*		*Raphanus sativus*		Nitrogen transformation	
	EC_{50}	Confidence interval	EC_{50}	Confidence interval	EC_{50}	Confidence interval	EC_{50}	Confidence interval
2-HEAF	6887	3805–12459	7166	6878–14257	3383	2464–4645	10014	4865–14157
2-HEAB	2012	437–4446	680	458–1009	1729	362–2495	7485	7082–7773
2-HDEAF	7793	1797–9136	3949	2317–6730	6430	3698–10179	8018	7500–8571
2-HDEAA	6415	4260–8316	996	692–1433	1584	508–4931	8272	7863–8704
2-HDEAPr	3891	2468–6025	3163	1951–5158	2128	1714–2640	8454	3973–9704
2-HDEAB	4174	2226–5247	1180	845–1648	3646	2761–4815	7014	6236–7888
2-HDEAiB	2313	1517–3528	1780	368–8584	2166	1847–2541	6487	6135–6858
2-HDEAPe	655	243–1067	503	294–1002	422	241–739	6639	6201–7108
2-HTEAB	1568	1149–2140	1261	995–1597	2013	1262–3210	6358	5983–6756
2-HTEAPe	1428	422–3655	2326	1930–2568	826	605–1128	6201	3763–8184
BMIM[Cl]	930	707–1223	1317	1120–1548	3742	3288–4258	555	426–723
OMIM[Cl]	150	84–362	561	367–857	371	316–435	321	216–321
BPy[Cl]	588	274–1216	2890	2458–3399	2774	2068–3721	663	566–775

Results for 2-HEAF, 2-HDEAPr and 2-HTEAPe are taken from Peric et al. (2011).

the first adverse effect can be spotted, and EC_{10} as a "safe" concentration, the analyzed PILs can be considered as low toxic and fairly environmentally safe. On the other hand, the analyzed AILs have all but two values for EC_{20} below 1000 mg kg^{-1}, and the EC_{10} are all but two below 500 mg kg^{-1}. The values of EC_{10} and EC_{20} and the confidence interval of dose–response curves for the analyzed ILs are shown in Supplementary material Table S1.

The Spearman's correlation coefficients were determined in order to get more information on toxicity of the analyzed PILs. The correlation between the complexity of the PILs molecule and their toxicity was investigated. For this analysis the molecular weight of PILs, the number of C-atoms in the cationic (amine) and anionic (acid) part of the molecule and EC_{50} values were used. The correlation analysis has shown that the elongation of the anionic part of the molecule causes higher toxicity in the case of the analyzed plants, with Pearson correlation coefficient being −0.955 ($p < 0.01$) for the *A. cepa*, −0.715 ($p < 0.05$) for *L. perenne* and −0.713 ($p < 0.05$) for *R. sativus*. There is also a negative correlation between the increase of the molecular weight and the obtained EC_{50} values for *A. cepa* and *L. perenne*, with PILs with higher molecular weight (the most complex ones) being the most toxic ones ($r = -0.716$, $p < 0.01$ and $r = -0.583$, $p < 0.05$ respectively). These findings are in concordance with the correlation analysis results for the aquatic plant *Lemna minor* (Peric et al., 2013), where the number of carbons in the anionic part of the molecule proved to be in negative correlation with EC_{50} values.

Apart from being more toxic than the PILs in the terms of lower EC_{50}, the AILs also caused chlorosis in the case of *R. sativus* and *L. perene* at concentrations $\geqslant$100 mg kg^{-1} (Fig. 1). A lower synthesis of chlorophyll is manifested as chlorosis, and it can be caused by nutrient deficiency (especially iron), higher soil salinity, damaged or compressed roots, atmospheric pollutants and toxic action of contaminants in the soil (Pessarakli, 2011). The electrical conductivity corresponding to the analyzed AILs was 0.879 dS m^{-1} as a maximum value, so the increased soil salinity can be discarded as a potential cause of chlorosis. The observed toxic effect of AILs could be due to root damage or blockage of the nutrient transport into the plant.

3.2. Soil microorganisms: Carbon transformation test

The cumulated respiration is graphically presented in the form of the respiration curves of the oxygen consumed during the 28 d of the carbon transformation test. The respiration curves obtained for the ILs can be divided into 4 groups according to their shape and distribution, and we have chosen one of each group to present the results of the cumulative respiration. The graph for 2-HDEAA is

Fig. 1. Chlorosis of the leaves of *Raphanus sativus* caused by [BMIM]Cl. (For interpretation to colours in figure, the reader is referred to the web version of this paper.)

representative for two more PILs: 2-HEAF and 2-HDEAF. The graph for 2-HEAB also represents 2-HDEAiB and 2-HTEAB. The 2-HDEAB graph also corresponds to 2-HDEAPr, 2-HDEAPe and 2-HTEAPe. And finally, the graph presented for [OMIM]Cl also corresponds to [BMIM]Cl and [BPy]Cl. The graphs in Fig. 2 show the cumulative respiration (CR) curves corresponding to control and the concentrations 1, 10, 100, 1000, 5000, and 10000 mg kg^{-1} for the representative ionic liquids. The graphs for 2-HEAF, 2-HDEAPr and 2-HTEAPe are published in Peric et al., 2011 and the graphs for the rest of the ILs can be found in Supplementary material, Figs. S3–S7.

In the case of 2-HEAF, 2-HDEAF and 2-HDEAA, none of the assayed concentrations produced values of accumulated oxygen below the control. This indicates that these ionic liquids show no toxicity towards soil microbiota, reflected as uninterrupted respiratory activity of the soil. The case of 2-HEAB, 2-HDEAiB and 2-HTEAB is similar in a sense that at the final point of the test, after 28 d, all of the values of the accumulated oxygen are above the control. The curves for the lowest concentrations (1, 10, and 100 mg kg^{-1}) show no difference when compared to the control, while the curve for the concentration of 1000 mg kg^{-1} of PILs shows a very strong response of the soil microbiota from the very start of the assay, with final accumulated oxygen values being between 123% and 135% of the control sample at the final point of the assay. In the case of higher concentrations (5000 and 10000 mg kg^{-1}) there is an initial inhibitory effect on the soil

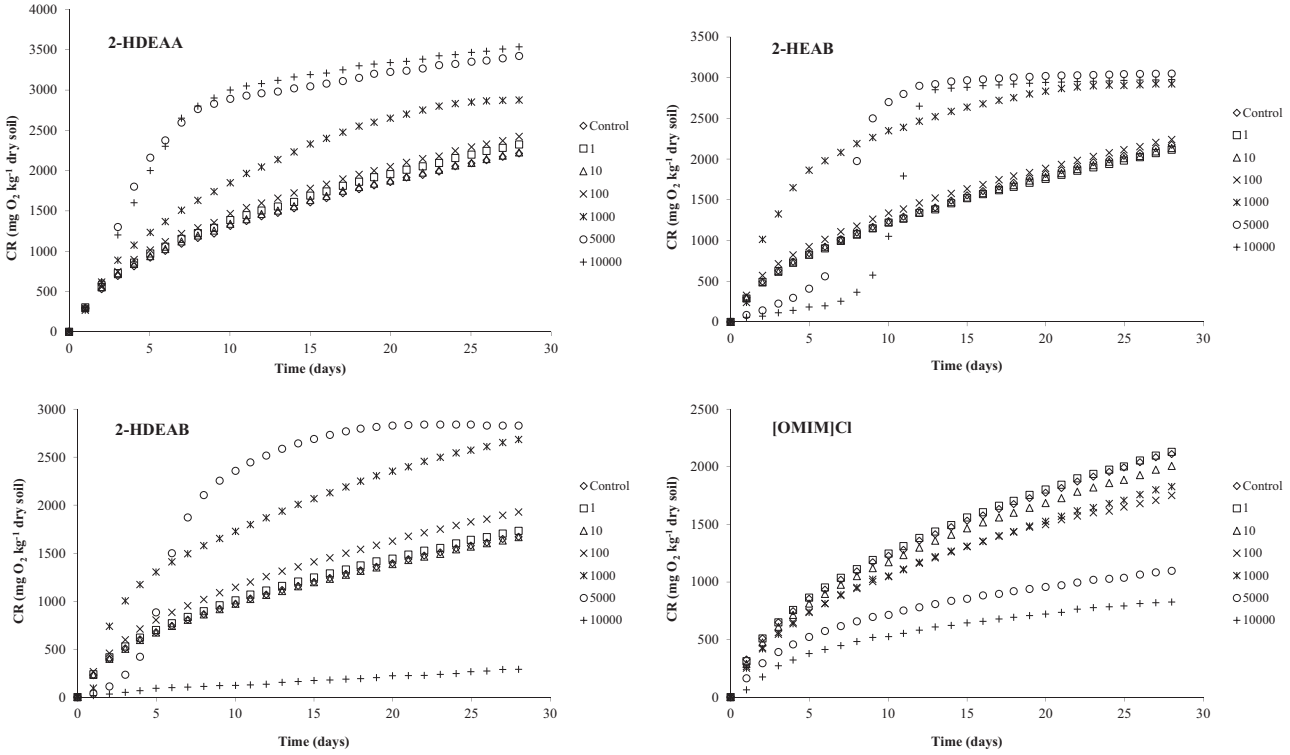

Fig. 2. Values of the cumulative respiration expressed as the cumulative oxygen consumed during 28 d of the respirometric assay for the control and different concentrations in mg kg^{-1} of the selected ILs. The values correspond to the averages of at least three replicates. The standard deviation is between 8 and 170 mg kg^{-1} for 2-HDEAA, between 8 and 78 mg kg^{-1} for 2-HEAB, between 8 and 53 mg kg^{-1} for 2-HDEAB and between 14 and 162 mg kg^{-1} for [OMIM]Cl after 28 d.

microbiota which manages to recuperate and start to respirate, with final percentage of the accumulating oxygen being between 132% and 142% of the value of the control. The third pattern of the PILs effect on the soil microbiota (the case of 2-HDEAPr, 2-HDEAB, 2-HDEAPe and 2-HTEAPe) implies little or no difference between the lowest concentrations (1, 10, and 100 mg kg^{-1}) and the control, and also the initial inhibition at the concentration of PILs 5000 mg kg^{-1}. But in the case of the highest concentration (10 000 mg kg^{-1}), the inhibition of the respiratory activity occurs throughout the whole assay, thus indicating toxicity for soil microbiota at this concentration. In the case of temporary inhibition and posterior recovery of the respiratory activity, the levels of the accumulated oxygen are higher than in the case of the control sample, which could be due to the degradation of labile organic matter coming from the microorganisms affected by the initial toxicity and/or due to the biodegradation of the analyzed ILs.

The AILs have a different behaviour. All of the curves are at the same level or lower than the control. There is no difference between the control samples and the lower concentrations (1, 10, and 100 mg kg^{-1}) in case of the [BMIM]Cl, but there is an inhibitory effect starting at 1000 mg kg^{-1}, which indicates that this compound shows toxicity towards the microorganisms involved in the C mineralization cycle. The inhibition of the respiratory activity in the case of [OMIM]Cl and [BPy]Cl starts at 100 mg kg^{-1} (Fig. 2). In general, the analyzed AILs have proven to be more toxic for the soil microbiota than the analyzed PILs.

Table 2 shows the results for the CR, BR, SIR and Q_R for the analyzed ILs.

The CR reflects the overall soil state (microbiota and nutrient availability) and the results show that at the lower concentrations of PILs (1, 10 and 100 mg kg^{-1}) there is no difference with the control samples. A significant ($p < 0.05$) increase of CR can be observed at 1000 and 5000 mg kg^{-1}. Only in the case of the highest concentration of 2-HDEAPr, 2-HDEAB, 2-HDEAPe and 2-HTEAPe (10 000 mg kg^{-1}) the inhibition of respiration occurs. When it comes to the AILs, significantly lower values of CR compared to the control can be seen starting at the concentration of 100 mg kg^{-1} in the case of [OMIM]Cl and [BPy]Cl and 1000 mg kg^{-1} in the case of [BMIM]Cl.

Basal respiration rate (BR) indicates the current biological activity which remains constant after the soil respiration is stabilized (once the labile carbon source is exhausted and the microbiota is adapted to the conditions of the incubation). This depends on the microbiota and nutritional state of the soil. Taking into account the fact that the same soil was used in all of the experiments, the nutritional state is the same for all of the samples, and the variable is the microbiota state, the results for the BR can indicate if the analyzed ILs cause inhibition or damage to the soil microbiota. According to the results obtained in the present study, 2-HDEAB and 2-HTEAB show significant decrease in BR values starting from 5000 mg kg^{-1}. The 2-HDEAPe and 2-HTEAPe show significant decrease at the highest concentration (10 000 mg kg^{-1}). In some cases there are low BR values when there is no inhibition of respiration (in concentrations of 5000 mg kg^{-1} for 2-HDEAB, and 5000 mg kg^{-1} and 10 000 mg kg^{-1} in the case of 2-HTEAB) or the BR value cannot be calculated because the stimulation of respiration is too high, like in the concentration of 1000, 5000 and 10 000 mg kg^{-1} for 2-HEAB, 5000 and 10 000 mg kg^{-1} for 2-HDEAiB, and 10 000 mg kg^{-1} in the case of 2-HDEAA. A significant increase of the BR is noted in the case of the concentrations of 5000 and 10 000 mg kg^{-1} for 2-HEAF, 1000, 5000 and 10 000 mg kg^{-1}, for 2-HDEAF, 5000 mg kg^{-1} for 2-HDEAPr, 1000 mg kg^{-1} for 2-HDEAiB, and 5000 mg kg^{-1} for 2-HTEAPe. All of the analyzed AILs have shown a stronger inhibition of BR than the PILs. A significant inhibition starts at 100 mg kg^{-1} in the case of [BPy]Cl and at 1000 mg kg^{-1} in the case of [BMIM]Cl and [OMIM]Cl.

The SIR is proportional to the active microbial biomass and is often used as an indicator of this parameter. Only the highest

Table 2
Average rate of cumulative respiration (CR) during the incubation period expressed as the % relative to the control soil; BR (mg O_2 h^{-1} kg^{-1} dry soil), SIR (mg O_2 h^{-1} kg^{-1} dry soil) and Q_R. The letters a, b and c indicate different homogeneous groups determined by Duncan test ($p < 0.05$) within rows.

	Control	Ionic liquid concentration					
		1	10	100	1000	5000	10000
2-HEAF							
% CR	100 a	93 a	89 a	95 a	120 b	281c	355 c
BR	1.35 a	1.05 a	1.24 a	1.40 a	1.14 a	2.78 b	6.92 c
SIR	11.43 a	10.00 a	9.11 a	11.79 a	10.71 a	15.00 b	28.57 c
Q_R	0.12	0.11	0.14	0.12	0.11	0.19	0.24
2-HEAB							
% CR	100 a	97 a	98 a	103 a	129 b	134 b	132 b
BR	1.79 b	1.81 b	1.75 b	1.75 b	nd	nd	nd
SIR	7.35 a	7.16 a	7.23 a	7.59 a	10.14 b	9.78 b	10.03 b
Q_R	0.24	0.25	0.24	0.23	nd	nd	nd
2-HDEAF							
% CR	100 a	104 a	101 a	105 a	110 a	108 a	110 a
BR	2.02 a	2.10 a	1.98 a	1.98 a	3.89 c	2.49 b	2.57 b
SIR	8.48 a	10.07 ab	9.54 ab	10.60 ab	13.25 ab	15.90 bc	20.14 c
Q_R	0.24	0.21	0.21	0.19	0.29	0.16	0.13
2-HDEAA							
% CR	100 a	105 a	101 a	109 a	130 b	158 c	156 c
BR	1.87 a	1.91 a	1.79 a	2.02 a	1.52 a	1.54 a	nd
SIR	7.72 a	10.90 b	12.26b	12.90 b	14.08 c	15.89 d	23.61 e
Q_R	0.24	0.17	0.15	0.16	0.11	0.10	nd
2-HDEAPr							
% CR	100 b	90 b	89 b	97 b	111 b	179 c	46 a
BR	1.40 a	1.32 a	1.09 a	1.20 a	1.63 a	3.77 b	1.22 a
SIR	12.57 b	10.84 b	11.27 b	12.70 b	20.29 c	25.86 c	2.48 a
Q_R	0.11	0.12	0.10	0.09	0.08	0.15	0.49
2-HDEAB							
% CR	100 b	104 b	100 b	115 c	161 d	163 d	18 a
BR	1.36 b	1.48 b	1.46 b	1.48 b	1.56 b	0.42 a	0.44 a
SIR	8.59 b	8.91 b	8.51 b	10.01 c	14.91 d	19.90 e	1.26 a
Q_R	0.16	0.14	0.09	0.05	0.04	0.02	0.35
2-HDEAiB							
% CR	100 a	100 a	105 a	104 a	135 b	142 b	133 b
BR	1.56 a	1.56 a	1.59 a	1.36 a	2.02 b	nd	nd
SIR	7.86 a	8.57 a	8.57 a	10.36 a	22.50 b	30.00 b	8.29 a
Q_R	0.20	0.18	0.19	0.13	0.10	nd	nd
2-HDEAPe							
% CR	100 a	106 a	113 a	114 a	133 b	138 b	18 c
BR	1.71 b	1.79 b	1.79 b	1.71 b	2.68 c	1.32 b	0.35 a
SIR	8.56 b	7.00 b	8.94 b	7.78 b	12.44 b	31.50 c	0.39 a
Q_R	0.20	0.26	0.20	0.22	0.22	0.04	0.90
2-HTEAB							
% CR	100 a	104 a	98 a	101 a	123 b	136 b	133 b
BR	1.79 b	1.79 b	1.59 b	1.63 b	1.51 b	0.19 a	0.16 a
SIR	7.51 a	7.76 a	8.51 a	16.27 b	17.52 b	22.03 b	17.52 b
Q_R	0.24	0.23	0.19	0.10	0.09	0.01	0.01
2-HTEAPe							
% CR	100 b	104 b	100 b	104 b	161 c	242 d	19 a
BR	1.71 b	1.55 b	1.59 b	1.48 b	1.42 b	3.18 c	0.36 a
SIR	10.60 b	10.28 b	10.93 b	10.93 b	24.43 c	34.70 d	0.63 a
Q_R	0.16	0.15	0.15	0.14	0.06	0.09	0.58
[BMIM]Cl							
% CR	100 c	94 bc	104 c	99 bc	86 b	69 a	63 a
BR	1.63 bc	1.44 abc	1.87 c	1.71 bc	1.48 abc	1.24 a	1.05 a
SIR	5.83 a	5.42 a	7.08 ab	7.92 ab	10.83 c	9.17 ab	8.75 ab
Q_R	0.28	0.27	0.26	0.22	0.14	0.14	0.12
[OMIM]Cl							
% CR	100 d	101 d	95 d	87 c	83 c	52 b	39 a
BR	1.63 c	1.59 c	1.56 bc	1.52 bc	1.24 b	0.66 a	0.41 a
SIR	6.54 ab	7.69 ab	8.57 ab	9.17 ab	10.00 b	6.35 a	5.77 a
Q_R	0.25	0.21	0.18	0.16	0.12	0.11	0.07
[BPy]Cl							
% CR	100 c	98 c	100 c	84 b	78 b	65 a	54 a
BR	2.28 c	2.43 c	2.36 c	1.79 b	1.30 a	1.22 a	0.98 a
SIR	9.73 b	9.44 b	9.16 b	9.75 b	9.73 b	8.02 ab	6.89 a
Q_R	0.23	0.26	0.26	0.18	0.13	0.15	0.13

Results for 2-HEAF, 2-HDEAPr and 2-HTEAPe are taken from Peric et al. (2011).
% CR = percentage of cumulative respiration, BR = basal respiration rate, SIR = substrate induced respiration, Q_R = respiratory activation quotient, nd = not determined.

concentration of 2-HDEAPr, 2-HDEAPe or 2-HTEAPe (10000 mg kg^{-1}) shows a substantial decrease in SIR values. The higher concentrations of PILs (1000, 5000 and 10000 mg kg^{-1}) generally produce an increase in the SIR values. The AILs show a moderate inhibition of SIR values at highest concentrations.

The increase of the CR values, found in the case of most of the PILs could indicate that these compounds are biodegraded by soil microorganisms, as confirmed by a preliminary biodegradation study (Peric et al., 2011). In contrast, the AILs have all of the CR values lower than the control, which can indicate that they do not have the same degradation potential as the PILs and that they inhibit the activity of soil microorganisms.

According to the ISO standards for the determination of abundance and activity of soil microflora using respiration curves, the values of Q_R which are higher than 0.30 indicate polluted soil, especially in the case of heavy metals (ISO, 2002). For the organic compounds this ratio can be altered if the compounds are susceptible to biodegradation. When it comes to PILs, only in the case of the highest concentrations (10000 mg kg^{-1}) of 2-HDEAPr, 2-HDEAB, 2-HDEAPe and 2-HTEAPe this effect can be noted. In some cases the calculated Q_R was below 0.30, even though there was a visible toxic effect the carbon transforming microbiota. As Simek et al. (2013) also concluded in their study, the meaning of the Q_R is not always fully clear, especially because of the complex physiological status of microbial communities in individual soil horizons, so we will not speculate about the meaning of these lower than expected values in the case of the AILs analyzed in our study.

Based on the significant differences in % CR, the LOAEC values for the tested PILs are high, being 10000 mg kg^{-1} for 2-HDEAB, 2-HDEAPr, 2-HDEAPe and 2-HTEAPe, and above 10000 mg kg^{-1} for 2-HEAF, 2-HEAB, 2-HDEAF, 2-HDEAA, 2-HDEAiB and 2-HTEAB. The tested AILs have shown lower values of LOAEC, of 100 mg kg^{-1} for [OMIM]Cl and [BPy]Cl and 1000 mg kg^{-1} for [BMIM]Cl. The AILs do not show a high inhibition potential for carbon mineralization processes, but have one order of magnitude lower values of LOAEC compared to the PILs.

The correlation analysis between the structure of PILs (cationic and anionic moiety size) and their toxic action in the case of the carbon transformation test shows that there is an inverse correlation between the increase of the number of carbons in the acid part of the PILs molecule and the values for % CR, BR and SIR (correlation coefficients r are −0.911, −0.621 and −0.565 respectively, with the level of significance $p < 0.05$). The same type of correlation was found for the molecular weight ($r = -0.721$ for CR, −0.761 for BR, $p < 0.01$ and $r = -0.588$ for SIR, $p < 0.05$), while the increase of complexity of the cationic part of the PILs molecules causes the decrease of BR values ($r = -0.670$, $p < 0.05$).

3.3. Soil microorganisms: Nitrogen transformation test

The values of EC_{50} (in mg kg^{-1}) calculated from the dose–response curves in the nitrogen transformation test can be seen in Table 1.

The EC_{50} values for the analyzed PILs are very high, ranging between 6201 and 10014 mg kg^{-1}. There is no toxic effect of the analyzed ILs on nitrogen transformation processes according to the Globally Harmonized System of classification and labelling of chemicals (EC_{50} > 100 mg kg^{-1}) (United Nations, 2006), but a clear difference in EC_{50} values between the two analyzed families was found. The values of EC_{50} for AILs are between 10 and 30 times lower than the EC_{50} values for PILs, with [OMIM]Cl being the most complex and the most toxic one.

The EC_{10} and EC_{20} values are also very high, being far over 100 mg kg^{-1} in the case of all of the analyzed PILs. The lowest value of EC_{10} obtained for the 2-HTEAPe is one order of magnitude higher than the Globally Harmonized System benchmark for chronic terrestrial toxicity (100 mg kg^{-1}). The rest of the EC_x values are almost all higher than 5000 mg kg^{-1}, the fact that clearly confirms that PILs have no toxic effect on the nitrifying microbiota. The AILs also have values of EC_{10} and EC_{20} which are higher than 100 mg kg^{-1}. And once again, there is a clear difference in the order of magnitude between the EC_{10} and EC_{20} values for PILs and AILs, being the ones for AILs 12–30 times lower than the ones for PILs. The values of EC_{10} and EC_{20} and the confidence interval of dose–response curves for the analyzed ILs are shown in Supplementary material Table S1.

The correlation analysis showed that there is a negative correlation between the increase of complexity of the amine part of the molecule (from mono-, over di-, and onto triethanolamine) and the EC_{50} values of the analyzed PILs, with values for the Pearson correlation coefficient being $r = -0.683$ ($p < 0.05$). The same correlation was found in the case of the elongation of the carbon alkyl chain in the anion moiety (the acid part of the molecule) and the increase of the molecular weight and the EC_{50} values obtained in the nitrogen transformation test, with r coefficient values being −0.850 and −0.878 respectively, with $p < 0.01$. This means that the more complex is the PILs molecule, there is a higher toxicity on the nitrifying microbiota.

4. Conclusions

Comparing the terrestrial ecotoxicity results for the representatives of two groups of ionic liquids, it can be seen that the analyzed AILs are more toxic than the new group of PILs. All of the AILs proved to be toxic for the onion, and the [OMIM]Cl for the radish and grass as well. In the case of PILs, only 2-HDEAPe can be classified as toxic for the three analyzed plants, with three other being classified as toxic for only one plant species. The shape of the soil respiration curves for PILs indicate that they stimulate the microbiota's activity, which can indicate that they are potentially biodegradable in soil. However, the AILs seem to be more resistant to biodegradation, as their respiration curves present inhibition of the microbiota's activity. None of the analyzed ionic liquids show inhibition of the nitrifying microbiota. According to the correlation analysis it can be observed that the molecular weight and the size of the acid (anionic part of the molecule) have more influence on the PILs toxicity than the amine (cationic part of the molecule). More complex ILs are the most toxic ones, with [OMIM]Cl being the most toxic in all of the tests performed. This comparative study indicates that ILs with simpler structure and with short alkyl side chains show lower toxicity and should be considered as a path to follow in the future synthesis of new ionic liquids.

Acknowledgments

B. Peric would like to thank the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development (Grant MAEC-AECID).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.02.043.

References

Álvarez, V.H., Iglesias, M., Dosil, N., Gonzalez-Cabaleiro, R., Martin-Pastor, M., Mattedi, S., Navaza, J.M., 2010. Brønsted ionic liquids for sustainable processes: synthesis and physical properties. J. Chem. Eng. Data 55, 625–632.

Bałczewski, P., Bachowska, B., Białas, T., Biczak, R., Wieczorek, W.M., Balińska, A., 2007. Synthesis and phytotoxicity of new ionic liquids incorporating chiral cations and/or chiral anions. J. Agric. Food Chem. 55, 1881–1892.

Bicak, N., 2005. A new ionic liquid: 2-hydroxy ethylammonium formate. J. Mol. Liq. 116, 15–18.
Coleman, D., Gathergood, N., 2010. Biodegradation studies of ionic liquids. Chem. Soc. Rev. 39, 600–637.
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.
Deive, F.J., Rodríguez, A., Varela, A., Rodrígues, C., Leitão, M.C., Houbraken, J.A.M.P., Pereiro, A.B., Longo, M.A., Sanromán, M.Á., Samson, R.A., Rebelo, L.P.N., Silva Pereira, C., 2011. Impact of ionic liquids on extreme microbial biotypes from soil. Green Chem. 13, 687–696.
Domínguez de María, P. (Ed.), 2012. Ionic Liquids in Biotransformations and Organocatalysis: Solvents and Beyond. John Wiley & Sons Inc., USA.
European Union, 2006. Regulation 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.
FAO (Food and Agriculture Organization), United Nations Educational, Scientific and Cultural Organization, 1998. World Reference Base for Soil Resources, Rome, Italy.
Fulladosa, E., Murat, J.C., 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.
Han, D., Row, K.H., 2010. Recent applications of ionic liquids in separation technology. Molecules 15, 2405–2426.
Harjani, J.R., Singer, R.D., Garcia, M.T., Scammells, P.J., 2009. Biodegradable pyridinium ionic liquids: design, synthesis and evaluation. Green Chem. 11, 83–90.
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. 16, 2802–2808.
Isnard, P., Flammarion, P., Roman, G., Babut, M., Bastien, P., Bintein, S., Esserméant, L., Férard, J.F., Schmitt, S., Saouter, E., Saroli, M., Thiébaud, H., Tomassone, R., Vindimian, E., 2001. Statistical analysis of regulatory ecotoxicity tests. Chemosphere 45, 659–669.
ISO, 2002. ISO guideline 17155: Soil Quality. Determination of Abundance and Activity of Soil Microflora Using Respiration Curves. International Organization for Standardization, Geneva, Switzerland.
Jastorff, B., Mölter, K., Behrend, P., Bottin-Weber, U., Filser, J., Heimers, A., Ondruschka, B., Ranke, J., Schaefer, M., Schröder, H., Stark, A., Stepnowski, P., Stock, F., Störmann, R., Stolte, S., Welz-Biermann, U., Ziegert, S., Thöming, J., 2005. Progress in evaluation of risk potential of ionic liquids-basis for an eco-design of sustainable products. Green Chem. 7, 362–372.
Khodadadi-Moghaddam, M., Habibi-Yangjeh, A., Gholami, M.R., 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.
Kokorin, A. (Ed.), 2011. Ionic Liquids: Applications and Perspectives. InTechOpen, New York.
Masten, S.A., 2004. Review of Toxicological Literature for Ionic Liquids. Integrated Laboratory Systems Inc., Research Triangle Park, NC, USA.
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 1-alkyl-3-methylimidazolium ionic liquids observed in an (eco) toxicological test battery. Green Chem. 9, 1198–1207.
Matzke, M., Thiele, T., Müller, A., Filser, J., 2008a. Sorption and desorption of imidazolium based ionic liquids in different soil types. Chemosphere 74, 568–574.
Matzke, M., Stolte, S., Arning, J., Uebers, U., Filser, J., 2008b. Imidazolium based ionic liquids in soils: effects of the side chain length on wheat (*Triticum aestivum*) and cress (*Lepidium sativum*) as affected by different clays and organic matter. Green Chem. 10, 584–591.
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.
Modelli, A., Sali, A., Galletti, P., Samorì, C., 2008. Biodegradation of oxygenated and non-oxygenated imidazolium-based ionic liquids in soil. Chemosphere 73, 1322–1327.
Mohammad Ali, I. (Ed.), 2012. Green Solvents II: Properties and Applications of Ionic Liquids. Springer, New York, USA.
Mrozik, W., Kotłowska, A., Kamysz, W., Stepnowski, P., 2012. Sorption of ionic liquids onto soils: experimental and chemometric studies. Chemosphere 88, 1202–1207.
OECD, 2000a. Guideline for Testing of Chemicals 216, Soil Microorganisms: Nitrogen Transformation Test. Organization for Economic Cooperation and Development, Paris, France.
OECD, 2000b. Guideline for Testing of Chemicals 217, Soil Microorganisms: Carbon Transformation Test. Organization for Economic Cooperation and Development, Paris, France.
OECD, 2006. Guideline for Testing of Chemicals 208, Terrestial Plants Test: Seedling Emergence and Seedling Growth Test. Organization for Economic Cooperation and Development, Paris, France.
Olivier-Bourbigou, H., Magna, L., Morvan, D., 2010. Ionic liquids and catalysis: recent progress from knowledge to applications. Appl. Catal. A 373, 1–53.
Peric, B., Martí, E., Sierra, J., Cruañas, R., Iglesias, M., Garau, M.A., 2011. Terrestrial ecotoxicity of short aliphatic protic ionic liquids. Environ. Toxicol. Chem. 30, 2802–2809.
Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M.A., Arning, J., Bottin-Weber, U., Stolte, S., 2013. (Eco)toxicity and biodegradability of short aliphatic protic ionic liquids. J. Hazard. Mater. 261, 99–105.
Pessarakli, M. (Ed.), 2011. Handbook of Plant and Crop Stress, third ed. CRC Press, Taylor & Francis Group, Boca Raton, FL, USA.
Pham, T.P.T., Cho, C.W., Yun, Y.S., 2010. Environmental fate and toxicity of ionic liquids: a review. Water Res. 44, 252–272.
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.
Simek, M., Virtanen, S., Simojoki, A., Chroňáková, A., Elhottová, D., Krištůfek, V., Yli-Halla, M., 2013. The microbial communities and potential greenhouse gas production in boreal acid sulphate, non-acid sulphate, and reedy sulphidic soils. Sci. Total Environ. 466–467, 663–672.
Stolte, S., Abdulkarim, S., Arning, J., Blomeyer-Nienstedt, A.K., Bottin-Weber, U., Matzke, M., Ranke, J., Jastorff, B., Thöming, J., 2008. Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazolium chloride and electrochemical wastewater treatment of poorly biodegradable compounds. Green Chem. 10, 214–224.
Studzińska, S., Buszewski, B., 2009. Study of toxicity of imidazolium ionic liquids to watercress (*Lepidium sativum* L.). Anal. Bioanal. Chem. 393, 983–990.
Studzińska, S., Sprynskyy, M., Buszewski, B., 2008. Study of sorption kinetics of some ionic liquids on different soil types. Chemosphere 71, 2121–2128.
Studzińska, S., Kowalkowski, T., Buszewski, B., 2009. Study of ionic liquid cations transport in soil. J. Hazard. Mater. 168, 1542–1547.
United Nations, 2006. Globally Harmonized System of classification and labeling of chemicals. UN/SCEGHS/12/INF5. New York, USA.
US EPA, 1971. Brucine Method for Determination of Nitrates. Washington, DC, USA.
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.
Werner, S., Haumann, M., Wasserscheid, P., 2010. Ionic liquids in chemical engineering. Annu. Rev. Chem. Biomol. Eng. 1, 203–230.
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.
Zhang, C., Wang, H., Malhotra, S.V., Dodge, C.J., Francis, A.J., 2010. Biodegradation of pyridinium-based ionic liquids by an axenic culture of soil *Corynebacteria*. Green Chem. 12, 851–858.
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.

3.4. Paper 4

QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP (QSAR) PREDICTION OF (ECO)TOXICITY OF SHORT ALIPHATIC PROTIC IONIC LÍQUIDS

Summary

The number of combinations of cations and anions forming ionic liquids (ILs) is practically infinite and they can be custom designed in order to be suited for a desired application. But the complete information on their environmental impact is not available. It is clearly not feasible to analyze the toxicity of so many compounds. The application of *in silico* methods such as QSAR could speed up the process of (eco)toxicological evaluation and predict the potential toxicity of ILs before they are considered for commercialization. Choosing an appropriate QSAR model can allow reliable prediction of toxicity, avoiding unnecessary animal experiments, which is also encouraged by European Chemicals Agency (ECHA) and the EU regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). In this study, a group contribution QSAR model was used in order to predict the (eco)toxicity of protic and aprotic ionic liquids (PILs and AILs respectively). Some of the representatives of a new family of short aliphatic PILs have been previously investigated on their (eco)toxicity, both in water and soil, together with some selected AILs. A QSAR prediction of the EC_{50} values was performed and compared with the experimentally determined EC_{50} in five (eco)toxicity tests (Microtox®, *Pseudokirchneriella subcapitata* and *Lemna minor* growth inhibition test, and Acetylcholinestherase inhibition and Cell viability assay with IPC-81 cells), obtaining high correlation coefficients. Additionally, a prediction of EC_{50} for this set of (eco)toxicity tests was made for eight representatives of a new family of PILs, whose toxicity has not been determined to date. The QSAR model applied in this study can allow the selection of potentially less toxic ILs amongst the existing ones (as in the case of aprotic ILs already in use), but it can also be very helpful in directing the synthesis efforts toward developing new "greener" ILs respectful with the environment (e.g. short aliphatic protic ILs).

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Quantitative structure-activity relationship (QSAR) prediction of (eco)toxicity of the short aliphatic protic ionic liquids

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Highlights

• The EC_{50} for different protic and aprotic ILs in 5 (eco)toxicity tests were compiled.
• Group contribution QSAR model was used to predict EC_{50} values for ionic liquids (ILs).
• The experimental and predicted EC_{50} values are well correlated.
• The EC_{50} values for eight previously untested protic ILs were predicted.

Abstract

Ionic liquids (ILs) are considered as a group of very promising compounds due to their excellent properties (practical non-volatility, high thermal stability and very good and diverse solving capacity). The ILs have a good prospect of replacing the traditional organic solvents in vast variety of applications. However, the complete information on their environmental impact is still not available. There is also an enormous number of possible combinations of anions and cations which can form ILs, the fact that requires a method allowing to predict the toxicity of existing and potential ILs. In this study, a group contribution QSAR model was used in order to predict the (eco)toxicity of protic and aprotic ILs. The representatives of a new family of short aliphatic protic ILs have been previously investigated on their (eco)toxicity, both in water and soil, as well as some selected aprotic ILs. A QSAR prediction of the EC_{50} was performed and compared with the experimentally determined EC_{50} in five (eco)toxicity tests (Microtox®, *Pseudokirchneriella subcapitata* and *Lemna minor* growth inhibition test, and Acetylcholinestherase inhibition and Cell viability assay with IPC-81 cells), obtaining high correlation coefficients. Additionally, a prediction of EC_{50} for these (eco)toxicity tests was made for the eight representatives of the new family of protic ILs, whose toxicity has not been determined to date. The QSAR model applied in this study can allow the selection of potentially less toxic ILs amongst the existing ones (as in the case of aprotic ILs), but it can also be very helpful in directing the synthesis efforts toward developing new "greener" ILs respectful with the environment (e.g. short aliphatic protic ILs).

Keywords: Ionic liquids; QSAR modelling; Group contribution; Aquatic ecotoxicity; Cytotoxicity; Acetylcholinesterase inhibition

1. Introduction

Ionic liquids (ILs) have gained a lot of attention in the past decades because of their properties, such as: negligible vapor pressure, high thermal stability and very high and versatile solving capacity. These properties make them interesting for many applications, usually aiming to replace volatile organic and toxic solvents, which have been traditionally used in laboratories and industries up to now (Petkovic et al., 2011). Even though ILs are practically non-volatile and thus cannot get into atmosphere and cause air pollution, many of them are soluble in water and can represent a danger for the environment if released with industrial and laboratory effluents. There are many studies that demonstrate the aquatic toxicity of the ILs (Pham et al., 2010). The main focus has been on so called aprotic ionic liquids (AILs), namely the ones derived from imidazolium and pyridinium. The protic ionic liquids (PILs) have come into the focus of the investigators only recently and they show a good potential, both in their applications and "greenness". It is considered that the possible number of ILs is about 10^{18}, and around one million can be easily synthesized in the laboratory. Although dramatic reductions in air pollution would result from the use of these new compounds, water pollution may increase because many ILs are water soluble and would inevitably be released into wastewater, groundwater and aquatic environments (Docherty et al., 2007). The ILs are chemical products, and that means that the requirements of a new European Union regulation on chemical substances REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) apply to ILs as well (EU, 2006).

It is very important to discover the hazardous potential of the ILs before putting them into use, and taking into account such a large potential number of ILs, it is important to save up time and money and possibly do a pre-application screening in order to determine the potential negative effect of the ILs on the environment. As the in depth studies of water and terrestrial ecotoxicity of ILs are either incomplete or missing, the prediction of the environmental impact of the new and existing, but not yet tested ILs is a necessity for the widespread application of the ILs. *In silico* methods such as QSAR (Quantitative Structure-Activity Relationship) can be used for the prediction of toxicity and thus reduce the costly and time consuming toxicity testing, either *in vitro* or *in vivo*. The use of the QSAR modelling for the prediction of toxicity is recognized by REACH regulation, stating that properties determined with the use of *in silico* methods are equivalent to laboratory testing. The QSAR modelling, as its name indicates, provides toxicity data based on the quantitative relationship between a chemical structure and its biological and/or toxicological activity, using the chemical descriptors generated from the molecular structure. These descriptors are statistically analyzed in order to develop a model that would describe the desired activity e.g. toxicity (Amberg, 2013). The QSAR

studies on toxicity of ILs have been far less frequent compared to other chemicals (Das and Roy, 2013).

A new family of short aliphatic PILs has been thoroughly investigated on their (eco)toxicity and biodegradability, both in water and soil, together with comparative studies regarding the most frequently used AILs (Peric et al., 2013, Peric et al., 2014). The aim of this study was to apply the QSAR model described by Luis et al. (2007) on the EC_{50} values of ILs obtained in different studies and thus be able to predict the EC_{50} of the PILs that have not been tested yet. A prediction of EC_{50} was done for five (eco)toxicity tests (Microtox® test with bacteria *Vibrio fischeri*, green algae *Pseudokirchneriella subcapitata* and aquatic plant *Lemna minor* growth inhibition test, Acetylcholinestherase inhibition and Cell viability assay with IPC-81 cells).

2. Material and methods

In order to perform the QSAR modelling, a database of the EC_{50} values of ILs for different (eco)toxicity tests was built. The experimentally obtained EC_{50} values for thirteen ILs, of which ten are PILs and three are AILs, are taken from Peric et al. (2013 and 2014). The remaining forty-two EC_{50} values were taken from the literature (Table 1). The structures of PILs and AILs included in this study are shown in Fig. 1 and Fig. 2 respectively. The Fig. 3 shows cations and anions of different ILs whose EC_{50} values were taken from the literature and were also used in the process of QSAR modelling. These ILs are derived from disubstituted imidazolium, pyridinium and pyrrolidinium and were selected in order to have a larger database of EC_{50} values. The PILs belong to a family of short aliphatic ionic liquids: 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxyethanolamine butanoate (2-HEAB), 2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine propionate (2-HDEAPr), 2-hydroxydiethanolamine butanoate (2-HDEAB), 2-hydroxydiethanolamine isobutanoate (2-HDEAiB), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), 2-hydroxytriethanolamine butanoate (2-HTEAB) and 2-hydroxytriethanolamine pentanoate (2-HTEAPe). The experimentally analyzed AILs are: 1-butyl-3-methylimidazolium chloride ($[C_4MIM][Cl]$), 1-methyl-3-octylimidazolium chloride ($[C_8MIM][Cl]$) and N-butylpyridinium chloride ($[C_4Py][Cl]$). Using the QSAR model, a prediction of EC_{50} values for the remaining eight ILs from the new family of PILs was made: 2-hydroxyethanolamine acetate (2-HEAA), 2-hydroxyethanolamine propionate (2-HEAPr), 2-hydroxyethanolamine isobutanoate (2-HEAiB), 2-hydroxyethanolamine pentanoate (2-HEAPe), 2-hydroxytriethanolamine formate (2-HTEAF), 2-hydroxytriethanolamine acetate (2-HTEAA) and 2-hydroxytriethanolamine propionate (2-HTEAPr), 2-hydroxytriethanolamine isobutanoate (2-HTEAiB).

Fig. 1.

Fig. 2.

Fig. 3.

3. Results and discussion

The QSAR model was based on the group contribution method, proposed by Luis et al. (2007) where molecular properties can be viewed as a sum of the contributions of its atoms and/or fragments. Every ILs has been divided into basic fragments/groups, which can contribute to toxicity. The groups were encoded in a Boolean manner (0/1), meaning that they are equal to 1 if the group is present in the ILs molecule and 0 if not. A detailed overview of all of the molecular descriptors can be found in the Table 1 and Table 2. Toxicity data has been expressed on molar basis in order to be comparable between structurally different ILs. The EC_{50} values were converted into a logarithmic scale and the toxicity was expressed as the calculated dimensionless ecotoxicity (Y*), with values between 0 and 1 according to the following formula:

$$Y^* = \frac{\log EC_{50\_max} - \log EC_{50}}{\log EC_{50\ max} - \log EC_{50\ min}}$$

where log EC_{50}_max and log EC_{50}_min are the highest and the lowest EC_{50} value in the EC_{50} database, respectively. The log EC_{50} is the logarithm of the experimentally obtained EC_{50} value. The predicted dimensionless ecotoxicity used in this model represents the sum of the contributions of all of the groups present in the ILs molecule, as presented in the following equation:

$$Y^* = \sum_i a_i \cdot A_i + \sum_j c_j \cdot C_j + \sum_k s_k \cdot S_k$$

The structure of the ILs is described by three main groups: Anions (A), Cations (C) and Substitutions (S) of the cation. The A_i and C_j are variables that have value of 1 o 0, depending if the anion or cation are present or not in the molecule. For example, the value will be 1 if pyridinium is present in the ILs. On the other hand, the S_k has the value of the number of C atoms in the alkyl chains (R_1 and R_2). The a_i, c_j and s_k are the coefficients of regression representing the contribution of each group to ILs toxicity. Table 1 shows the group descriptors used in the QSAR modelling, the experimentally obtained value for the log EC_{50} in μmol L^{-1} for the ILs in the inhibition of the *Vibrio fischeri* luminescence assay (Microtox®) and the calculated dimensionless toxicity (Y*). The structure of the ILs has been described based on the main contributions of anions, cations and the side alkyl chains. Anions include the ones who appear in the AILs molecule: chloride (Cl^-), bromide (Br^-), tetrafluoroborate (BF_4^-), hexafluorophosphate

(PF_6^-), dicyanamide $N(CN_2)_2^-$, bis-trifluoromethan- sulfonimide $N(CF_3SO_2)_2^-$; and anions belonging to the PILs molecule: formate, acetate, propionate, butanoate, isobutanoate and pentanoate. The anions were grouped into four main descriptors, based on the similar contribution: A_1 (Cl^- and Br^-), A_2 (BF_4^- and PF_6^-), A_3 ($N(CN_2)_2^-$ and $N(CF_3SO_2)_2^-$) and A_4 (number of C atoms in the PILs anions). The AILs cations are imidazolium, pyridinium, and pyrrolidinium (Im, Py, and Pyr, respectively) and the PILs cations are monoethanolamine, diethanolamine or triethanolamine (Amine). Alkyl side chain substituents of the heterocyclic ring, from propyl to decyl (R_1) and the methyl substituent (R_2) were also considered.

Table 1

The data set of 55 EC_{50} values was fitted to the QSAR model by multiple linear regression using IBM SPSS Statistics 20 software. The calculated dimensionless toxicity obtained from experimental EC_{50} data and the predicted dimensionless ecotoxicity obtained after QSAR modelling were plotted and the data plot graph is shown in the Fig. 4.

Fig. 4.

A good fitting was achieved, with n=55, R^2=0.9184 and 10 descriptors. The distribution of residuals is presented in the Table 3. Residuals were calculated as the differences between the calculated dimensionless ecotoxicity and the one predicted by the QSAR method (taking into account the absolute values).

The distribution of the residuals and the Figure 4 indicate that there are practically no outliners. The Table 3 shows that 84% of the residuals are lower than 0.10. Both the most and the least toxic ILs were well predicted by the model, with the residuals being lower than 0.10. This means that there is a deviation of ±10% in the log EC_{50} ($\mu mol\ L^{-1}$) calculated applying the equation:

$$\log EC_{50}\ (\mu mol\ L^{-1}) = 4.55 - (4.55+0.18) \cdot Y^*$$

This equation was also used to predict the EC_{50} values for the eight PILs whose toxicity has not yet been tested.

All of the molecular descriptors are presented and explained in the Table 2, together with the corresponding group contributions and confidence intervals for the QSAR model. The anions were grouped in four groups in order to avoid the overfitting that may be caused by an excess of descriptors. The analysis of the contributions of each of the descriptors (Table 1) provides information on the influence of the structural groups on the ILs toxicity. The anions of the AILs have a negative contribution to toxicity. The contributions of anions show very small differences between three groups of AILs anions. The contribution of the cations is positive, which means that the presence of

either of these cations in the AILs molecules leads to an increase in toxicity. Many studies confirmed that the influence of cations on ILs toxicity is stronger than the one of anions (Ranke et al., 2004; Matzke et al., 2007; Stolte et al., 2007). Imidazolium, pyridinium and pyrrolidinium cation contribute around 27%, 17% and 3% to the toxicity, respectively. The imidazolium's higher contribution to toxicity and lower toxicity of pyrrolidinium cation compared to imidazolium and piridinium has been also found by other authors (Stolte et al., 2007; Pham et al., 2008, Latała et al., 2009). On the other hand, the cations and anions of the PILs do not exhibit a high overall influence on the ILs toxicity, if they are analyzed in the present set of ILs. This coincides with the fact that the PILs showed much lower toxicity than the AILs in the Microtox® test. The amines have a stronger influence on toxicity towards *Vibrio fischeri* in the Microtox® test as the contribution of the amine is five times higher than the one for the acid part of the PILs molecule. These results are in agreement with those reported by Peric et al. (2013) where a higher correlation between the complexity of the cationic moiety and toxicity was found.

The contributions for the number of carbon atoms in long alkyl side chains indicates that with every carbon added to the side chain, an increase of about 13% in toxicity of AILs is produced. The presence of the short chain also has a positive influence on toxicity. These two results are in agreement with other authors findings that the substitution of the cation ring and the increase of the side alkyl chain length leads to an increase of toxicity (Ranke et al., 2004; Wells and Coombe, 2006, Pham et al., 2010; Radošević et al., 2013).

Table 2

In summary, the here presented QSAR model has 10 descriptors and could allow the prediction of the (eco)toxicity of existing and potential ILs, resulting from the possible combinations of 11 types of anions, 6 types of cations and 8 types of different substituents of the cationic moiety.

Using this QSAR model, EC_{50} values for ILs that have not been experimentally analyzed can be predicted. This was done for the 8 representatives of the new family of short aliphatic PILs. The results can be found in the Table 5.

Having achieved a good fitting with the Microtox® test results, the same QSAR model was used for four more tests in order to predict the ILs (eco)toxicity: aquatic ecotoxicity (toxic effect on algae and aquatic plant *Lemna minor*), inhibition of acetylcholinesterase (AChE) and cytotoxicity (IPC-81 cells). The fitting plots for the calculated and predicted Y* can be seen in the Fig. 5.

Fig. 5.

In case of these four (eco)toxicity tests a good fitting was also achieved, with R^2 being 0.866 for the Test of inhibition of algal growth (n=32), 0.901 for the *Lemna minor* growth inhibition test (n=24), 0.9918 for the Acetylcholinestherase inhibition test (n=61) and 0.9799 for the Cytotoxicity test with IPC-81 cell line (n=45).

The distribution of residuals for the QSAR modelling for the four (eco)toxicity tests is presented in the Table 3.

Table 3

The group contributions and confidence intervals are shown in the Table 4. All but one (eco)toxicity test (Acetylcholinestherase inhibition) shows negative, very low or no contribution of the anions to the toxicity, meaning that the selected anions do not play a key role in AILs toxicity, which, as previously mentioned, coincides with the literature findings. The contribution of the cations is the strongest in the case of the Acetylcholinesterase inhibition test, both for AILs and PILs. There is also a positive influence of anions for this test. This can be explained with the different toxicity mode of action between the Acetylcholinesterase inhibition test and the other three tests. While in Algal and *Lemna minor* growth inhibition test and Cell viability assay with IPC-81 the toxicity is based on the membrane disruption, the Acetylcholinesterase inhibition test involves binding to the enzyme's active center. As initially found by Stock et al. (2004), acetylcholinesterase can be inhibited by ILs containing a cation with positively charged nitrogen and certain lipophilicity. The study of Arning et al. (2008) revealed that the pyridinium derived ILs show stronger inhibition than the imidazolium ones, which has also been confirmed in this study, with the contribution to toxicity of pyridinium being two times higher than the one for imidazolium. The anions do not show inhibitory effect on the enzyme activity with only exception of the fluoride and fluoride containing anions (Matzke et al., 2007), also confirmed by the results of this study. We also found a stronger correlation of PILs toxicity with cationic moiety in our previous study (Peric et al., 2013), as presented in the Table 4.

Table 4

Pyrrolidinium cation showed the lowest contribution to the ILs toxicity, as found by Stolte et al. (2007). The statistical models applied by Roy et al. (2014) also demonstrated that aromatic ILs are more toxic than non-aromatic and aliphatic ones.

Also in case of these four tests, the cations and anions of the PILs do not exhibit a high overall influence on the ILs toxicity, if they are analyzed in the present set of ILs. Also in case of these four tests, the PILs showed much lower toxicity than the AILs in all of the (eco)toxicity tests. The amines have a stronger influence on toxicity towards acetylcholinesterase, and the acids in the anionic moiety have a stronger contribution to the toxicity towards *P. subcapitata*, *L. minor* and IPC-81. These results are also in agreement with those reported by Peric et al. (2013) where good correlation between the

acid moieties and toxicity was found for these tests. It can be seen that both PILs cation and anion contribute to toxicity in the Test of *Lemna*'s growth inhibition, which coincides with our findings that *L. minor* is the most sensitive species in the case of the aquatic ecotoxicity tests (Peric et al., 2013).

Once again, the results for group contribution indicate that there is an increasing toxicity with an increase in the alkyl chain length. The influence of the long side alkyl chains is predominant, as the short alkyl chains show negative, very low o zero influence in the AILs toxicity. This influence also coincides with the strong relationship between the (eco)toxicity of ILs and its hydrophobic character described in the literature (Ranke et al., 2007, Stolte et al., 2007, Cvjetko Bubalo et el., 2014), meaning that longer the alkyl chain, the higher the hydrophobic character of the ionic liquid, the fact that facilitates the ILs molecule's penetration through cell membranes and thus potentiates their toxicity. A correlation between an increasing chain length of the side chains connected to the cationic head groups and an enhanced inhibitory potential of the ILs was found in other author's studies (Stock et al., 2004, Arning et al., 2008) and confirmed in this study.

As in the case of Microtox®, from the QSAR model, the EC_{50} values for the eight representatives of the new family of short aliphatic PILs that have not been experimentally obtained were predicted. The results for the four toxicity tests (Algal and *Lemna minor* growth inhibition test, and Acetylcholinestherase inhibition and Cell viability assay with IPC-81 cells) can be found in the Table 5. The model proposed by Luis et al. (2007), although initially designed for the AILs, seems to be adequate for the PILs too.

Table 5

4. Conclusions

The model based on group contribution used in this work allows the prediction of the EC_{50} values of aprotic and protic ILs. A good correlation between the experimental and predicted EC_{50} data was obtained and the EC_{50} values for eight new, until the date untested PILs, were predicted. This model can also help to determine the positive or negative influence of different structural groups on the (eco)toxicity and in that way "guide" the synthesis of the new ILs toward more sustainable ones. Literature findings of the predominant influence of cations on toxicity and the increase of toxicity with the elongation of the side alkyl chains in AILs molecule were confirmed by the results of this study. In the case of new PILs, both anionic and cationic part of the molecule have influence on (eco)toxicity, and it is the elongation of the acid in the anionic moiety which has more influence on the overall toxicity in three of the five (eco)toxicity tests (algae, aquatic plants and cytotoxicity). The QSAR models can estimate not only the toxicity of the existing ILs, but also offer the ability to predict the effects of new ILs

that could be synthesized in order to suite the desired application. *In silico* models are also a lot quicker than the standard laboratory toxicity tests and thus can save up time and money. This kind of QSAR model can be of useful for the designers of ILs, because it allows them to evaluate the toxicity of an ILs with the desired application potential.

Acknowledgments

B. Peric would like to thank the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development (Grant MAEC-AECID).

The authors would like to thank Francesc Fàbrega for his advice on the initial QSAR modelling and Dr. Jesús Gómez for his valuable suggestions and comments.

References:

Amberg, A., 2013. *In silico* methods, in: Vogel, H.G., Maas, J., Hock, F.J., Mayer, D. (Eds.), Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays. Springer, Berlin Heidelberg, pp. 1273–1296.

Arning, J., Stolte, S., Böschen, A., Stock, F., Pitner, W.-R., Welz-Biermann, U., Jastorff, B., Ranke, J., 2008. Qualitative and quantitative structure activity relationships for the inhibitory effects of cationic head groups, functionalized side chains and anions of ionic liquids on acetylcholinesterase. Green Chem. 10, 47–58.

Couling, D.J., Bernot, R.J., Docherty, K.M., Dixon, J.K., Maginn, E.J., 2006. Assessing the factors responsible for ionic liquid toxicity to aquatic organisms via quantitative structure-property relationship modeling. Green Chem. 8, 82–90.

Cvjetko Bubalo, M., Radošević, K. Radojčić Redovniković, I., Halambek, J., Gaurina Srček, V, 2014. A brief overview of the potential environmental hazards of ionic liquids. Ecotoxicol. Environ. Saf. 99, 1–12.

Das, R.N., Roy, K., 2013. Advances in QSPR/QSTR models of ionic liquids for the design of greener solvents of the future. Mol. Divers. 17, 151–96.

Docherty, K.M., Dixon, J.K., Kulpa, C.F., 2007. Biodegradability of imidazolium and pyridinium ionic liquids by an activated sludge microbial community. Biodegradation. 18, 481–493.

Docherty, K.M., Kulpa, C.F., 2005. Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. Green Chem. 7, 185–189.

European Union, Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). 2006. Off. J. Eur. Union. 396, 1–856.

Garcia, M.T., Gathergood, N., Scammells, P.J., 2005. Biodegradable ionic liquids. Part II: effect of the anion and toxicology. Green Chem. 7, 9–14.

Latała, A., Nędzi, M., Stepnowski, P., 2009. Toxicity of imidazolium and pyridinium based ionic liquids towards algae. *Chlorella vulgaris*, *Oocystis submarina* (green algae) and *Cyclotella meneghiniana*, *Skeletonema marinoi* (diatoms). Green Chem. 11, 580–588.

Luis, P., Ortiz, I., Aldaco, R., Irabien, A., 2007. A novel group contribution method in the development of a QSAR for predicting the toxicity (*Vibrio fischeri* EC50) of ionic liquids. Ecotoxicol. Environ. Saf. 67, 423–429.

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 1-alkyl-3-methylimidazolium ionic liquids observed in an (eco)toxicological test battery. Green Chem. 9, 1198–1207.

Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M.A., Arning J., Bottin-Weber U., Stolte S., 2013. (Eco)toxicity and biodegradability of short aliphatic protic ionic liquids. J. Hazard. Mater. 261, 99–105.

Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M.A., 2014. A comparative study of the terrestrial ecotoxicity of selected protic and aprotic ionic liquids. Chemosphere. 108, 418–425.

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

Pham, T.P.T., Cho, C.-W., Min, J., Yun, Y.-S., 2008. Alkyl-chain length effects of imidazolium and pyridinium ionic liquids on photosynthetic response of *Pseudokirchneriella subcapitata*. J. Biosci. Bioeng 105, 425–428.

Pham, T.P.T., Cho, C.W., Yun, Y.S., 2010. Environmental fate and toxicity of ionic liquids: a review. Water Res. 44, 252–272.

Radošević, K., Cvjetko, M., Kopjar, N., Novak, R., Dumić, J., Gaurina Srček, V., 2013. *In vitro* cytotoxicity assessment of imidazolium ionic liquids: biological effects in fish Channel Catfish Ovary (CCO) cell line. Ecotoxicol. Environ. Saf. 92, 112–118.

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 length in acute *Vibrio fischeri* and WST-1 cell viability assays. Ecotoxicol. Environ. Saf. 58, 396–404.

Ranke, J., Müller, A., Bottin-Weber, U., Stock, F., Stolte, S., Arning, J., Störman, R., Jastroff, B., 2007. Lipophilicity parameters for ionic liquid cations and their correlation to in vitro cytotoxicity. Ecotoxicol. Environ. Saf. 67, 430–438.

Ranke, J., Stolte, S., Störmann, R., Arning, J., Jastorff, B., 2007. Design of sustainable chemical products – the example of ionic liquids. Chem. Rev. 107, 2183–2206

Roy K., Das R.N., Popelier P.L.A, 2014. Quantitative structure–activity relationship for toxicity of ionic liquids to Daphnia magna: Aromaticity vs. lipophilicity. Chemosphere, 112, 120–127.

Stock, F., Hoffmann, J., Ranke, J., Störmann, R., Ondruschka, B., Jastorff, B., 2004. Effects of ionic liquids on the acetylcholinesterase – a structure-activity relationship consideration. Green Chem. 6, 286–290.

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

Wells, A.S., Coombe, V.T., 2006. On the freshwater ecotoxicity and biodegradation properties of some common ionic liquids. Org. Pro. Res. Dev. 10, 794–798.

Figure captions

Fig. 1. Structures and abbreviations of the studied PILs.

Fig. 2. Structures and abbreviations of the analyzed AILs.

Fig. 3. Structures of AILs used in QSAR modelling.

Fig. 4. Calculated and predicted dimensionless ecotoxicity data plot for Microtox® test.

Fig. 5. Calculated and predicted dimensionless ecotoxicity data plot for Algal and *Lemna minor* growth inhibition and Cell viability assay with IPC-81 cells.

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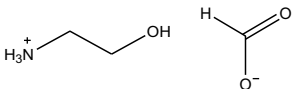
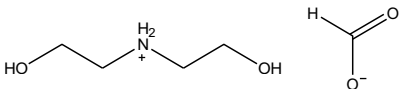
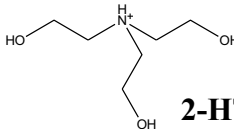
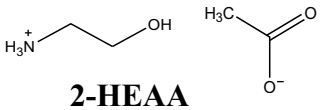
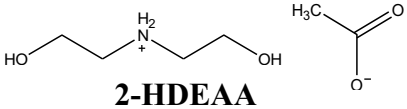
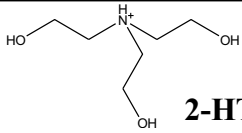
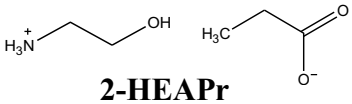
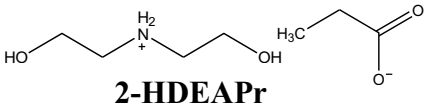
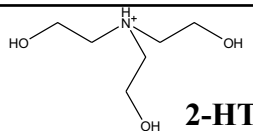
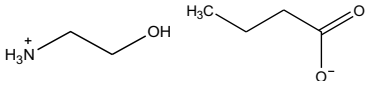
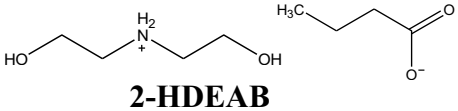
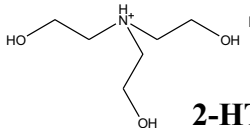
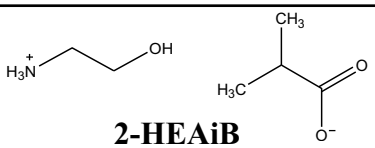
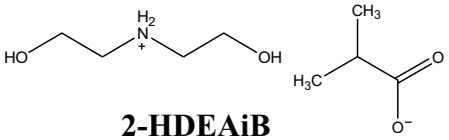
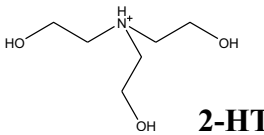
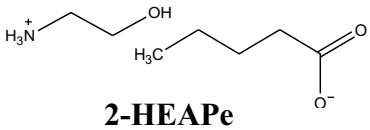
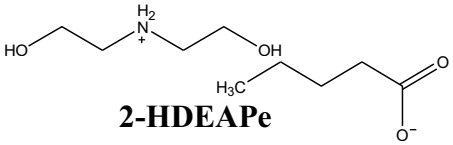
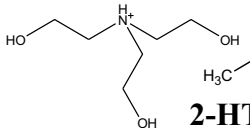
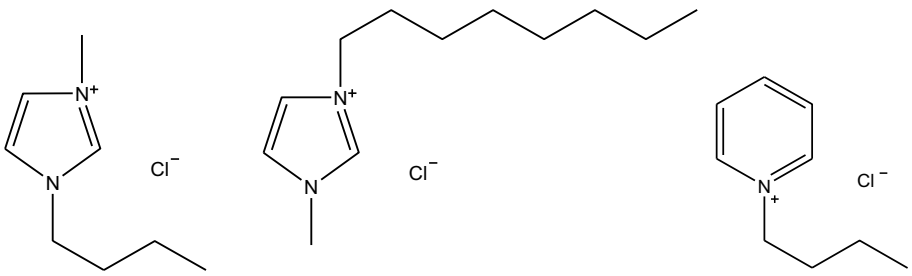
Formic acid	 **2-HEAF**	 **2-HDEAF**	 **2-H**
Acetic acid	 **2-HEAA**	 **2-HDEAA**	 **2-H**
Propionic acid	 **2-HEAPr**	 **2-HDEAPr**	 **2-HT**
Butiric acid	 **2-HEAB**	 **2-HDEAB**	 **2-H**
Isobutiric acid	 **2-HEAiB**	 **2-HDEAiB**	 **2-HT**
Pentanoic acid	 **2-HEAPe**	 **2-HDEAPe**	 **2-HT**

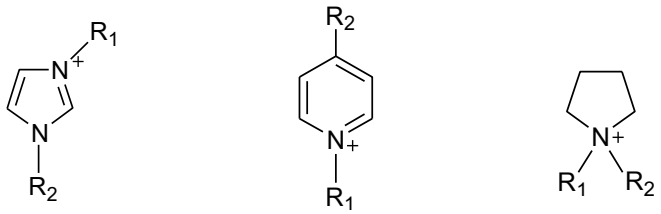
Fig. 1.



[C_4MIM][Cl] [C_8MIM][Cl] [C_4Py][Cl]

Fig. 2.

Disubstituted cations



Imidazolium (Im) Pyridinium (Py) Pyrrolidinium (Pyr)

Anions

Cl^-, Br^-, BF_4^-, PF_6^-, $(CN)_2N^-$, $(CF_3SO_2)_2N^-$

Fig. 3.

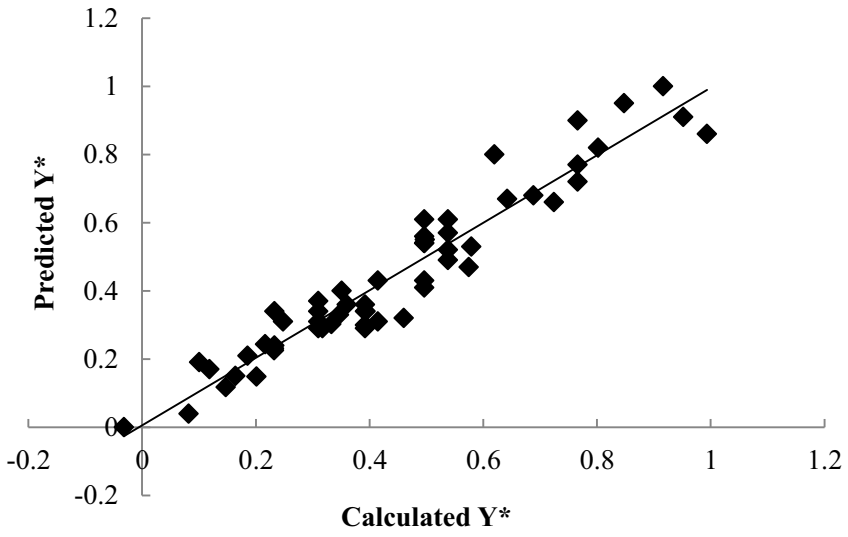


Fig. 4.

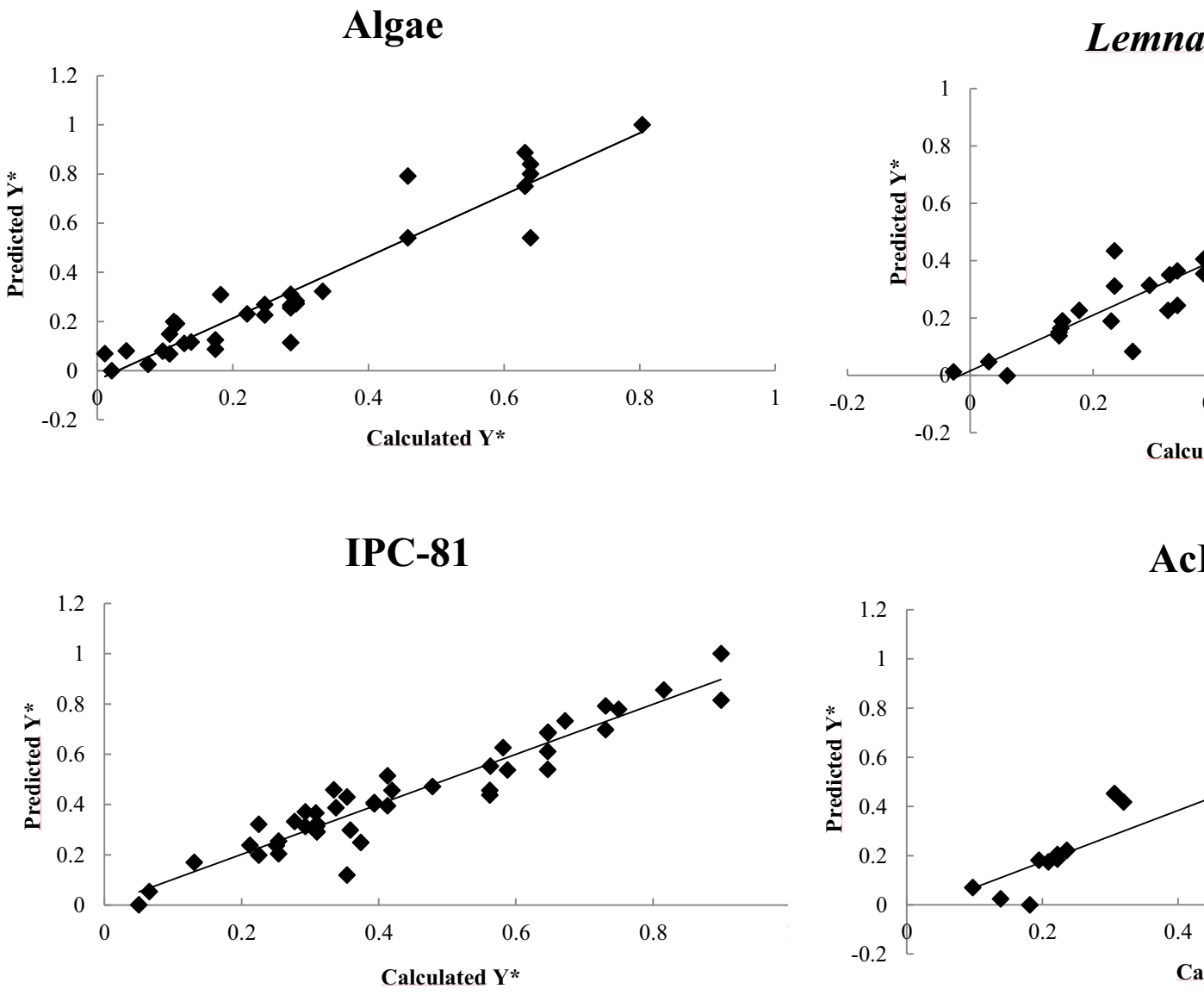

Algae
Predicted Y*
Calculated Y*
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.2
0.4
0.6
0.8
1
Lemna
Predicted Y*
Calcu
-0.2
0
0.2
IPC-81
Predicted Y*
Calculated Y*
Acl
Predicted Y*
Ca

Fig. 5.

Table 1
Ionic liquid toxicities in μmol L^{-1} ($logEC_{50}$) for the Microtox® test, calculated dimensionless ecotoxicity (Y*)

No.	Compound	log EC_{50} (μmol L^{-1})	Y*	A_1	A_2	A_3	A_4	Im	Py
1	$[C_2MIM][Cl]$	4.55	0.00	1	0	0	0	1	0
2	$[C_2MIM][Cl]$	4.33	0.05	1	0	0	0	1	0
3	$[C_3MIM][BF_4]$	3.94	0.13	0	1	0	0	1	0
4	$[C_3MIM][PF_6]$	3.60	0.20	0	1	0	0	1	0
5	$[C_4MIM][Cl]$	3.71	0.18	1	0	0	0	1	0
6	$[C_4MIM][Cl]$	2.95	0.34	1	0	0	0	1	0
7	$[C_4MIM][Cl]$	3.34	0.26	1	0	0	0	1	0
8	$[C_4MIM][Cl]$	3.21	0.28	1	0	0	0	1	0
9	$[C_4MIM][Br]$	3.35	0.25	1	0	0	0	1	0
10	$[C_4MIM][Br]$	3.27	0.27	1	0	0	0	1	0
11	$[C_4MIM][Br]$	3.07	0.31	1	0	0	0	1	0
12	$[C_4MIM][BF_4]$	3.55	0.21	0	1	0	0	1	0
13	$[C_4MIM][BF_4]$	3.10	0.31	0	1	0	0	1	0
14	$[C_4MIM][(CF_3SO_2)_2N]$	3.39	0.25	0	0	1	0	1	0
15	$[C_4MIM][(CN)_2N]$	3.67	0.19	0	0	1	0	1	0
16	$[C_5MIM][BF_4]$	3.18	0.29	0	1	0	0	1	0
17	$[C_6MIM][Cl]$	1.94	0.55	1	0	0	0	1	0
18	$[C_6MIM][Cl]$	2.32	0.47	1	0	0	0	1	0
19	$[C_6MIM][Cl]$	2.18	0.50	1	0	0	0	1	0
20	$[C_6MIM][Cl]$	2.91	0.34	1	0	0	0	1	0
21	$[C_6MIM][Br]$	1.42	0.66	1	0	0	0	1	0
22	$[C_6MIM][BF_4]$	3.15	0.30	0	1	0	0	1	0

23	$[C_6MIM][PF_6]$	2.17	0.50	0	1	0	0	1	0
24	$[C_6MIM][PF_6]$	3.07	0.31	0	1	0	0	1	0
25	$[C_8MIM][Cl]$	1.19	0.71	1	0	0	0	1	0
26	$[C_8MIM][Cl]$	1.01	0.75	1	0	0	0	1	0
27	$[C_8MIM][Cl]$	0.30	0.90	1	0	0	0	1	0
28	$[C_8MIM][BF_4]$	1.41	0.66	0	1	0	0	1	0
29	$[C_8MIM][PF_6]$	0.95	0.76	0	1	0	0	1	0
30	$[C_{10}MIM][Cl]$	0.5	0.86	1	0	0	0	1	0
31	$[C_{10}MIM][BF_4]$	-0.18	1.00	0	1	0	0	1	0
32	$[C_4Py][Cl]$	3.41	0.24	1	0	0	0	0	1
33	$[C_4Py][Cl]$	2.64	0.40	1	0	0	0	0	1
34	$[C_4Py][Cl]$	3.24	0.28	1	0	0	0	0	1
35	$[C_4Py][Br]$	3.40	0.24	1	0	0	0	0	1
36	$[C_4Py][Br]$	2.73	0.38	1	0	0	0	0	1
37	$[C_4Py][(CN)_2N]$	2.61	0.41	0	0	1	0	0	1
38	$[C_4MPy][Br]$	2.12	0.51	1	0	0	0	0	1
39	$[C_4MPy][Br]$	2.75	0.38	1	0	0	0	0	1
40	$[C_4MPy][(CN)_2N]$	1.99	0.54	0	0	1	0	0	1
41	$[C_6MPy][Cl]$	1.44	0.66	1	0	0	0	0	1
42	$[C_6MPy][Br]$	1.48	0.65	1	0	0	0	0	1
43	$[C_4MPyr][(CF_3SO_2)_2N]$	2.54	0.42	0	0	1	0	0	0
44	$[C_6MPyr][Cl]$	2.99	0.33	1	0	0	0	0	0
45	$[C_8MPyr][Br]$	0.25	0.91	1	0	0	0	0	0
46	2-HEAF	3.82	0.16	0	0	0	1	0	0
47	2-HEAB	4.18	0.08	0	0	0	4	0	0
48	2-HDEAF	3.72	0.17	0	0	0	1	0	0
49	2-HDEAA	4.03	0.11	0	0	0	2	0	0
50	2-HDEAPr	3.56	0.21	0	0	0	3	0	0

51	2-HDEAB	3.62	0.20	0	0	0	4	0	0
52	2-HDEAiB	3.64	0.19	0	0	0	4	0	0
53	2-HDEAPe	3.23	0.28	0	0	0	5	0	0
54	2-HTEAB	3.32	0.26	0	0	0	4	0	0
55	2-HTEAPe	3.26	0.27	0	0	0	5	0	0

References: 5, 15, 16, 22, 32, 35, 37 and 39 from Couling et al., (2006); 6, 9, 21, 33, 36, 38, 40 and 45 from D
13, 18, 25 and 29 from Garcia et al. (2005); 1, 17, 41, 42 and 44 from Luis et al. (2007); 2, 14, 26 and 43 fron
19, 20, 23, 24, 28, 30 and 31 from Ranke et al., (2007); 8, 27, 34 and 46-55 from Peric et al., 2013.

Table 2
Significance and contribution of the descriptors used in the QSAR modelling.

Group	Molecular descriptor	Comments	Contribution
Anion	A_1	Influence of anions: chloride (Cl^-), bromide (Br^-). Value is 1 if it exists and 0 if not.	-0.269
	A_2	Influence of anions: hexafluorophosphate (PF_6^-), tetrafluoroborate ($BF4^-$). Value is 1 if it exists and 0 if not.	-0.300
	A_3	Influence of anions: dicyanamide $N(CN_2)_2^-$, bis-trifluoromethan- sulfonimide $N(CF_3SO_2)_2^-$. Value is 1 if it exists and 0 if not.	-0.251
	A_4	Influence of number of carbons in PILs anion. Value of A_4: 1 to 5	0.016
Cation	Im - Imidazolium	Influence of imidazolium cation. Value = 1 if it exists and 0 if not.	0.274
	Py - Pyridinium	Influence of pyridinium cation. Value = 1 if it exists and 0 if not.	0.175

	Pyr - Pyrrolidinium	Influence of pyrrolidinium cation. Value = 1 if it exists and 0 if not.	0.035
	Amine	Influence of amine. Value = 1, 2 or 3 in function of the complexity of the PILs cation	0.085
Cation subsitution	R_1	Influence of number of carbons in long chains of the AILs molecule. Value of R_1: 0 to 10	0.132
	R_2	Influence of number of carbons in short chains of the AILs molecule. Value of R_1: 1 if methyl group exists and 0 if not.	0.112

Table 3

Distribution of residuals between the calculated and predicted dimensionless (eco)toxicity for Microtox® t
inhibition test, Acetylcholinesterase inhibition and Cell viability assay with IPC-81 cells.

	Microtox		Algae		*Lemna minor*		AChE		IPC-81	
Range	Residuals	%	Residuals	%	Residuals	%	Residuals	%	Residuals	%
<0,10	46	84	23	72	20	83	53	87	41	91
0,10-0,20	8	14	4	12	4	17	8	13	3	7
>0,20	1	2	5	16	0	0	0	0	1	2
<0,28	55	100	32	100	24	100	61	100	45	100

Table 4
The group contributions to calculated dimensionless ecotoxicity.

		Algae			*Lemna minor*			AChE		
Group	Molecular descriptor	Contribution	Confidence interval (95%)		Contribution	Confidence interval (95%)		Contribution	Confidence inte (95%)	
Anion	A_1	-0.098	-0.225	0.029	-0.244	-0.357	-0.131	0.023	-0.031	0.0
	A_2	-0.091	-0.233	0.041	-0.231	-0.349	-0.113	0.019	-0.037	0.0
	A_3	-0.051	-0.182	0.080	-0.419	-0.541	-0.297	0.000	0.000	0.0
	A_4	0.032	-0.048	0.111	0.087	0.003	0.174	0.014	-0.020	0.0
Cation	Im	0.075	-0.064	0.214	0.005	-0.023	0.033	0.361	0.312	0.4
	Py	0.000	0.000	0.000	0.048	-0.061	0.157	0.699	0.629	0.7
	Pyr	0.000	0.000	0.000	0.085	0.035	0.140	0.000	0.000	0.0
	Amine	-0.010	-0.146	0.126	-0.057	-0.206	0.092	0.084	0.027	0.1
Substitution	R_1	0.086	0.048	0.124	0.141	0.057	0.224	0.018	0.010	0.0
	R_2	-0.036	-0.187	0.114	0.000	0.000	0.000	0.000	0.000	0.0

Table 5

The predicted values of log EC_{50} (μmol L^{-1}) for Microtox® test, Algal and *Lemna minor* growth inhibition t and Cell viability assay with IPC-81 cells for eight representatives of the new family of PILs.

	Microtox	Algae	*L. minor*	AChE	IPC-81
2-HEAA	4.19	3.94	3.22	4.36	4.12
2-HEAPr	4.11	3.68	2.94	4.31	3.81
2-HEAiB	4.03	3.43	2.67	4.26	3.49
2-HEAPe	3.96	3.18	2.40	4.21	3.17
2-HTEAF	4.26	4.19	3.49	4.41	4.44
2-HTEAA	3.35	4.10	3.57	3.74	4.25
2-HTEAPr	3.27	3.85	3.30	3.69	3.93
2-HTEAiB	3.19	3.59	3.03	3.64	3.61

DISCUSSION

4. DISCUSSION

Aquatic, cellular and terrestrial toxicity, together with potential biodegradability in water and soil of selected PILs and AILs was determined in this thesis. For all ILs tested dose-response curves were obtained and EC_{50} values were calculated. The classification of the hazard potential of the ILs was done according to the Globally Harmonized System of Classification and Labelling of Chemicals (United Nations, 2006 and 2013), both for aquatic and terrestrial environment. The classification criteria for hazardous substances according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), both for aquatic and terrestrial environment, can be found in the Table 4. For the tests of inhibition of acetylcholinesterase and cytotoxicity towards IPC-81, the tested ILs were classified according to the UFT/Merck Ionic Liquids Biological Database (last accessed: 15th of July 2014). The classification criteria are presented in the Table 5.

Table 4. The classification criteria for hazardous substances for aquatic and terrestrial environment according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Hazard category (GHS 2013)	**EC_{50} (mg L^{-1})**	**Hazard communication**
Aquatic environment		
Acute 1	≤ 1	Very toxic for aquatic life
Acute 2	>1 but ≤ 10	Toxic
Acute 3	>10 but ≤ 100	Harmful
	> 100	Non-toxic
Hazard category (GHS 2006)	**EC_{50} (mg kg^{-1})**	**Hazard communication**
Terrestrial environment		
Substances hazardous to soil micro-organisms		
Chronic 1	≤ 1	Very toxic for terrestrial life
Chronic 2	>1 but ≤ 10	Toxic
Chronic 3	>10 but ≤ 100	Harmful
	> 100	Non-toxic
Substances hazardous to soil dwelling macro-organisms: invertebrates and plants (soil exposure)		
Acute 1	≤ 10	Very toxic for terrestrial life
Acute 2	>10 but ≤ 100	Toxic
Acute 3	>100 but ≤ 1000	Harmful
	> 1000	Non-toxic

Table 5. The classification of toxicity exhibited in Acetylcholinesterase inhibition test and the Test of toxicity towards IPC-81 cell line according to UFT/Merck Ionic Liquids Biological Database.

EC_{50} (mmol L^{-1})	
Acetylcholinesterase inhibition	
< 0,01	very high inhibition potential
0,01 - 0,1	high inhibition potential
0,1 - 1	moderate inhibition potential
> 1	low inhibition potential
Cytotoxicity towards IPC-81	
< 0,001	very high cytotoxicity
0,001 - 0,1	high cytotoxicity
0,1 - 5	moderate cytotoxicity
> 5	low cytotoxicity

The overview of the effects for the of the analyzed ILs in different (eco)toxicity and biodegradability tests is presented in the table 6.

Table 6. The overview of the effects of the analyzed ILs in different (eco)toxicity and biodegradability tests.

	GHS (2013) aquatic environment			OECD 301 guideline	GHS (2006) terrestrial environment					UFT Merck ILs Biological Database	
	V. fischeri	*P. subcapitata*	*L. minor*	Biodegradability in water	*A. cepa*	*L. perenne*	*R. sativus*	Soil micro-organisms	Soil respiration curves	AChE	IPC-81
2-HEAF	non-toxic	non-toxic	non-toxic	readily biodegradable	non-toxic	non-toxic	non-toxic	non-toxic	Type1	no inhibition	low cytotoxicity
2-HEAB	non-toxic	non-toxic	Acute 3	readily biodegradable	non-toxic	Acute 3	non-toxic	non-toxic	Type 2	no inhibition	moderate cytotoxicity
2-HDEAF	non-toxic	non-toxic	non-toxic	potentially biodegradable	non-toxic	non-toxic	non-toxic	non-toxic	Type 1	no inhibition	non-cytotoxic
2-HDEAA	non-toxic	non-toxic	non-toxic	readily biodegradable	non-toxic	Acute 3	non-toxic	non-toxic	Type 1	no inhibition	low cytotoxicity
2-HDEAPr	non-toxic	non-toxic	non-toxic	readily biodegradable	non-toxic	non-toxic	non-toxic	non-toxic	Type 3	no inhibition	low cytotoxicity
2-HDEAB	non-toxic	non-toxic	Acute 3	readily biodegradable	non-toxic	non-toxic	non-toxic	non-toxic	Type 3	no inhibition	moderate cytotoxicity
2-HDEAiB	non-toxic	non-toxic	Acute 3	readily biodegradable	non-toxic	non-toxic	non-toxic	non-toxic	Type 2	no inhibition	moderate cytotoxicity
2-HDEAPe	non-toxic	non-toxic	non-toxic	readily biodegradable	Acute 3	Acute 3	Acute 3	non-toxic	Type 3	low inhibition potential	low cytotoxicity
2-HTEAB	non-toxic	non-toxic	non-toxic	readily biodegradable	non-toxic	non-toxic	non-toxic	non-toxic	Type 2	low inhibition potential	moderate cytotoxicity
2-HTEAPe	non-toxic	non-toxic	non-toxic	readily biodegradable	non-toxic	non-toxic	Acute 3	non-toxic	Type 3	low inhibition potential	moderate cytotoxicity
[BMIM]Cl	non-toxic	Acute 3	Acute 3	not readily biodegradable	Acute 3	non-toxic	non-toxic	non-toxic	Type 4	high inhibition potential	moderate cytotoxicity
[OMIM]Cl	Acute 1	Acute 2	Acute 2	not readily biodegradable	Acute 3	Acute 3	Acute 3	non-toxic	Type 4	high inhibition potential	high cytotoxicity
[BPy]Cl	non-toxic	Acute 3	Acute 3	not readily biodegradable	Acute 3	non-toxic	non-toxic	non-toxic	Type 4	high inhibition potential	moderate cytotoxicity

According to the Globally Harmonized System of Classification and Labeling of chemicals (United Nations, 2013), most of the PILs can be classified as non-toxic for **aquatic organisms**, because their EC_{50} values are higher than 100 mg L^{-1}, whilst all of the AILs can be placed into some of the acute toxicity according to their EC_{50} for two out of three tests performed. Based on the results of the Microtox® test, the [OMIM]Cl can be classified as very toxic for aquatic organisms, belonging to the category Acute 1, and the other two AILs have EC_{50} values higher than 100 mg L^{-1}. All of the analyzed PILs have proven to be non-toxic for *Vibrio fischeri* and *Pseudokirchneriella subcapitata*. In the test of inhibition of the growth of *P. subcapitata*, the [OMIM]Cl can be classified as toxic (Acute 2 category) and [BMIM]Cl and [BPy]Cl as harmful for aquatic organisms (Acute 3 category). The aquatic plant *Lemna minor* proved to be more sensitive to the presence of PILs than green alga *P. subcapitata* or marine bacteria *V. fischeri*. The 2-HEAB, 2-HDEAB and 2-HDEAiB fit into the category of harmful for the aquatic environment (Acute

3) in the test with *L. minor*. This fact indicates higher sensitivity of *L. minor* towards PILs derived from acids with longer alkyl chain, especially the butyric one, which was also confirmed by the correlation analysis. All of the AILs show toxic effect towards *L. minor*. The [OMIM]Cl can be classified as toxic (Acute 2 category), and the other two analyzed AILs as harmful for the aquatic environment (Acute 3 category). If the contaminant has higher lipophilicity, the toxic effect on algae and bacteria is stronger, because their lipid cell membrane is the point of entry for the toxicants. The cell wall is known to play a critical role in the transport of materials into and out of algal cells, including toxicants (Sena et al., 2010). The studies published on the toxic mode of action of ILs generally consider membrane accumulation and disruption and surface activity, with subsequent accumulation within cells (Ranke et al., 2006; Luczak et al., 2010). The increase of lipophilicity with the increase of the length of the long alkyl substituents facilitates increased membrane interactions (Megaw et al., 2013). Stolte et al. (2007b) attributed the acute toxic effects of ionic liquids to side chain length or lipophilicity of the ILs cation, causing membrane interaction similar to polar narcosis. There is a similarity between the chemical structure and the mode of action for some surfactants and ILs. Like surfactants, the ILs could potentially increase membrane permeability leading to cell narcosis (Sena et al., 2010). So, as the AILs have higher lipophilicity than PILs, their stronger toxic effect on algae and bacteria is not surprising. But the case of *L. minor* is different, as it takes nutrients from the water mainly via fronds, which means that the more hydrophilic compounds will be more available to *L. minor*. Also, the duration of the test in case of *L. minor* is substantially longer than in case of algae and bacteria, which can also lead to higher toxicity of ILs. These findings concur with those published by other authors (Cedergreen and Madsen, 2003; Mohammad et al., 2005; Stolte et al., 2007a). The stronger effect of PILs anion on toxicity towards *L. minor* and an increase of toxicity with the increase of the number of carbons in the anionic part of the PILs molecule was proved by both correlation and QSAR analysis (See Chapter 3.2 and 3.4.).

According to OECD 301 guidelines (1992) for the manometric respirometry test, the compounds which reach the **biodegradation** level higher than 60% of Theoretical Oxygen Demand within 28 days of testing can be considered as "readily biodegradable". The analyzed PILs showed much better biodegradation rates in water than AILs. After 28 days of the test, the biodegradation rates of most of the PILs were higher than 60%. Three of the tested PILs do not fit into "readily biodegradable" criteria: 2-HDEAF, 2-HTEAB and 2-HTEAPe. As the results of 59 and 57% for the 2-HTEAB and 2-HTEAPe respectively are very close to the 60% limit, they also can be considered as potentially readily biodegradable. The 2-HDEAF is the only PIL with the biodegradation level substantially below 60%, being 13% its biodegradation rate. The levels of biodegradation are usually dependent on compound's stability and toxicity, as proven in the case of the analyzed AILs, with a biodegradation rate being around 1%, indicating their resistance to water biodegradation, which will be confirmed in the test of carbon mineralization in soil.

The OECD 301 guideline gives a possibility of an additional testing of the biodegradability, e.g. by chromatographic methods. That's why the ionic chromatography quantification was periodically performed during the manometric respirometry tests. The goal was to determine if there are any remains of the cationic moiety after 5 and 14 days and at the end of the test. The primary degradation of 2-HEAF, 2-HEAB, 2-HDEAF and 2-HDEAPr was complete within 5 days, while the other PILs molecules (except the 2-HDEAPe) were completely mineralized during the period between the fifth and the twenty-eighth day. After the finalization of the water respirometry test, on the 28th day, the only PIL that was not completely metabolized by the activated sludge microbial community was 2-HDEAPe, remaining at the available concentration of 38%. The rest of the PILs were completely degraded after 28 days, as no cationic moiety could be detected in water samples. This additional analysis confirms that all of the PILs are potentially degradable in water, because even for the 2-HDEAF a 100% biodegradation of the cation was found.

In a study performed by Stolte et al. (2008), imidazolium and pyridinium ILs with short alkyl side chains ([BMIM]Cl and [BPy]Cl) were highly resistant to mineralization during the incubation period. A primary biodegradation could be detected for the [OMIM]Cl after 28 days, but only concerning the eight carbon side chain that underwent several oxidations and decarboxylations to be reduced to an acetate, while the aromatic ring was left intact, so the primary biodegradation was not in fact complete, as in case of the PILs.

The terrestrial toxicity and biodegradability in soil is a quite neglected feature when it comes to ILs and the data available on the ecotoxicological effects of ionic liquids on **terrestrial plants** is scarce. The studies published up to date dealing with the phytotoxicity of ILs have been mainly focused on the aquatic plants (Zhu et al., 2009 and Pham et al., 2010). As far as PILs are concerned, only one representative showed toxic effect for all of the three analyzed species, the 2-HDEAPe. The 2-HDEAPe proved to be harmful (category Acute 3) in the test with *Allium cepa*. The EC_{50} value for 2-HDEAA was 996 mg kg^{-1}, very close to the 1000 mg kg^{-1} limit of the GHS (United Nations, 2006), that it can be considered as practically harmless for the terrestrial plants. *Lolium perenne* was the most sensitive species of the analyzed terrestrial plants, with three PILs showing toxic effects (2-HEAB, 2-HDEAA and 2-HDEAPe) being classified as harmful for terrestrial plants (category Acute 3). The 2-HDEAPe and 2-HTEAPe can be classified as harmful for the terrestrial plants (category Acute 3) in the test with *Raphanus sativus*. The most toxic of the PILs were the ones with pentanoic acid, which indicates that the PILs with longer alkyl chains have higher toxicity. The higher toxicity of longer alkyl chains has also been confirmed in the case of [OMIM]Cl and [BMIM]Cl, where [OMIM]Cl has a longer side alkyl chain and it was more toxic in all of the performed studies. These results are in agreement with the findings of other authors (Ranke et al., 2004; Matzke et al., 2008; Pham et al., 2010). The analyzed PILs are less toxic for plants than the analyzed AILs. *A. cepa* proved to be the most sensitive species to the toxic effect of AILs, with the three of them being classified as harmful for terrestrial plants (category Acute 3). In case of the *R. sativus* and *L. perenne*, only [OMIM]Cl classified as harmful. The higher toxicity of AILs towards terrestrial plans is also confirmed via chlorosis of the

leaves of *R. sativus* and *L. perenne* in the concentrations ≥ 100 mg kg^{-1} (See Chapter 3.3.). The observed toxic effect of AILs could be caused by root damage or blockage of the nutrient transport from soil into plants.

The information available in literature on ILs **effect on soil microbiota and biodegradation in soil** is non-sufficient, with a limited number of analyzed ILs: three derived from imidazolium and one from pyridinium (Modelli et al., 2008 and Zhang et al., 2010). The results of this study indicate that there is no toxic effect of the analyzed PILs and AILs on soil microorganisms involved in the carbon and nitrogen mineralization cycles. Due to no toxic effects (PILs) or very low toxic effect (AILs), no EC_{50} values could be calculated. The very high EC_{50} or no possibility of calculating them is no surprise, taking into account that the tests were carried out in soil matrix. Soil has a great capacity of blocking and neutralizing the toxic effect chemicals and retaining the contamination within. The heterotrophic soil microbiota is diverse and contains many microbial strains. So, if any of them is affected by a chemical added to soil, the other ones carry on with they function and thus it is very difficult to obtain low EC_{50} in soil (Van Elsas et al., 2012).

A pattern in the form of the cumulative respiration (CR) curves obtained after Carbon transformation test was observed and the curves were classified in 4 types, corresponding to the analyzed ILs. Three of them describe the effects of PILs and the forth one is representative for AILs. In the case of the first type (2-HEAF, 2-HDEAF and 2-HDEAA), there is no toxic effect towards the soil microbiota and all of the values of accumulated oxygen are above the control after 28 days of testing. The second type (2-HEAB, 2-HDEAiB and 2-HTEAB) also shows an uninterrupted respiratory activity of the soil, but with some differences in the intensity of the response of the soil microbiota. The higher concentrations (5000 and 10000 mg kg^{-1}) show an initial inhibitory effect on the soil microbiota which manages to recuperate and start to respirate, with final percentage of the accumulating oxygen being above the one of the control. In the third type (2-HDEAPr, 2-HDEAB, 2-HDEAPe and 2-HTEAPe), the curves present little or no difference between the accumulated oxygen for the lowest concentrations (1, 10, and 100 mg kg^{-1}) and the

control, and there is also the initial inhibition at the concentration of 5000 mg kg^{-1} and posterior recuperation of respiration with the final values of the accumulated consumed oxygen being above the control. The possible cause for this behavior of the microbiota (the initial inhibition and posterior recuperation and stimulation of respiration) could be due to a degradation of labile organic matter proceeding from the microorganisms affected by the initial toxicity and/or to the biodegradation of the substance itself (Martí et al., 2011). The highest concentration (10000 mg kg^{-1}) presents inhibition of the respiratory activity throughout the whole assay, which is a clear indicator of toxicity for soil microbiota at this concentration. These findings were confirmed by the ion chromatography quantification results. On the 28^{th} day of the carbon transformation test, none of the compounds, but 2-HDEAPe, could be detected in the aqueous extracts of soil. All of these findings indicate a potential biodegradation of PILs in soil.

In the case of AILs, a different pattern of behavior was found. The fourth type of the respiration curves shows no difference between the values of accumulated oxygen for the control samples and the lower concentrations (1, 10, and 100 mg kg^{-1}) in case of the [BMIM]Cl and [BPy]Cl, but there is an inhibitory effect starting at 1000 mg kg^{-1}, the fact which indicates that these compounds show some toxicity towards the microorganisms involved in the carbon mineralization cycle. The [OMIM]Cl was the most toxic of the three AILs, with the inhibition of the respiratory activity starting already at 100 mg kg^{-1}. Overall, in case of this test, the analyzed AILs have also proven to be more toxic for the soil microbiota than the analyzed PILs and have no biodegradation potential, but, still did not show an inhibition strong enough to provide EC_{50} values.

The results for the nitrogen transformation test show very high EC_{50} for all of the analyzed PILs, thus indicating no toxicity for the nitrogen mineralizing soil microbiota. All of the EC_{50} for the AILs are also higher than 100 mg kg^{-1}, which according to the GHS means that there is no toxic effect of the analyzed ILs on nitrogen mineralization processes. But, the EC_{50} for AILs were significantly lower than the ones for PILs.

The **additional toxicity tests** of acetylcholinesterase inhibition (AChE) and cellular toxicity towards IPC-81 rat promyelocytic leukemia cell line were done in order to get a more in-depth evaluation of the ILs toxicity. The AChE is involved in one of the key mechanisms of neurotransmission in nearly all higher organisms, including humans. The AChE is an (eco)toxicologically relevant molecular target and its inhibition assay can be performed in a microtiter plate and therefore represents a fast and cost effective screening tool for early toxicity testing of industrial chemicals (Arning et al., 2008). Cell cultures provide a quick and convenient insight on the biological activities of chemicals too (Ranke et al., 2004).

In the Acetylcholinesterase inhibition test, the PILs did not exhibit toxicity, with EC_{50} values ranging from 1.27 (low inhibitory potential) to 59.02 mmol L^{-1} (no inhibitory potential). The AILs presented a high inhibition potential, with EC_{50} values between 0.04 and 0.08 mmol L^{-1}. The OMIM[Cl] is the most toxic one of the three AILs. There is a difference of several orders of magnitude when comparing EC_{50} of PILs and AILs, with the ones for PILs being higher. Another way to make the evaluation of toxicity of the ILs analyzed would be comparing their EC_{50} values with the one for aldicarb (2-methyl-2-(methylthio)propanal O-(N-methylcarbamoyl)oxime), with the value of 0.0049 mmol L^{-1} (UFT/Merck database). Aldicarb belongs to the family of carbamates and is a potent inhibitor of the enzyme acetylcholinesterase, mainly used as an insecticide. Compared with aldicarb, the PILs did not show any toxic effect, while the AILs, although less toxic than aldicarb, proved to be potent inhibitors of the acetylcholinesterase. When it comes to cytotoxicity towards IPC-81, the EC_{50} values for the analyzed PILs ranged between 1.76 and 30.95 mmol L^{-1}, while the AILs had EC_{50} between 0.1 and 5 mmol L^{-1}. One of the PILs, 2-HDEAF, showed no cytotoxic effect. The 2-HEAB, 2-HDEAB, 2-HDEAiB, 2-HTEAB and 2-HTEAPe have a moderate inhibitory potential. The AILs proved to be more toxic than the PILs, with OMIM[Cl] being the most toxic one. Their EC_{50} indicate moderate ([BMIM]Cl and [BPy]Cl) and high ([OMIM]Cl) cytotoxicity. In the study of Ranke et al. (2006) a good correlation between the lipophilicity parameter of the cation and the observed cytotoxicity for a set of approximately 70 ionic liquids was found, and it explains the higher toxicity of the more lipophilic AILs compared to PILs.

The PILs in general show very little or no toxicity. The 2-HEAF, 2-HDEAF, 2-HDEAPr and 2-HTEAB proved to be non-toxic in all of the ecotoxicity tests. The 2-HEAB proved to be harmful for *Lemna minor* and *Lolium perenne*, a fact which indicates that it is hazardous for plants. Two of the three PILs that proved to be toxic for *L. minor* (2-HDEAB and 2-HDEAiB) showed no adverse effects in other ecotoxicity tests and exhibited moderate cytotoxicity. The third representative of PILs toxic to *L. minor*, 2-HDEAB, exhibited toxicity in terrestrial plant seedling inhibition test towards *L. perenne* and also showed moderate cytotoxicity potential, as well as being very close to the limit value of toxicity in the test of inhibition of algal growth, which indicates that it would pose a threat if found in the aquatic environment. In contrast, the 2-HDEAPe showed toxicity toward all of the analyzed higher plants and has a Type 3 of soil respiration curves, which indicates that it is a potentially hazardous compound for the terrestrial life. The other PIL that demonstrated to be toxic for plants, 2-HTEAPe, apart from being harmful for *Raphanus sativus*, also has a Type 3 soil respiration curves, so, based on his EC_{50} values, it can also be potentially hazardous for the terrestrial life if found in higher concentrations in soil, but less toxic than the 2-HDEAPe. The 2-HTEAB exhibited moderate cytotoxicity, but, overall can be considered as non-ecotoxic. The analyzed PILs are potentially biodegradable in water and soil.

In contrast to PILs, the AILs proved to be toxic in almost all of the performed tests. The [BMIM]Cl and [BPy]Cl are toxic for *P. subcapitata*, *L. minor* and *A. cepa*. The most toxic representative, both in aquatic and terrestrial environment, is the [OMIM]Cl. Apart for the demonstrated ecotoxicity in all of the performed tests, it exhibited high inhibition potential towards AChE and high cytotoxicity. Even though being soluble, it is the most lipophilic one of all of the analyzed ILs, therefore the high toxicity in all of the tests where the test organisms' lipid cell membrane was a target spot for the entrance of AILs. Also, the AILs with its positively charged nitrogen and a long alkyl chain in its structure resemble acetylcholine more than PILs, so the high inhibition potential of AChE is no surprise. The analyzed AILs are not biodegradable in water and less biodegradable than PILs in soil.

One of the REACH requirements is to prove that the analyzed compound is not a PBT chemical. The PBTs are chemicals that are toxic, persist in the environment and bioaccumulate in food chains and, as a result, pose a risk to human health and ecosystems (ECHA, 2012b). According to REACH (Annex XIII), a substance fulfils the persistence criterion when its half-life in water is higher than 40 days and the half-life in soil is higher than 120 days. Only AILs could meet this criteria, as the [BMIM]Cl and [BPy]Cl do not exhibit any biodegradation after 31 days (Stolte et al., 2008). In soil the AILs also exhibit slight inhibition of the carbon mineralization activity, but as the test lasted for 28 days, further and longer lasting analysis is needed to confirm fully these hypotheses. The PILs are not persistent, as they biodegrade both in water and soil. As both PILs and AILs have very low or negative values of $\log K_{ow}$ (Ropel et al., 2005 and Peric et al., unpublished results), they don't have the potential to be bioaccumulative. According to REACH, if the substance is documented to fulfill the persistence and bioaccumulation criteria, at least acute testing should be performed in order to determine whether the substance meets the screening criteria for toxicity. The [OMIM]Cl would be a serious candidate for a toxic category, as it exhibited toxic potential in all of the acute toxicity tests.

A **correlation analysis** was performed for all the results of the (eco)toxicity tests. The Spearman's correlation coefficients were obtained in order to relate the structure and toxicity/biodegradability of the PILs and thus get more information on their toxic effects and possible biodegradation. The molecular weight of PILs, the number of C-atoms in the cationic (amine) and anionic (acid) part of the molecule, as possible factors of influence, were correlated with the EC_{50} values and percentage of biodegradation. The results for the ecotoxicity tests in both compartments (water and soil) show that the elongation of alkyl chains in the anionic part of the molecule and the increase of the molecular weight are in direct correlation with higher toxicity. The anionic part of the PILs molecule seems to have more influence on the toxicity than the cationic part, in contrast to AILs, as it showed significant negative correlation with EC_{50} for all of the performed tests. The increase of the molecular weight also has a strong influence on toxicity, which indicates that the complexity of the PILs molecule has a positive influence on potential toxicity. The percentage of biodegradation during the first 5 days of the

ready biodegradability test is positively influenced by the elongation of the anionic part of the molecule. In the later stages of the test, getting closer to the 28th day, it is the complexity of the amine part of the molecule that reduces the biodegradability rate. The test of inhibition of acetylcholinesterase shows strong influence of the three studied factors, being all of them inversely correlated with EC_{50} values. Nevertheless, the PILs have low inhibitory potential for this enzyme. A positive influence of the anionic part of the molecule on the toxicity of PILs was also found in the case of the test performed on IPC-81 cells. The correlation analyses performed by other authors confirm the aforementioned data. Stolte et al. (2007a) found correlation between lipophilicity and toxicity of AILs molecule for bacteria and algae. In the case of the rat leukemia cell line, a correlation between a HPLC derived lipophilicity parameter and the observed cytotoxicity (Stolte et al., 2007b) was established. A correlation between an elongation of the side chains connected to the cationic head groups and an enhanced inhibitory potential on the enzyme acetylcholinesterase was found by Ventura et al. (2012).

In the case of the carbon transformation test an inverse correlation between the increase of number of carbons in the acid part of the PILs molecule and the values for percentage of cumulative respiration, basal respiration and substrate induced respiration were obtained. The increase of the molecular weight causes the decrease of these three parameters, while the only feature that is influenced by the increase of complexity of the cationic part of the PILs molecules are the basal respiration values, having a negative Spearman's correlation coefficient. The clear indication that the higher complexity of the PILs molecule leads to the lower activity of the soil microbiota was found in the case of the nitrogen transformation test, where the complexity of the amine and acid part of the molecule, together with the increase of the molecular weight show a negative correlation with the EC_{50} values. However, the PILs did not show any toxic effect towards the nitrifying microbiota.

There is a huge number of possible ILs and it is not economically feasible to test the toxicity of all of them. A good way to avoid the expensive and extensive toxicity testing is the use of ***in silico* QSAR methods** for the prediction of toxicity, which

are a lot quicker and can cut down the investigation costs. The QSAR modelling based on the group contribution (Luis et al., 2007) was performed for five tests: Microtox® test, *P. subcapitata* and *L. minor* growth inhibition test, and Acetylcholinesterase inhibition and Cell viability assay with IPC-81 cells. The results indicated the same tendency as obtained with correlation analysis, with both cationic and anionic part contributing to PILs toxicity, in the case of the performed tests of aquatic and cellular toxicity. The selected AILs were also included in the model, and the findings confirmed those previously published by other authors. Namely, the moiety principally contributing to toxicity was the cationic one, with the anionic moiety having marginal o no effect on toxicity (Ranke et al., 2004, Matzke et al., 2007 and Stolte et al., 2007a). The number of carbons in side alkyl chains showed strong influence on ILs toxicity, meaning that the increase of the side alkyl chain leads to an increase in toxicity, a well-studied fact in literature (Ranke et al., 2004, Wells and Coombe, 2006, Pham et al., 2010 and Radošević et al., 2013). The cations and anions of the studied PILs did not exhibit a high overall influence on the ILs toxicity, being analyzed in a set of different AILs. This coincides with the fact that the PILs showed much lower toxicity than the AILs in all of the toxicity tests.

Applying the group contribution QSAR model, the EC_{50} values for the eight representatives of the new family of short aliphatic PILs that had not been previously analyzed for their (eco)toxicity were predicted. The results for the five toxicity tests (Microtox® test, Algal and *Lemna minor* growth inhibition test, and Acetylcholinesterase inhibition and Cell viability assay with IPC-81 cells) can be found in Chapter 3.4. The overview of the effects of the PILs, based on the predicted EC50, is presented in the Table 7.

Table 7. The overview of the effects based on predicted toxicity for Microtox® test, Algal and *Lemna minor* growth inhibition test, Acetylcholinesterase inhibition and Cell viability assay with IPC-81 cells for eight representatives of the new family of PILs.

	Microtox®	**Algae**	***L. minor***	**AChE**	**IPC-81**
2-HEAA	non-toxic	non-toxic	non-toxic	no inhibition	low cytotoxicity
2-HEAPr	non-toxic	non-toxic	non-toxic	no inhibition	low cytotoxicity
2-HEAiB	non-toxic	non-toxic	Acute 3	no inhibition	moderate cytotoxicity
2-HEAPe	non-toxic	non-toxic	Acute 3	no inhibition	moderate cytotoxicity
2-HTEAF	non-toxic	non-toxic	non-toxic	no inhibition	low cytotoxicity
2-HTEAA	non-toxic	non-toxic	non-toxic	low inhibition potential	low cytotoxicity
2-HTEAPr	non-toxic	non-toxic	non-toxic	low inhibition potential	low cytotoxicity
2-HTEAiB	non-toxic	non-toxic	non-toxic	low inhibition potential	moderate cytotoxicity

The toxicities predicted by means of QSAR modelling show that, as previously found for the rest of the representatives of the new family of short aliphatic PILs, the remaining eight members of the family also show low or no toxicity. In the case of Microtox® test and Algal growth inhibition test, all of the EC_{50} values are far above the threshold of toxicity of 100 mg L^{-1}. The only toxic effect can be seen in the *L. minor* growth inhibition test, where two of the PILs, 2-HEAiB and 2-HEAPe have EC_{50}<100 mg L^{-1}, and according to the GHS classification it can be classified as harmful for the aquatic environment (category Acute 3). Once again, the PILs with the longer carbon chain acid in their anionic part of the molecule turned out to be more toxic (butyric and pentanoic) and *L. minor* proved to be the most sensitive species in the aquatic toxicity testing, as in case of the rest of the experimentally analyzed PILs. None of the PILs showed inhibitory activity towards acetylcholinesterase. The 2-HEAiB, 2-HEAPe and 2-HTEAiB have a moderate inhibitory potential towards IPC-81 cells, while the other PILs have very low or no toxic effect, according to the UFT/Merck Ionic Liquids Biological Database classification criteria.

CONCLUSIONS

5. CONCLUSIONS

As a result of the performed investigation of (eco)toxicity and biodegradability of selected short aliphatic PILs and a comparative study of (eco)toxicity and biodegradability of PILs and AILs, the following conclusions have been reached:

- The PILs analyzed in the present study have shown no toxicity in the performed aquatic toxicity tests, with the exception of three PILs in the case of duckweed growth inhibition test. The OMIM[Cl] proved to be the most toxic in all of the aquatic toxicity tests, being classified as very toxic or toxic for the aquatic life, and the other two representatives of AILs proved to be harmful for the aquatic life.

- Only 2-HDEAPe can be classified as harmful for the three analyzed plants, with three other PILs being classified as harmful for only one plant species. The analyzed AILs proved to be harmful for the onion, and the [OMIM]Cl for the radish and grass as well. None of the analyzed ionic liquids proved to be toxic for nitrifying and heterotrophic microbiota, but there are visible differences between EC_{50} values for the two groups of ILs in the nitrogen transformation test and in the shape of the respiration curves.

- In the manometric respirometry test, the PILs showed high rates of biodegradation in water. The shape of the soil respiration curves for PILs indicate that they in general stimulate the microbiota's activity, which can indicate potential biodegradability in soil. These facts, together with the results of the ionic chromatography quantification, indicate that the analyzed PILs are potentially biodegradable in water and soil. In contrast, the AILs seem not to be biodegradable in water and show some inhibition of the soil microbiota's activity, indicating resistance to biodegradation in general.

- In the case of inhibition of acetylcholinesterase tests, none of the PILs exhibited inhibition, unlike the AILs, which exhibited high inhibitory potential towards the enzymatic activity. In the cytotoxicity test, none of the analyzed ionic

liquids showed very high toxicity, being the [OMIM]Cl once again the most toxic one.

- According to the correlation analysis, the molecular weight and the size of the acid (anionic part of the molecule) have more influence on the PILs toxicity than the amine (cationic part of the molecule). In contrast, for AILs the cationic part of the molecule is the dominant one in terms of toxicity. The comparative study of (eco)toxicity and biodegradability of AILs and PILs indicate that more complex ILs are the most toxic ones. This fact, together with other authors' findings, leads to the conclusion that ionic liquids with simpler structure, without bulky organic cations and with short alkyl side chains show lower toxicity and should be considered as a path to follow in the future synthesis of new ionic liquids.

- The QSAR modelling based on group contribution showed that in case of the analyzed PILs both anionic and cationic part of the molecule have influence on their toxicity, with the elongation of the acid in the anionic moiety being slightly more influent. Regarding the AILs, the QSAR modelling confirmed the literature findings that the cations have a predominant influence on their toxicity, as well as the increase of toxicity with the elongation of the side alkyl chains.

- The eight representatives of the new family of PILs, whose EC_{50} were predicted by means of QSAR modelling, just like the experimentally analyzed PILs, have a very good "green" potential.

- The low toxicity and good biodegradability of PILs observed during this study, together with their low production cost, simple synthesis and numerous possible applications, suggests that they could have a good prospect for a wider use. The comparative study indicates that short aliphatic PILs can be considered as environmentally safer alternatives for more toxic AILs which are frequently used at present. The complete and in-depth determination of the overall sustainability and actual "greenness" of ILs requires more testing and it is preferable to do it simultaneously with the development of new ILs and their potential technological applications.

REFERENCES

6. REFERENCES

Amberg, A., 2013. *In silico* methods, in: Vogel, H.G., Maas, J., Hock, F.J., Mayer, D. (Eds.), Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays. Springer, Berlin Heidelberg, pp. 1273–1296.

Arning, J., Stolte, S., Böschen, A., Stock, F., Pitner, W.R., Welz-Biermann, U., Jastorff, B., Ranke, J. 2008. Qualitative and quantitative structure activity relationships for the inhibitory effects of cationic head groups, functionalized side chains and anions of ionic liquids on acetylcholinesterase. Green Chem. 10, 47–58.

Azevedo, A.M., Ribeiro, D.M., Pinto, P.C., Lúcio, M., Reis, S., Saraiva, M.L. 2013. Imidazolium ionic liquids as solvents of pharmaceuticals: influence on HSA binding and partition coefficient of nimesulide. Int. J. Pharm. 443, 273–278.

Bałczewski, P., Bachowska, B., Bialas, T., Biczak, R., Wieczorek, W. M., Balińska, A. 2007. Synthesis and phytotoxicity of new ionic liquids incorporating chiral cations and/or chiral anions. J. Agric. Food Chem. 55, 1881–1892.

Barrosse-Antle, L.E., Bond, A.M., Compton, R.G., O'Mahony, A.M., Rogers, E.I., Silvester, D.S. 2010. Voltammetry in room temperature ionic liquids: comparisons and contrasts with conventional electrochemical solvents. Chem. Asian J. 5, 202–230.

Bernot, R. J., Brueseke, M.A., Evans-White, M.A., Lamberti, G.A. 2005a. Acute and chronic toxicity of imidazolium-based ionic liquids on *Daphnia magna*. Environ. Toxicol. Chem. 24, 87–92.

Bernot, R.J., Kennedy, E.E., Lamberti, G.A. 2005b. Effects of ionic liquids on the survival, movement, and feeding behavior of the freshwater snail, *Physa acuta*. Environ. Toxicol. Chem. 24, 1759–1765.

Biçak, N. 2005. A new ionic liquid: 2-hydroxy ethylammonium formate. J. Mol. Liq. 116, 15–18.

Biçak, N., Filiz Senkal, B., Sezer, E. 2005. Preparation of organo-soluble polyanilines in ionic liquid. Synthetic Met. 155, 105–109.

Blaise, C., Férard, J.F. (Eds.) 2005. Small-scale Freshwater Toxicity Investigations. Volume I. Toxicity test methods, Springer Science & Business, The Netherlands.

CAS registry http://www.cas.org/content/chemical-substances (last accessed: September 10th 2014)

Cedergreen, N., Madsen, T.V. 2003. Light regulation of root and leaf NO_3^- uptake and reduction in the floating macrophyte *Lemna minor*. New Phytol. 161, 449–457.

Chiappe, C., Pieraccini, D. 2005. Ionic liquids: solvent properties and organic reactivity. J. Phys. Org. Chem. 18, 275–297.

Cho, C.W., Pham, T.P.T., Jeon, Y.C., Vijayaraghavan, K., Choe, W.S., Yun, Y.S. 2007. Toxicity of imidazolium salt with anion bromide to a phytoplankton *Selenastrum capricornutum*: effect of alkyl chain length. Chemosphere 69, 1003–1007.

Cho, C.W., Jeon, Y.C., Pham, T.P.T., Vijayaraghavan, K., Yun, Y.S. 2008. The ecotoxicity of ionic liquids and traditional organic solvents on microalga *Selenastrum capricornutum*. Ecotoxicol. Environ. Saf. 71, 166–171.

Cho, C.W., Ranke, J., Arning, J., Thöming, J., Preiss, U., Jungnickel, C., Diedenhofen, M., Krossing, I., Stolte, S. 2013. *In silico* modelling for predicting the cationic hydrophobicity and cytotoxicity of ionic liquids towards the *Leukemia* rat cell line, *Vibrio fischeri* and *Scenedesmus vacuolatus* based on molecular interaction potentials of ions. SAR QSAR Environ Res. 24, 863–882.

Costello, D.M., Brown, L.M., Lamberti, G.A. 2009. Acute toxic effects of ionic liquids on zebra mussel (*Dreissena polymorpha*) surviving and feeding. Green Chem. 11, 548–553.

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.

Cota, I., Gonzalez-Olmos, R., Iglesias, M., Medina, F. 2013. New Brønsted Ionic Liquids: Synthesis, Thermodinamics and Catalytic Activity in Aldol Condensation Reactions, in: Ionic Liquids - New Aspects for the Future, Kadokawa J. (Ed.), InTech. DOI: 10.5772/45605.

Cota, I., Medina, F., Gonzalez-Olmos, R., Iglesias, M. 2014. Alanine-supported protic ionic liquids as efficient catalysts for aldol condensation reactions. C. R. Chimie, 17, 18–22.

Couling, D.J., Bernot, R.J., Docherty, K.M., Dixon, J.K., Maginn, E.J. 2006. Assessing the factors responsible for ionic liquid toxicity to aquatic organisms via quantitative structure-property relationship modeling. Green Chem. 8, 82–90.

Cvjetko Bubalo, M., Hanousek, K., Radošević, K., Gaurina Srček, V., Jakovljević, T., Radojčić Redovniković, I. 2014. Imidiazolium based ionic liquids: effects of different anions and alkyl chains lengths on the barley seedlings. Ecotoxicol. Environ. Saf. 101, 116–23.

Das, R.N., Roy, K. 2013. Advances in QSPR/QSTR models of ionic liquids for the design of greener solvents of the future. Mol. Divers. 17, 151–96.

Deive, F.J., Rodríguez, A., Varela, A., Rodrígues, C., Leitão, M.C., Houbraken, J.A.M.P., Pereiro, A.B., Longo, M.A., Sanromán, M.Á., Samson, R.A., Rebelo, L.P.N., Silva Pereira, C. 2011. Impact of ionic liquids on extreme microbial biotypes from soil. Green Chem. 13, 687–696.

De Souza, R.L., Faria de, E.L., Figueiredo, R.T., Freitas, S.L., Iglesias. M., Mattedi, S., Zanin, G.M., dos Santos, O.A., Coutinho, J.A.P., Lima, Á.S., Soares, C.M. 2013. Protic ionic liquid as additive on lipase immobilization using silica sol-gel. Enzyme Microb. Technol. 52, 141–150.

Dobler, D., Schmidts, T., Klingenhöfer, I., Runkel, F. 2013. Ionic liquids as ingredients in topical drug delivery systems. Int. J. Pharm. 441, 620–627.

Docherty, K.M., Kulpa, C.F. 2005. Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. Green Chem. 7, 185–189.

Docherty, K.M., Dixon, J.K., Kulpa, C.F. 2007. Biodegradability of imidazolium and pyridinium ionic liquids by an activated sludge microbial community. Biodegradation 18, 481–493.

Domínguez de María, P. (Ed.) 2012. Ionic Liquids in Biotransformations and Organocatalysis: Solvents and Beyond, John Wiley & Sons Inc., USA.

Dos Santos, R.F., Falcade, T., Antonini, L.M., Ortega V., M.R. Mattedi, S., Iglesias, M., Malfatti, C.F. 2014. Ionic liquids (IL) corrosion on A285 carbon steel. Dyna 184, 122–128.

Du, F.Y., Xiao, X.H., Luo, X.J., Li, G.K. 2009. Application of ionic liquids in the microwave-assisted extraction of polyphenolic compounds from medicinal plants. Talanta 78, 1177–1184.

Earle, M.J., Seddon, K.R. 2000. Ionic liquids: green solvents for the future. Pure Appl. Chem., 72, 1391–1398.

ECHA http://echa.europa.eu/ (last accessed: August 14th 2014)

ECHA. 2008. Guidance on Information Requirements and Chemical Safety Assessment Chapter R6: QSARs and grouping of chemicals, Helsinki, Finland.

ECHA. 2010. Practical Guide 10: How to avoid unnecessary testing on animals, Helsinki, Finland.

ECHA. 2012a. Practical Guide 5: How to report QSARs, Helsinki, Finland.

ECHA. 2012b. Guidance on information requirements and chemical safety assessment Chapter R.11: PBT Assessment, Helsinki, Finland.

Endres, F. 2012. Interfaces of ionic liquids. Phys. Chem. Chem. Phys. 14, 5008–5009.

European Union. 2006. Regulation 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.

Florindo, C., Araújo, J.M., Alves, F., Matos, C., Ferraz, R., Prudêncio, C., Noronha, J.P., Petrovski, Ž., Branco, L., Rebelo, L.P., Marrucho, I.M. 2013. Evaluation of solubility and partition properties of ampicillin-based ionic liquids. Int. J. Pharm. 456, 553–559.

Forsyth, S.A., Pringle, J.M., MacFarlane, D.R. 2004. Ionic liquids - An overview. Aust. J. Chem. 57, 113–119.

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

Frade, R.F.M., Rosatella, A.A., Marques, C.S., Branco, L.C., Kulkarni, P.S., Mateus, N.M.M., Afonso, C.A.M., Duarte, C.M.M. 2009. Toxicological evaluation on human colon carcinoma cell line (CaCo-2) of ionic liquids based on imidazolium, guanidinium, ammonium, phosphonium, pyridinium and pyrrolidinium cations. Green Chem. 11, 1660–1665.

Garcia, M.T., Gathergood, N., Scammells, P.J. 2005. Biodegradable ionic liquids. Part II: effect of the anion and toxicology. Green Chem. 7, 9–14.

Gathergood, N., Garcia, M.T., Scammells, P.J. 2004. Biodegradable ionic liquids: part I. Concept, preliminary targets and evaluation. Green Chem. 6, 166–175.

Gathergood, N., Scammells, P.J., Garcia, M.T. 2006. Biodegradable ionic liquids. Part III: the first readily biodegradable ionic liquids. Green Chem. 8, 156–160.

Greaves, T.L., Weerawardena, A., Fong, C., Krodkiewska, I., Drummond, C.J. 2006. Protic Ionic Liquids: Solvents with Tunable Phase Behavior and Physicochemical Properties. Phys. Chem. B 110, 22479-22487.

Greaves, T.L., Drummond, C.J. 2008. Protic Ionic Liquids: Properties and Applications. Chem. Rev. 108, 206–237.

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

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

Harjani, J.R., Singer, R.D., Garcia, M.T., Scammells, P.J. 2008. The design and synthesis of biodegradable pyridinium ionic liquids. Green Chem. 10, 436–438.

Hough, W.L., Rogers, R.D. 2007a. Ionic liquids then and now: from solvents to materials to active pharmaceutical ingredients. Bull. Chem. Soc. Jpn., 80 (12), 2262–2269.

Hough, W.L., Smiglak, M., Rodríguez, H., Swatloski, R.P., Spear, S.K., Daly, D.T., Pernak, J., Grisel, J.E., Carliss, R.D., Soutullo, M.D., Davis, J.H. Jr., Rogers, R.D. 2007b. The third evolution of ionic liquids: active pharmaceutical ingredients. New J. Chem., 31, 1429–1436.

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

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. 16, 2802–2808.

Iglesias, M., Andrade, R.S., Mazzer, H., Cardozo, L. 2014. Textile dyeing using protic ionic liquids. Poster presented at Expoquimia, 30 September - 3 October 2014, Barcelona.

IL Thermo NIST Standard Reference Database. http://ilthermo.boulder.nist.gov/ (last accessed: June 20th 2014)

Jaitely, V., Karatas, A., Florence, A.T. 2008. Water-immiscible room temperature ionic liquids (RTILs) as drug reservoirs for controlled release. Int. J. Pharm. 354, 168–173.

Jastorff, B., Störmann, R., Ranke, J., Mölter, K., Stock, F., Oberheitmann, B., Hoffmann, W., Hoffmann, J., Nüchter, M., Ondruschka, B., Filser, J. 2003. How hazardous are ionic liquids? Structure–activity relationships and biological testing as important elements for sustainability evaluation. Green Chem. 5, 136–142.

Jastorff, B., Mölter, K., Behrend, P., Bottin-Weber, U., Filser, J., Heimers, A., Ondruschka, B., Ranke, J., Schaefer, M., Schröder, H., Stark, A., Stepnowski, P., Stock, F., Störmann, R., Stolte, S., Welz-Biermann, U., Ziegert, S., Thöming, J. 2005. Progress in evaluation of risk potential of ionic liquids-basis for an eco-design of sustainable products. Green Chem. 7, 362–372.

Jastorff B., Störmann R., Ranke J. 2007. Thinking in structure-activity-relationships - a way forward towards sustainable chemistry. Clean: Soil, Air, Water. 35, 399–405.

Jin, R., Fan, L., An, X. 2011. Microwave assisted ionic liquid pretreatment of medicinal plants for fast solvent extraction of active ingredients. Sep. Purif. Technol. 83, 45–49.

Kaiser, K.L.E., Palabrica, V.S. 1991. *Photobacterium phosphoreum*, toxicity data index. Water Poll. Res. J. Can. 26, 361–431.

Keskin, S., Kayrak-Talay, D., Akman, U., Hortaçsu, O. 2007. A review of ionic liquids towards supercritical fluid applications. J. Supercrit. Fluids 43,150–180.

Khodadadi-Moghaddam, M., Habibi-Yangjeh, A., Gholami, M.R. 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.

Kruger, E. 2008. Ionic liquids as media for electro-organic synthesis. PhD thesis, Nelson Mandela Metropolitan University, Port Elisabeth, South Africa.

Kumar, S., Ruth, W. R., Sprenger, B., Kragl, U. 2006. On the biodegradation of ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate, Chim. Oggi 24, 70–72.

Kumar, R.A., Papaiconomou, N., Lee, J.M., Salminen, J., Clark, D.S., Prausnitz, J.M. 2009. *In vitro* cytotoxicities of ionic liquids: Effect of cation rings, functional groups, and anions. Environ. Toxicol. 24, 388–395.

Larson, J.H., Frost, P.C., Lamberti, G.A. 2008. Variable toxicity of ionic liquid-forming chemicals to *Lemna minor* and the influence of dissolved organic matter. Environ. Toxicol. Chem. 27, 676–681.

Latała, A., Stepnowski, P., Nędzi, 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.

Liu, T., Sui, X., Zhang, R., Yang, L., Zu, Y., Zhang, L., Zhang, Y., Zhang, Z. 2011. Application of ionic liquids based microwave-assisted simultaneous extraction of carnosic acid, rosmarinic acid and essential oil from *Rosmarinus officinalis*. J Chromatogr A. 1218(47), 8480–8489.

Luis, P., Ortiz, I., Aldaco, R., Irabien, A. 2007. A novel group contribution method in the development of a QSAR for predicting the toxicity (*Vibrio fischeri* EC_{50}) of ionic liquids. Ecotoxicol. Environ. Saf. 67, 423–429.

Luis, P., Garea, A., Irabien, A. 2010. Quantitative structure-activity relationships (QSARs) to estimate ionic liquids ecotoxicity EC_{50} (*Vibrio fischeri*). J. Mol. Liq. 152, 28–33.

Luczak, J., Jungnickel, C., Lacka, I., Stolle, S., Hupka, J. 2010. Antimicrobial and surface activity of 1-alkyl-3-methylimidazolium derivatives. Green Chem. 12, 593–601.

Luo, Y.R., San-Hu, W., Li, X.Y., Yun, M.X., Wang, J.J., Sun, Z.J. 2010. Toxicity of ionic liquids on the growth, reproductive ability, and ATPase activity of earthworm. Ecotoxicol. Environ. Saf. 73(5), 1046–50.

Martí, E., Sierra, J., Cáliz, J., Montserrat, G., Vila, X., Garau, M.A., Cruañas, R. 2011. Ecotoxicity of chlorophenolic compounds depending on soil characteristics. Sci. Total Environ. 409, 2707–2716.

Masten, S.A. 2004. Review of Toxicological Literature for Ionic Liquids, Integrated Laboratory Systems Inc., Research Triangle Park, NC, USA.

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 1-alkyl-3-methylimidazolium ionic liquids observed in an (eco)toxicological test battery. Green Chem. 9, 1198–1207.

Matzke, M., Stolte, S., Arning, J., Uebers, U., Filser, J. 2008. Imidazolium based ionic liquids in soils: effects of the side chain length on wheat (*Triticum aestivum*) and

cress (*Lepidium sativum*) as affected by different clays and organic matter. Green Chem. 10, 584–591.

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.

Megaw, J., Busetti, A., Gilmore, B.F. 2013. Isolation and characterization of 1-alkyl-3-methylimidazolium chloride ionic liquid-tolerant and biodegrading marine bacteria. PLoS ONE 8(4): e60806. DOI: 10.1371/journal.pone.0060806.

Modelli, A., Sali, A., Galletti, P., Samorì, C. 2008. Biodegradation of oxygenated and non-oxygenated imidazolium-based ionic liquids in soil. Chemosphere 73, 1322–1327.

Mohammad Ali, I. (Ed.), 2012. Green Solvents II: Properties and Applications of Ionic Liquids, Springer, New York, USA.

Mohammad, M., Kishimoto, T., Itoh, K., Suyama, K., Yamamoto, H., 2005. Comparative sensitivity of *Pseudokirchneriella subcapitata* vs. *Lemna* sp. to eight sulfonylurea herbicides. Bull. Environ. Contam. Toxicol. 75, 866–872.

Neumann, J., Steudte, S., Cho, C.-W., Thöming, J., Stolte, S. 2014. Biodegradability of 27 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium ionic liquid cations under aerobic conditions. Green Chem. 16, 2174–2184.

OECD. 1992. Guidelines for the Testing of Chemicals, Guideline 301: Ready biodegradability, Paris, France.

Olivier-Bourbigou, H., Magna, L., Morvan, D. 2010. Ionic liquids and catalysis: Recent progress from knowledge to applications, Applied Catalysis A: General 373, 1–56.

Pham, T.P.T., Cho, C.W., Yun, Y.S. 2010. Environmental fate and toxicity of ionic liquids: A review. Water Res. 44, 252–272.

Pretti, C., Chiappe, C., Pieraccini, D., Gregori, M., Abramo, F., Monni, G., Intorre, L. 2006. Acute toxicity of ionic liquids to the zebrafish (*Danio rerio*). Green Chem. 8, 238–240.

Pretti, C., Chiappe, C., Baldetti, I., Brunini, S., Monni, G., Intorre, L. 2009. Acute toxicity of ionic liquids for three freshwater organisms: *Pseudokirchneriella*

subcapitata, *Daphnia magna* and *Danio rerio*. Ecotoxicol. Environ. Saf. 72, 1170–1176.

Quijano, G., Couvert, A., Amrane, A., Darracq, G., Couriol, C., Le Cloirec, P., Paquin, L., Carrie, D. 2011. Toxicity and biodegradability of ionic liquids: new perspectives towards whole-cell biotechnological applications. Chem. Eng. J. 174, 27-32.

Radošević, K., Cvjetko, M., Kopjar, N., Novak, R., Dumić, J., Gaurina Srček, V. 2013. In vitro cytotoxicity assessment of imidazolium ionic liquids: biological effects in fish Channel Catfish Ovary (CCO) cell line. Ecotoxicol. Environ. Saf. 92, 112–118.

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 length in acute *Vibrio fischeri* and WST-1 cell viability assays. Ecotox. Environ. Saf. 58, 396–404.

Ranke, J., Cox, M., Müller, A., Schmidt, C. 2006. Sorption, cellular distribution, and cytotoxicity of imidazolium ionic liquids in mammalian cells - influence of lipophilicity. Toxicol. Environ. Chem. 88: 273–285

Ranke, J., Stolte, S., Störmann, R., Arning, J., Jastorff, B. 2007a. Design of sustainable chemical products – the example of ionic liquids. Chem. Rev. 107, 2183–2206.

Ranke, J., Stock, F., Müller, A., Stolte, S., Störmann, R., Bottin-Weber, U., Jastorff, B. 2007b. Lipophilicity parameters for ionic liquid cations and their correlation to *in vitro* cytotoxicity. Ecotoxicol. Environ. Saf. 67, 430–438.

Rogers, R.D., Seddon, K.R. 2002. Ionic liquids: industrial applications for green chemistry, American Chemical Society, Washington D.C.

Rogers, R.D., Zhang, S.J., Wang, J.J. 2012. Preface: An International Look at Ionic Liquids. Sci. China: Chem. 55 (8), 1475–1477.

Ropel, L., Belvèze, L.S., Aki, S.N.V.K., Stadtherr, M.A., Brennecke, J.F. 2005. Octanol-water partition coefficients of imidazolium-based ionic liquids, Green Chem. 7, 83–90.

Roth, M. 2009. Partitioning behaviour of organic compounds between ionic liquids and supercritical fluids. J. Chromatogr. A 1216, 1861–1880.

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.

Schuster, D., Laggner, C., Langer, T. 2005. Why drugs fail - a study on side effects in new chemical entities. Curr. Pharm. Des. 11, 3545–3559.

Sena, D.W., Kulacki, K.J., Chaloner, D.T., Lamberti, G.A. 2010. The role of the cell wall in the toxicity of ionic liquids to the alga *Chlamydomonas reinhardtii*. Green Chem. 12, 1066–1071.

Sheldon, R. 2001. Catalytic reactions in ionic liquids. Chem. Commun. 23, 2399–2407.

Składanowski, A.C., Stepnowski, P., Kleszczyński, K., Dmochowska, B. 2005. AMP deaminase *in vitro* inhibition by xenobiotics. A potential molecular method for risk assessment of synthetic nitro- and polycyclic musks, imidazolium ionic liquids and N-glucopyranosyl ammonium salts. Environ. Toxicol. Pharmacol. 19, 291–296.

Stasiewicz, M., Mulkiewicz, E., Tomczak-Wandzel, R., Kumirska, J., Siedlecka, E., Gołebiowski, M., Gajdus, J., Stepnowski, P. 2008. Assessing toxicity and biodegradation of novel, environmentally benign ionic liquids (1-alkoxymethyl-3-hydroxypyridinium chloride, saccharinate and acesulfamates) on cellular and molecular level. Ecotoxicol. Environ. Saf. 71, 157–165.

Stock, F., Hoffmann, J., Ranke, J., Störmann, R., Ondruschka, B., Jastorff, B. 2004. Effects of ionic liquids on the acetylcholinesterase - a structure-activity relationship consideration. Green Chem. 6, 286–290.

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

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

Stolte, S., Abdulkarim, S., Arning, J., Blomeyer-Nienstedt, A.-K., Bottin-Weber, U., Matzke, M., Ranke, J., Jastorff, B., Thöming, J. 2008. Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazoliumchloride and electrochemical wastewater treatment of poorly biodegradable compounds. Green Chem. 10, 214–224.

Studzińska, S., Buszewski, B. 2009. Study of toxicity of imidazolium ionic liquids to watercress (*Lepidium sativum* L.). Anal. Bioanal. Chem. 393, 983–990.

Swatloski, R.P., Holbrey, J.D., Memon, S.B., Caldwell, G.A., Caldwell, K.A., Rogers, R.D. 2004. Using *Caenorhabditis elegans* to probe toxicity of 1-alkyl-3-methylimidazolium chloride based ionic liquids. Chem. Commun, 668–669.

Tokuda, H., Hayamizu, K., Ishii, K., Susan, M.A.B.H., Watanabe, M.J. 2004. Physicochemical Properties and Structures of Room Temperature Ionic Liquids. 1. Variation of Anionic Species. J. Phys. Chem. B 108, 16593–16600.

Tokuda, H., Hayamizu, K., Ishii, K., Susan, M.A.B.H., Watanabe, M.J. 2005. Physicochemical Properties and Structures of Room Temperature Ionic Liquids. 2. Variation of Alkyl Chain Length in Imidazolium Cation. J. Phys. Chem. B 109, 6103–6110.

Tokuda, H., Ishii, K., Susan, M.A.B.H., Tsuzuki, S., Hayamizu, K., Watanabe, M.J. 2006. Physicochemical Properties and Structures of Room-Temperature Ionic Liquids. 3. Variation of Cationic Structures. J. Phys. Chem. B 110, 2833–2839.

UFT/Merck Ionic Liquids Biological Effects Database: http://www.il-eco.uft.uni-bremen.de (last accessed: July 15th 2014)

United Nations. 2006. Globally Harmonized System of classification and labeling of chemicals. UN/SCEGHS/12/INF5. New York, USA.

United Nations. 2013. Globally Harmonized System of classification and labeling of chemicals (GHS), 5th revised edition, New York and Geneva.

Van Elsas, J.D., Jansson, J.K., Trevors, J.T. (Eds.) 2012. Modern Soil Microbiology, second edition. CRC Press, Taylor&Francis Group, Boca Raton, Florida, USA.

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

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

Visser, A.E., Swatloski, R.P., Reichert, W.M., Mayton, R., Sheff, S., Wierzbicki, A., Davis J.H., Rogers, R.D. 2002. Task-specific ionic liquids incorporating novel cations for the coordination and extraction of Hg^{2+} and Cd^{2+}: synthesis, characterization, and extraction studies. Environ. Sci. Technol. 36(11), 2523–2529.

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

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.

Wasserscheid, P., Welton, T. (Eds.) 2007. Ionic liquids in synthesis, 2nd edition. Wiley-VCH, Weinheim, Germany.

Web of science® https://webofknowledge.com (last accessed: July 22nd 2014)

Wells, A.S., Coombe, V.T. 2006. On the freshwater ecotoxicity and biodegradation properties of some common ionic liquids. Org. Pro. Res. Dev. 10, 794–798.

Welton, T. 1999. Room temperature ionic liquids. Solvents for synthesis and catalysis. Chem. Rev. 99, 2071–2083.

Werner, S., Haumann, M., Wasserscheid, P. 2010. Ionic liquids in chemical engineering. Annu. Rev. Chem. Biomol. Eng. 1, 203–230.

Wilkes, J.S. 2002. A short history of ionic liquids - from molten salts to neoteric solvents. Green Chem. 4, 73–80.

Yu, M., Li, S.M., Li, X.Y., Zhang, B.J., Wang, J.J. 2008. Acute effects of 1-octyl-3-methylimidazolium bromide ionic liquid on the antioxidant enzyme system of mouse liver. Ecotoxicol. Environ. Saf. 71, 903–908.

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.

Zhang, C., Wang, H., Malhotra, S.V., Dodge, C.J., Francis, A.J. 2010. Biodegradation of pyridinium-based ionic liquids *by an* axenic culture *of* soil *Corynebacteria.* Green Chem. 12, 851–858.

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.

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AVALUACIÓ DE L'(ECO)TOXICITAT I LA BIODEGRADABILITAT DE LÍQUIDS IÒNICS PRÒTICS ALIFÀTICS DE CADENA CURTA

BREZANA PERIC

2014

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

PROGRAMA DE DOCTORAT
Ciències i tecnologies del medi ambient

AVALUACIÓ DE L'(ECO)TOXICITAT I LA BIODEGRADABILITAT DE LÍQUIDS IÒNICS PRÒTICS ALIFÀTICS DE CADENA CURTA

EVALUATION OF (ECO)TOXICITY AND BIODEGRADABILITY OF SHORT ALIPHATIC PROTIC IONIC LIQUIDS

Memòria presentada per Brezana Peric per optar al títol de doctor per la Universitat de Barcelona

Tutora i directora: Dra. Maria Antonia Garau Guasch

Director: Dr. Jordi Sierra i Llopart

Doctoranda: Brezana Peric

2014

AGRAÏMENTS

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En primer lugar, quiero agradecer al Ministerio de Asuntos Exteriores y de Cooperación (MAEC) y a la Agencia Española de Cooperación Internacional para el Desarrollo (AECID) por haberme concedido la beca MAEC-AECID, sin la cual este doctorado no se hubiera podido realizar.

Un agraïment molt especial a la Teresa Maria de Manuel Ochoa, que no ha sigut només la responsable de la gestió de beques, sinó també una gran amiga, donant-me suport i ànims durant anys, sentint tots els meus problemes i aguantant fins i tot ploreres.

Dono les gràcies als meus directors de tesi, la Dra. M. Antonia Garau i el Dr. Jordi Serra, pels anys d'una col·laboració meravellosa i per la seva ajuda i els seus consells, dins i fora del laboratori.

També m'agradaria donar les gràcies als altres membres del meu equip: Dr. Robert Cruañas, per haver-me donat l'oportunitat de fer el projecte de tesi al Laboratori d'Edafologia, per la seva infinita paciència en escoltar totes les meves idees, dubtes i problemes, i la Dra. Esther Martí pels seus valuosos suggeriments i els seu suport moral. Agraeixo al Màrius per la seva ajuda amb l'anàlisi cromatogràfic.

Gràcies a tot el personal de la Unitat d'Edafologia, heu estat meravellosos! Aquests sis anys han sigut com un somni gràcies a vosaltres. Un agraïment especial a l'Angelito de mi alma. Miquel, gràcies per haver estat sempre a prop i per haver-me ajudat amb qualsevol problema o dubte que he tingut durant aquests anys, i també per fer-me sentir menys sola i més com a casa, sobre tot compartint el "castell" durant els dies festius i les vacances. Cèlia, Silvana i Miquelet, gràcies per totes les rialles i xerrades.

Al equip de la Unitat de Fisiologia Vegetal dono les gràcies per tots els grans moments que hem passat junts: Xavi, Jean, Laura, Míriam, Strahil, Kosta, Nati i molts altres que he arribat a conèixer durant el meu doctorat.

Durant la meva estada al Centre d'Investigacions Ambientals i Tecnologia Sostenible (UFT) de la Universitat de Bremen vaig tenir alguns col·laboradors boníssims i em vaig trobar amb gent de tot el món. Mencionaré només alguns membres del personal de l'UFT: Stefan, Jürgen, Ulrike, Jen, Andrea, Hà, Cho, Jan, els dos Antjes, Ruth... Gràcies a tots per l'estada que va ser una experiència única i inoblidable, malgrat el fred polar. Magda i Alke, gràcies per fer-me sentir com a casa. Albert, Brittany i Catherine gràcies per ajudar-me a sobreviure la fredor del nord.

També m'agradaria donar les gràcies als meus col·legues del Grup d'Anàlisi i Gestió Ambiental de la Universitat Rovira i Virgili, especialment a la Neus, pel bon rotllo durant els congressos i per haver-me permès realitzar el test Microtox® al seu laboratori.

José Luis, gràcies per ser el millor cap del món. Gràcies per la teva comprensió, consells i xerrades. He après molt de tu. Glòria i Francisco, gràcies per ser-hi cada vegada que necessitava ajuda o algú amb qui parlar. Moltes gràcies a la resta de l'equip d'informàtica també.

Gràcies a la M. Àngels, la Pepita i l'Ana per haver tingut molta paciència i per haver-me ajudat i guiat pels laberints administratius i tècnics.

Gràcies al Jihad i al Roy per ser tan "pura vida" i unes persones i amics estupends. També vull agrair a la Laura, que ha estat una bona amiga més enllà de les parets de la nostra unitat. Un agraïment especial a la meva meravellosa amiga mexicana Vanessa, vaig gaudir molt del nostre any a Barcelona! Moltes "chachi piruli" gràcies a la Isabel, la meva "profunda inspiració", que no només ha enriquit el meu vocabulari espanyol, sinó també tres anys de la meva vida a Barcelona. Moltes gràcies a la Ziortza, sempre admiraré el seu caràcter basc implacable. A l'Anita-farmacèutic-cantant-actriu-Balança li dec moltes gràcies, per ser una bona amiga des del primer dia i per tenir sempre uns consell i punts de vista sensats. Senyor G., gràcies per tots els bitllets d'últim segon i per gairebé 25 anys d'amistat. Abuelit@, gràcies per tots els xats, converses i correus interminables, i per creure sempre en mi. Gràcies a Jeca^2, Caca, Milena, Danijela, Dušica i la resta del meu BJ&BG equip. També expresso la meva gratitud a Marija, Rajka i Gocita pel seu suport i preocupació. Un agraïment molt especial a Ljubiša, per ser-hi sempre per a mi durant gairebé dues dècades.

A tots els meus professors de català i d'altres llengües agraeixo tot el que m'han ensenyat. També agraeixo a tots els meus companys de classe de diversos cursos als que he assistit al llarg del doctorat per la seva amistat que en ha durat mooolt més de les classes.

Un gran i molt especial agraïment a l'equip de Professorat. Nois, vosaltres sou els millors! Gràcies per fer-me sentir com a un membre més de l'equip i per totes les converses durant les sessions de cafè&begudes els dissabtes després dels partits.

Neus i Paco, la meva família "adoptiva", gràcies per ser-hi sempre. Neus, gràcies per la teva paciència i el teu interès, per les llargues hores de conversa, i per tenir sempre alguna cosa agradable i reconfortant a dir. Paco, ets el gat més preciós de tot l'univers!

Micifú&Kitties, gràcies pel vostre afecte i pel suport incondicional, sou els millors!!!

Per acabar, voldria agrair a la meva família per la seva comprensió i suport al llarg dels anys del doctorat, i en especial a la meva mare, a qui dedico aquesta tesi.

És impossible esmentar a tothom aquí, i per això vull agrair a totes les persones que han passat per la meva vida durant el meu doctorat.

ÍNDEX

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INTRODUCCIÓ

1. INTRODUCCIÓ

1.1. Líquids iònics

Els líquids iònics (ILs) són un grup de compostos bastant nou i prometedor, que ha generat un gran interès com a possibles substituts, ambientalment benignes, de dissolvents orgànics. Un líquid iònic (IL) es defineix generalment com una sal composta enterament d'ions (cations i anions) amb un punt de fusió per sota de 100°C (Rogers and Seddon, 2002). Els líquids iònics són líquids en un interval ampli de temperatures, i alguns d'ells ho són fins i tot a temperatura ambient (Ranke et al., 2007a). Aquest ILs s'anomenen RTILs (líquids iònic a temperatura ambient, segons las seves sigles en anglès Room Temperature Ionic Liquids). La majoria dels líquids iònics utilitzats fins ara tenen un catió orgànic (imidazoli[a], piridini[b], pirrolidini[c], piperidini[d], amoni[e], fosfoni[f], etc), substituït amb cadenes alquíliques de diferent longitud (de C_1 a C_{22}) i anions inorgànics com ara $[Cl]^-$, $[Br]^-$, $[I]^-$, $[N(CN)_2]^-$, $[BF_4]^-$, $[PF_6]^-$, etc. (Figura 1). Actualment s'estan desenvolupant nous ILs i contínuament s'estan descobrint les seves noves aplicacions.

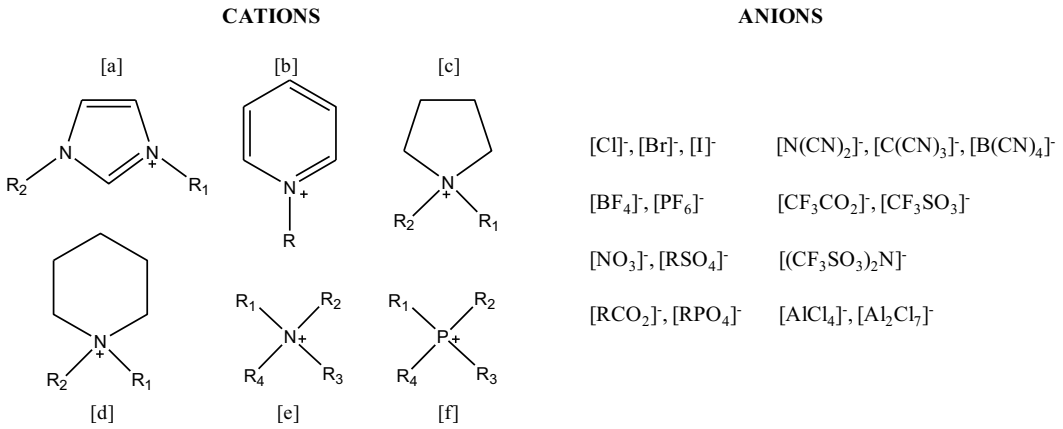


Figura 1. Els principals ions presents en l'estructura d'ILs.

Els líquids iònics no són compostos descoberts recentment, sinó que es coneixen des de fa gairebé un segle. La primera observació documentada de líquids iònics va ser un producte format durant la reacció de Friedel-Crafts anomenat "oli vermell" a meitat del segle XIX. L'estructura de l'oli vermell més tard es va

identificar per RMN com un producte intermedi estable compost per un carbocatió i un anió tetracloroaluminat (Forsyth et al., 2004). El primer exemple d'un líquid iònic a temperatura ambient va ser el nitrat d'etilamoni $[EtNH_3^+(NO_3)]^-$ amb un punt de fusió de 12ºC. Va ser descrit per Paul Walden el 1914 i s'obtingué mitjançant la neutralització d'etilamina amb àcid nítric concentrat. Un dels primers RTILs, l'1,3-dialquilimidazoli va ser descrit a principis de 1980 per Wilkes i col·laboradors, que el varen obtenir barrejant el clorur d'1-etil-3-metilimidazoli amb el triclorur d'alumini (Wilkes, 2002). Els ILs derivats d'organoaluminats tenen un ventall limitat d'aplicacions a causa de l'alta reactivitat de l'anió cloraluminat amb l'aigua (Kruger, 2008). A principis de 1990, Wilkes informà de dos líquids iònics nous: tetrafluoroborat d'1-butil-3-metilimidazoli i hexafluorofosfat d'1-butil-3-metilimidazoli (Wilkes, 2002). El catió 1-etil-3-metilimidazoli va ser el més estudiat fins a l'any 2001, i avui en dia, les sals de l'1-3-dialquilimidazoli són el tipus d'ILs més utilitzats i investigats (Keskin et al., 2007).

El gran nombre de líquids iònics existents i potencials i les seves nombroses aplicacions fan que la seva classificació sigui una tasca molt difícil a causa dels diferents criteris que es podrien utilitzar (característiques químiques, físiques o estructurals, aplicacions industrials, etc). Així, d'acord amb les propietats químiques, els ILs es poden dividir en pròtics (PILs, segons les seves sigles en anglès: Protic Ionic Liquids) i apròtics (AILs, segons les seves sigles en anglès: Aprotic Ionic Liquids). El tret distintiu entre ambdós és que els PILs tenen un protó disponible per a l'enllaç d'hidrogen (Greaves and Drummond, 2008), mentre que els AILs no el tenen.

No obstant això, per a alguns autors, la forma més útil per a la seva classificació es basa en les propietats que han condicionat l'evolució del seu ús. Segons Hough et al. (2007a) la primera generació inclou ILs amb propietats físiques úniques, com ara pressió de vapor molt baixa i alta estabilitat tèrmica. Els ILs de segona generació tenen un ús potencial com a materials funcionals (materials energètics, lubricants, agents complexants d'ions metàl·lics, etc) que fan servir propietats físiques i químiques "a mida". La tercera i més recent generació d'ILs inclou propietats biològiques combinades amb propietats físiques i químiques

seleccionades. Aquesta tercera generació d'ILs amb propietats biològiques pot ser un gran avanç per a la indústria farmacèutica, ja que està obrint moltes possibilitats per principis actius farmacèutics (APIs, segons les seves sigles en anglès: Active Pharmaceutical Ingredients), en forma de líquids iònics (IL-API). La majoria dels APIs són sals cristal·lines que presenten alguns problemes relacionats amb la dissolució, el transport, la biodisponibilitat i el control del polimorfisme, que podrien reduir l'activitat farmacològica (Schuster et al., 2005 i Hough et al., 2007b). Una possible manera de superar els inconvenients d'un fàrmac amb un grup actiu iònic és canviar l'ió complementari per un altre capaç d'aportar propietats fisicoquímiques d'IL a la nova substància i així modificar les seves propietats farmacocinètiques inicials.

Cota i col·laboradors (2007) varen sintetitzar una nova família de PILs amb cations i anions diferents dels que s'han utilitzat fins ara i, és la que ha estat objecte d'aquesta tesi. Aquests PILs es componen d'amines substituïdes (mono, di i trietanolamina) com a cations, i àcids orgànics alifàtics (fòrmic, acètic, propiònic, butíric, isobutíric i pentanoic) com a anions (Cota et al., 2007). També s'han analitzat uns dels més utilitzats AILs, derivats de l'imidazoli i del piridini (Masten, 2004, Werner et al., 2010, Domínguez de Maria, 2012 i Mohammad Ali, 2012). Concretament, els PILs analitzats són: formiat de 2-hidroxietanolamina (2-HEAF), butanoat de 2-hidroxietanolamina (2-HEAB), formiat de 2-hidroxidietanolamina (2-HDEAF), acetat de 2- hidroxidietanolamina (2-HDEAA), propionat de 2-hidroxidietanolamina (2-HDEAPr), butanoat de 2-hidroxidietanolamina (2-HDEAB), isobutanoat de 2-hidroxidietanolamina (2-HDEAiB), pentanoat de 2-hidroxidietanolamina (2-HDEAPe), butanoat de 2-hidroxitrietanolamina (2-HTEAB) i pentanoat de 2-hidroxitrietanolamina (2-HTEAPe). Els AILs analitzats són: clorur d'1-butil-3-metilimidazoli ([BMIM]Cl), clorur d'1-metil-3-octilimidazoli ([OMIM]Cl) i clorur de N-butilpiridini ([BPy]Cl). Les estructures dels PILs analitzats es mostren en la Figura 2 i les estructures dels AILs en la Figura 3.

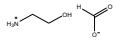
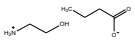
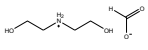
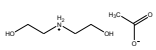
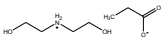
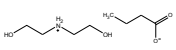
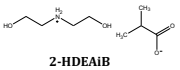
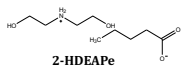
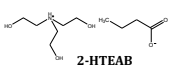
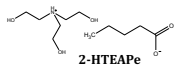
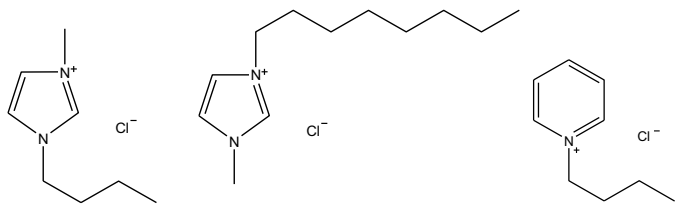
	Àcid fòrmic	Àcid acètic	Àcid propiònic	Àcid butíric	Àcid isobutíric	Àcid pentanoic
Monoetanolamina	 2-HEAF			 2-HEAB		
Dietanolamina	 2-HDEAF	 2-HDEAA	 2-HDEAPr	 2-HDEAB	 2-HDEAiB	 2-HDEAPe
Trietanolamina				 2-HTEAB		 2-HTEAPe

Figura 2. Estructures i abreviacions dels PILs analitzats.



[BMIM]Cl **[OMIM]Cl** **[PBy]Cl**

Figura 3. Estructures i abreviacions dels AILs analitzats.

L'interès pels ILs està creixent ràpidament, fet que es manifesta en el nombre de publicacions que està augmentant constantment any rere any. Més del 95% de tots els estudis s'han publicat durant els darrers vint anys, i el nombre de publicacions ha augmentat d'una manera espectacular en l'última dècada. Els 20 treballs publicats el 1994 augmentaren a gairebé 50 el 1997, i els ILs van ser considerats com una mena de compostos exòtics en aquell moment. Però 5 anys més tard, el 2002, ja hi havia més de 500 articles publicats, i que varen ser més de 2500 el 2008. Al 2011, més de 3000 publicacions tractaven aquest tipus de compostos, augmentant a gairebé 4000 el 2013 i aquest nombre segueix creixent a tot el món, a causa de la investigació constant sobre la síntesi i possibles aplicacions dels ILs, tant els existents com els nous (Fig. 4). Aquest nombre s'ha superat enguany. S'ha produït un notable desenvolupament dels grups de recerca de la Xina en aquest camp, amb més de 1200 articles publicats en 2011 procedents de grups de recerca xinesos (Barrosse-Antle et al., 2010, Endres, 2012, Rogers et al., 2012, Web of Science®).

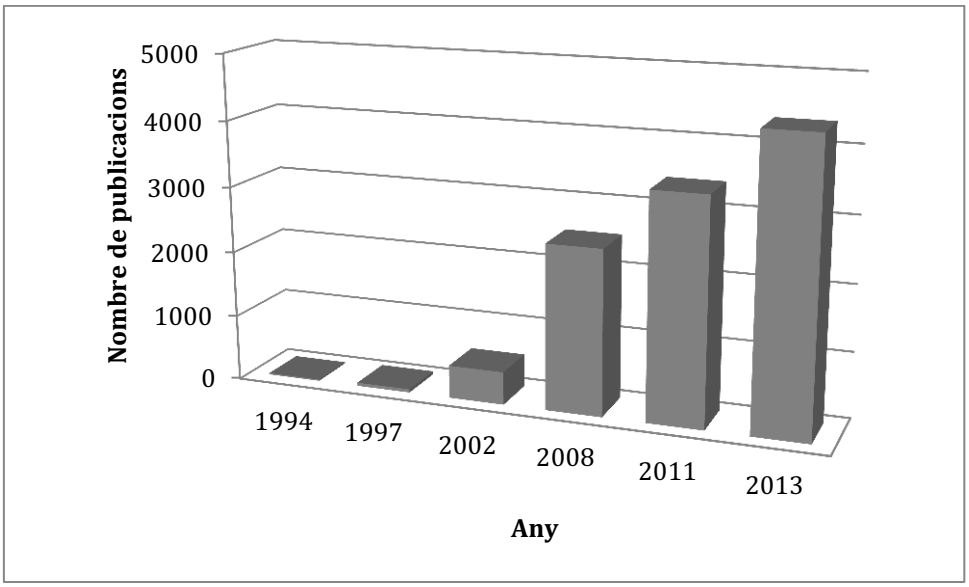


Figura 4. Evolució del nombre de publicacions sobre líquids iònics entre 1994 i 2013.

1.2. Propietats i aplicacions dels líquids iònics

Les propietats fisicoquímiques dels líquids iònics, com en el cas d'altres compostos, depenen de forces intermoleculars i intramoleculars i, conseqüentment, de l'estructura del catió i de l'anió. Un nombre significatiu de treballs de recerca s'ha realitzat per establir la relació entre les propietats fisicoquímiques dels ILs i l'estructura dels seus cations i anions (Tokuda et al., 2004, 2005, 2006, Greaves, et al., 2006). En general, els ILs tenen una pressió de vapor insignificant i per tant no són volàtils i no s'espera contaminació atmosfèrica com a conseqüència del seu ús. Els ILs també són tèrmicament estables i per aquest motiu es poden utilitzar en processos químics que requereixen entrada de calor. Aquestes i altres característiques (Taula 1), com ara la no inflamabilitat, els fan útils per a moltes aplicacions, i també fan que compleixin molts dels requisits de la química verda.

Taula 1. Algunes característiques fisicoquímiques dels líquids iònics (IL Thermo NIST Standard Reference Database).

Punt de descomposició	150 – 500°C
Conductivitat elèctrica a 25°C	< 0.6 S m^{-1} (valor màxim 11.9)
Inflamabilitat	No inflamables
Punt de fusió	< 100°C (valor màxim 239°C)
Rang de temperatures de la fase líquida	-96 – 300°C
Conductivitat tèrmica	0.117 – 0.199 W/m/K
Pressió de vapor	Insignificant
Viscositat	0.013 – 0.22 Pa·s (valor màxim 1.02)

Hi ha literalment milions d'estructures diferents que es poden formar mitjançant la combinació de diferents cations i anions, i el nombre de possibles combinacions s'estima que és de l'ordre de 10^{18} (Visser et al., 2002 i Chiappe i Pieraccini, 2005). Aquesta enorme quantitat de possibles ILs permetria, d'acord amb les característiques fisicoquímiques, seleccionar el més adequat per a una aplicación concreta. En particular, els líquids iònics a temperatura ambient (RTILs) són sovint anomenats "dissolvents de disseny", ja que és possible crear un IL amb una propietat determinada. Els RTILs s'han utilitzat per a nombroses aplicacions, i el seu desenvolupament continua a una velocitat considerable a causa de les seves propietats físiques i químiques peculiars, com ara l'elevada estabilitat tèrmica i química, manca d'inflamabilitat, baixa volatilitat i alta solubilitat modificable en diversos compostos orgànics (Earle i Seddon, 2000). A causa de la seva baixa volatilitat, molts dels RTILs s'han utilitzat com a alternatives més "verdes" a solvents orgànics convencionals tòxics i volàtils, aprofitant les seves propietats úniques (Wasserscheid i Welton, 2007). Aquest conjunt de propietats permet el disseny de sistemes de reacció molt atractius que poden resoldre alguns dels principals inconvenients dels mètodes de síntesi que actualment s'utilitzen o per a l'obtenció de nous procediments per a la fabricació de diversos productes (Welton, 1999 i Wasserscheid i Welton, 2007). Polaritat, hidrofília/hidrofòbia i altres propietats dels líquids iònics es poden ajustar mitjançant una combinació adequada de cations i anions (Sheldon, 2001).

A mesura que s'anaven descobrint les propietats dels líquids iònics, hi va haver un creixent interès en la seva aplicació com a medi de reacció en una àmplia varietat de transformacions químiques que fins fa poc només podien dur-se a terme fent servir dissolvents orgànics. La bibliografia descriu nombrosos usos de ILs, com per exemple: medis de reacció per a moltes transformacions orgàniques (Sheldon, 2001), en separacions i extraccions (Han i Armstrong, 2007), com electròlits per electroquímica (Hapiot i Lagrost, 2008), en nanotecnologia (Ichikawa et al., 2007), en biotecnologia (Van Rantwijk i Sheldon, 2007), en processos d'enginyeria (Greaves and Drummond, 2008), en aplicacions biotecnològiques (Quijano et al., 2011), en absorció de gasos (CO_2) (Rogers i Seddon, 2002), com a catalitzadors en síntesi orgànica (Olivier-Bourbigou et al., 2010), i en reaccions de polimerització radical i organometàl·lica (Zhu et al., 2009). Altres autors han descrit una aplicació específica dels ILs en l'extracció de principis actius de plantes medicinals (Du et al., 2009, Jin et al., 2011 i Liu et al., 2011). Els líquids iònics també tenen un potencial en gran mesura inexplorat per ser utilitzats en la indústria farmacèutica. Es poden utilitzar com a emmagatzematge de fàrmacs per a un alliberament controlat (Jaitely et al., 2008), dissolvents de productes farmacèutics (Azevedo et al., 2013) o com a ingredients en sistemes d'administració de fàrmacs via tòpica (Dobler et al., 2013). Per tal de superar els problemes associats amb la baixa solubilitat en aigua, i per tant baixa biodisponibilitat dels principis actius farmacèutics (APIs), els líquids iònics que contenen APIs estan sent investigats, com en el cas dels líquids iònics derivats de l'ampicil·lina (Florindo et al., 2013). Algunes de les aplicacions dels ILs es presenten a la Figura 5.

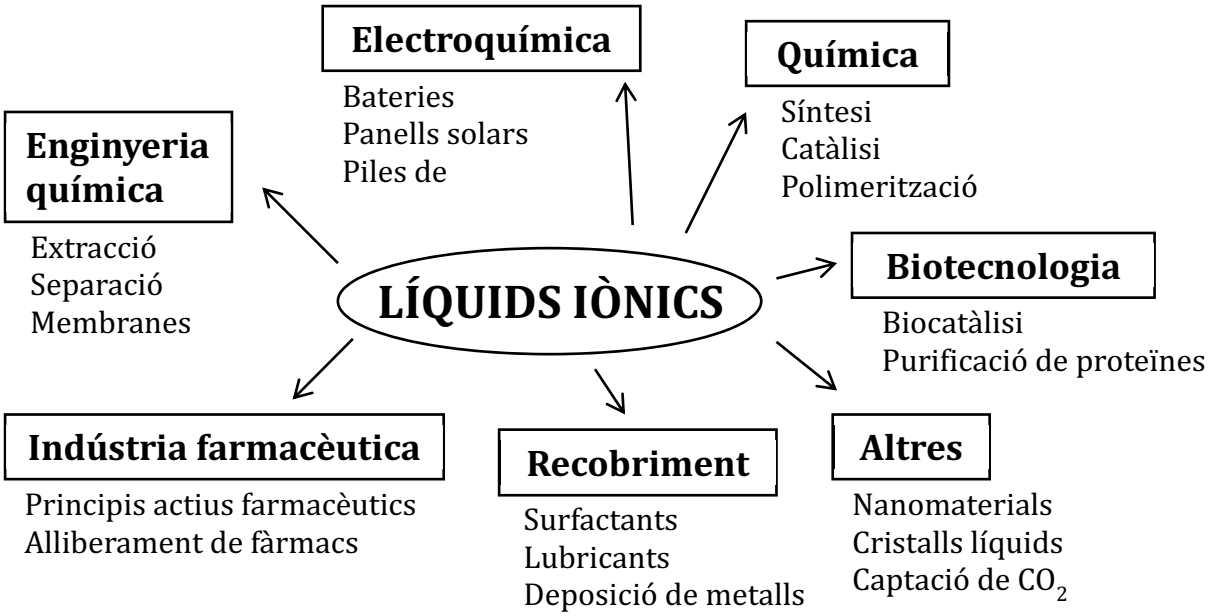


Figura 5. Algunes de les possibles aplicacions dels líquids iònics (adaptat de Pham et al., 2010).

Les possibles aplicacions per a la nova família dels PILs estudiada són nombroses. El 2-HEAF té la capacitat de dissoldre moltes sals inorgàniques, compostos hidroxilats i alguns polímers insolubles, com ara la polianilina i el polipirrol (Bicak, 2005). També, el 2-HEAF s'ha considerat com a un dissolvent potencial en la preparació de polianilinas organosolubles amb pesos moleculars raonables (Bicak et al., 2005 i Cota et al., 2007). S'ha revisat l'aplicació i les perspectives d'un sistema de dissolvents de dues fases, compost per líquids iònics i fluids supercrítics amb èmfasi en el diòxid de carboni supercrític (Roth, 2009). Alguns representants d'aquesta família, com ara el 2-HDEAF, el 2-HDEAA i el 2-HDEAPe, han estat analitzats per la seva potencial activitat catalítica en reaccions de condensació aldòlica (Iglesias et al., 2010 i Cota et al., 2013) i per a la hidrogenació catalítica heterogènia de ciclohexà i acetona catalitzada per Pt/Al_2O_3 amb metanol, etanol i propanol (Khodadadi-Moghaddam et al., 2009). Altres PILs, també derivats de la 2-hidroxietanolamina i d'àcids orgànics, s'han estudiat per tal de desenvolupar un nou procés per a l'absorció de CO_2 (Yuan et al., 2007). Recentment s'ha estudiat el potencial efecte corrosiu dels PILs en l'estructura de metall dels sistemes d'absorció de CO_2 i s'han trobat uns índex de corrosió molt baixos (especialment per al 2-HEAA) en el cas de plaques carbòniques d'acer A285,

que és un material àmpliament utilitzat en operacions d'absorció (Dos Santos et al., 2014). De Souza i col·laboradors (2013) han aconseguit la immobilització de la lipasa de *Burkholderia cepacia* amb la tècnica sol-gel usant PILs derivats de la monoetanolamina com a additius per protegir contra la inactivació de la lipasa a causa de l'alliberament d'alcohol i la contracció del gel. En un altre estudi, el mateix conjunt de PILs s'ha immobilitzat sobre alanina com a suport, obtenint catalitzadors heterogenis actius, que han mostrat un excel·lent potencial catalític en condensacions citral-acetona i benzaldehid-acetona (dues reaccions d'interès per a la indústria de química fina) i poden ser reciclats i reutilitzats durant tres cicles consecutius sense cap pèrdua significativa de la seva activitat (Cota et al., 2014). Una possible aplicació dels PILs (2-HEAA, 2-HDEAA, 2-HEAPr i 2-HDEAPr) que encara s'està desenvolupant, implica la recerca d'un mètode alternatiu per al tenyit de fibres de cotó utilitzant els PIL com a substituts de l'aigua (Iglesias et al., 2014).

1.3. Ecotoxicitat de líquids iònics

Encara que els ILs tenen un perfil potencialment "verd", són bàsicament productes químics, i com a tal, han de complir amb els requisits de la legislació vigent sobre productes químics a la Unió Europea anomenada Registre, Avaluació, Autorització i Restricció de Substàncies Químiques (Registration, Evaluation, Authorization, and Restriction of Chemical Substances - REACH) (European Union, 2006). Tenint en compte el possible ús comercial dels ILs, un aspecte molt important que ha de ser estudiat amb la suficient profunditat és el seu impacte potencial negatiu sobre el medi ambient i els éssers humans. El REACH regula la seguretat dels productes químics, la fabricació, la toxicitat, la biodegradabilitat, el transport i l'ús en els sectors industrials. Aquesta legislació és aplicable a les substàncies fabricades o importades a la Unió Europea en quantitats d'1 tona o més a l'any. El REACH va entrar en vigor l'1 de juny de 2007 i exigeix a tots els fabricants i importadors de substàncies químiques que les registrin en l'Agència Europea de Productes Químics (ECHA, segons las seves sigles en anglès European Chemicals Agency). El registre significa que un fabricant o importador ha de

presentar un expedient de registre a l'ECHA. La tercera data límit d'inscripció serà el 31 maig 2018 per a totes les substàncies produïdes, importades o utilitzades en quantitat d'1 a 100 tones per any, i, per tant, podria afectar els líquids iònics, si són produïts i utilitzats a gran escala.

Els fabricants i importadors han de presentar dos documents: un expedient tècnic, per a les substàncies fabricades o importades en quantitats d'1 tona o més a l'any, i, a més, un informe sobre la seguretat química, per a les substàncies en quantitats de 10 tones o més per any. L'expedient tècnic conté informació sobre les propietats i la classificació d'una substància, així com sobre els usos i orientacions sobre l'ús segur. La informació necessària per determinar les propietats de les substàncies varia en funció de la quantitat en què es fabrica o importa la substància. Com més gran sigui la quantitat, es requereix més informació sobre les propietats intrínseques de la substància química. L'informe de seguretat química documenta els perills i la classificació d'una substància i l'avaluació per si la substància és PBT (Persistent, Bioacumulable i Tòxica) o mPmB (molt Persistent, molt Bioacumulable). L'informe de seguretat química també inclou escenaris d'exposició (European Union, 2006).

Els requisits per a la informació ambiental es poden dividir en dos tipus d'informació principals:

- Informació sobre l'ecotoxicitat: toxicitat aquàtica a llarg termini (invertebrats), toxicitat a curt termini o a llarg termini per als organismes terrestres, toxicitat a llarg termini per als organismes dels sediments, toxicitat a llarg termini per a la reproducció d'aus.
- Comportament mediambiental: degradació biòtica (test de simulació), informació sobre els productes de degradació, bioacumulació i adsorció-desorció.

Tot i que els líquids iònics es consideren com no volàtils i per tant no poden contribuir a la contaminació de l'aire, la solubilitat en aigua de molts dels líquids iònics no es gens despreciable. El possible alliberament dels líquids iònics al medi aquàtic i terrestre pot conduir a la contaminació de l'aigua i del sòl, i els riscos relacionats amb aquests tipus de contaminació. Per tant, és important avaluar

l'ecotoxicitat, la biodegradabilitat, la bioacumulació i el destí mediambiental d'aquests productes químics. Tenint en compte la possible utilització i comercialització dels líquids iònics, haurien de passar l'avaluació del REACH, que recomana que els assaigs per a l'avaluació de la toxicitat potencial dels productes químics es facin d'acord amb les directrius de l'OCDE. Aquests assaigs es poden dividir en tres grups: efectes sobre els sistemes biòtics, efectes sobre la salut humana, i degradació i acumulació.

1.3.1. Ecotoxicitat aquàtica

Diferents publicacions han investigat diverses propietats dels líquids iònics i els seus efectes sobre els organismes aquàtics. Investigacions toxicològiques realitzades durant la darrera dècada indiquen que els líquids iònics podrien no ser tan "verds" com es considerava inicialment.

Les algues verdes són organismes ecològicament rellevants i representen la base de la cadena alimentària. L'assaig fet amb algues té un avantatge sobre les proves realitzades, per exemple, amb peixos o invertebrats, perquè dóna una resposta a nivell de població. Les algues tenen un cicle de vida curt que les fa ideals per als estudis toxicològics, ja que poden respondre ràpidament als canvis ambientals (Blaise i Ferard, 2005). Cho i col·laboradors (2008) han trobat que la toxicitat de l'1-butil-3-metilimidazoli, de l'1-butil-3-metilpiridini, de l'1-butil-1-metilpirrolidini, del tetrabutilamoni i dels bromurs de tetrabutilfosfoni per la *Pseudokirchneriella subcapitata* era entre dos i quatre ordres de magnitud major que la dels dissolvents orgànics estudiats (metanol, dimetilformamida i 2-propanol). Aquest grup també va investigar una altra sèrie de líquids iònics basats en l'imidazoli i varen arribar a la conclusió que la toxicitat dels líquids iònics incrementa amb l'augment de la longitud de la cadena lateral (Cho et al., 2007). Latała et al. (2005) varen examinar l'efecte de líquids iònics derivats de l'imidazoli sobre altres espècies d'algues del Bàltic (*Oocystis submarina* i *Cyclotella meneghiniana*). Ells van trobar que la resposta de les dues espècies era molt diferent, sent *Oocystis submarina* la més resistent a la toxicitat dels ILs, i que la

toxicitat es reduïa en les aigües més salines. Matzke et al. (2007) van investigar l'efecte tòxic de líquids iònics derivats de l'imidazoli amb C_4 i C_8 en les cadenes laterals sobre *Scenedesmus vacuolatus* sp. Els seus resultats van confirmar les conclusions d'altres autors, és a dir, que la toxicitat depèn en gran mesura de l'efecte de la cadena lateral.

Els resultats dels estudis de toxicitat de ILs cap *Daphnia magna* (puça d'aigua dolça) van demostrar el vincle entre la toxicitat i la longitud de la cadena alquílica dels líquids iònics assajats que contenien imidazoli, piridini o amoni quaternari com a cations (Couling et al., 2006, Wells i Coombe, 2006, Samori et al., 2007 i Pretti et al., 2009). Altres autors (Bernot et al., 2005a) indiquen que els ILs derivats de l'imidazoli presenten una toxicitat aguda per *Daphnia magna* més alt que el benzè i alguns dissolvents organoclorats (tri i tetraclorometà).

En altres estudis sobre el cargol *Physa acuta* amb ILs derivats de l'imidazoli i del piridini (Bernot et al., 2005b) es van obtenir resultats similars i també amb *Dreissena polymorpha* (musclo zebra) (Costello et al., 2009). En aquests treballs també es va demostrar una relació positiva entre la longitud de la cadena alquílica i la toxicitat dels líquids iònics, així com una sensibilitat més baixa de *Physa acuta* que *Daphnia magna* front als ILs.

Quant a la toxicitat dels líquids iònics pels peixos zebra (*Danio rerio*), Pretti et al. (2006) varen avaluar la toxicitat de líquids iònics utilitzats habitualment (derivats de l'imidazoli i del piridini) i la de nous ILs AMMOENG® (derivats d'amoni quaternari). Els resultats d'aquest estudi varen posar de manifest que els ILs poden causar en peixos un efecte completament diferent del que s'esperava en base a les seves estructures químiques. L'imidazoli, el piridini i el pirrolidini poden ser considerats com no altament letals pels peixos zebra. D'altra banda, algunes sals d'amoni mostren valors de CL_{50} (Concentració Letal 50, la que produeix la mort del 50% dels animals durant un període determinat) notablement menors que els de dissolvents orgànics i amines terciàries. També varen demostrar que les espècies de peixos són menys sensibles a la toxicitat dels ILs en comparació amb altres espècies que pertanyen a nivells tròfics inferiors.

La llentia d'aigua, *Lemna minor*, és una planta aquàtica que sovint s'ha utilitzat per estudiar la fitotoxicitat dels ILs (Jastorff et al., 2005, Matzke et al., 2007, Stolte et al., 2007a i Larson et al., 2008). En general, els compostos de l'1-alquil-3-metilimidazoli amb cadenes alquíliques més llargues són més tòxics per *Lemna minor* que els que tenen cadenes més curtes. Els cations imidazoli i piridini amb grups butil tenien valors de CE_{50} (Concentració Efectiva 50, la que produeix una reducció de 50% del creixement de la planta) similar, mentre que el catió amoni té un valor de CE_{50} molt més gran i per tant és menys tòxic.

Microtox® és un assaig de toxicitat estandarditzat que es basa en la reducció de la bioluminescència dels bacteris marins, *Vibrio fischeri*, quan s'exposen a diferents concentracions d'una substància tòxica. És una prova ràpida, sensible, reproduïble, ecològicament rellevant i assequible econòmicament. Es reconeix i s'utilitza àmpliament com un assaig estàndard per a determinar la toxicitat aquàtica. Docherty i Kulpa (2005) varen investigar la toxicitat de líquids iònics derivats de l'imidazoli i del piridini i van trobar que la hidrofòbia, que correspon a l'augment de la longitud de la cadena alquílica del catió del IL, provoca un augment de la toxicitat. Aquests resultats concorden amb els d'altres autors (Ranke et al., 2004 i Matzke et al., 2007). Couling i col·laboradors (2006) varen ampliar el ventall de ILs investigats i varen observar que els compostos derivats d'amoni quaternari són menys tòxics que els derivats de l'imidazoli i del piridini. En comparació amb alguns dissolvents industrials habitualment usats com ara fenol, toluè i benzè, els ILs de cadena llarga (> C_4) són més tòxics. Els valors de CE_{50} corresponents a l'assaig de Microtox® per als dissolvents orgànics utilitzats amb més freqüència es presenten a la Taula 2.

Taula 2. Toxicitat aguda de dissolvents orgànics per a *Vibrio fischeri* (Kaiser i Palabrica, 1991).

Dissolvent	**log CE_{50} ($\mu mol\ L^{-1}$)**
Metanol	3.5
Acetonitril	2.77
Acetona	2.52
Benzè	2.03
Fenol	1.49

En termes generals, tots els assaigs de toxicitat aquàtica varen mostrar que la part catiònica de la molècula era responsable de la toxicitat dels ILs. En la majoria dels casos no hi va haver influència de la part aniònica de la molècula sobre la toxicitat. Només l'anió bis[(trifluorometil)sulfonil]imida $[(CF_3SO_2)_2N]^-$ va mostrar una toxicitat més elevada que els altres ILs (Ranke et al., 2004, Matzke et al., 2007 i Stolte et al., 2007a). Es va demostrar que les cadenes laterals tenien una gran influència sobre la toxicitat. Com més llarga i més ramificada sigui la cadena lateral, més tòxic és el líquid iònic. Els líquids iònics més tòxics acostumen a tenir una cadena alquílica amb més de vuit carbonis (Wells i Coombe, 2006, Pham et al., 2010 i Radošević et al., 2013). Aquests resultats apunten al fet que alguns líquids iònics poden ser més tòxics que els dissolvents orgànics d'ús comú, així que podrien ser no tan "verds" com s'esperava.

A la Taula 3 es presenta la toxicitat dels ILs a diferents nivells de complexitat biològica.

Taula 3. Toxicitat aquàtica dels diferents ILs expressada com a $logCE_{50}$ (μmol L^{-1}).

Líquid iònic	*Vibrio fischeri*	*P. subcapitata*	*Lemna minor*	*Daphnia magna*
$[C_4MIM]Cl$	2.95 [1]	2.34 [6]	2.82 [6]	1.93 [4]
$[C_4MIM]Br$	3.07 [2]	3.46 [7]	N.D.	1.57 [4]
$[C_4MIM]BF_4$	3.55 [2]	N.D.	2.49 [10]	1.68 [4]
$[C_4MIM]PF_6$	3.07 [3]	2.20 [8]	N.D.	1.85 [4]
$[C_6MIM]Br$	1.42 [4]	2.57 [9]	N.D.	0.78 [4]
$[C_6MIM]BF_4$	3.18 [2]	N.D.	N.D.	N.D.
$[C_8MIM]Cl$	1.19 [3]	1.46 [8]	N.D.	N.D.
$[C_8MIM]BF_4$	1.41 [2]	N.D.	N.D.	N.D.
$[C_4Py]Cl$	3.18 [5]	N.D.	2.32 [5]	N.D.
$[C_4MPy]Br$	2.12 [1]	3.46 [7]	N.D.	1.76 [4]
$[C_4MPyr]Cl$	>4.30 [5]	3.67 [7]	N.D.	N.D.
$[C_4Pip]Br$	4.27 [5]	3.27 [5]	0.47 [5]	N.D.

Abreviacions: 1-butil-3-metilimidazoli (C_4MIM), 1-hexil-3-metilimidazoli (C_6MIM), 1-octil-3-metilimidazoli (C_8MIM), 1-butilpiridini (C_4Py), 1-butil-3-metilpiridini (C_4MPy), 1-butil-3-metilpirrolidini (C_4MPyr), 1-butil-3-metilpiperidi (C_4Pip). N.D. no disponible.

Resultats procedents de: [1] Docherty and Kulpa, 2005; [2] Ranke et al., 2004; [3] Garcia et al., 2005; [4] Couling et al., 2006; [5] Stolte et al., 2005; [6] Matzke et al., 2007; [7] Cho et al., 2008; [8] Wells and Coombe, 2006; [9] Cho et al., 2007; [10] Jastorff et al., 2005.

1.3.2. Ecotoxicitat terrestre

Els estudis sobre els efectes dels ILs sobre els organismes del sòl i sediments han sigut molt limitats fins al moment. En els estudis realitzats, s'han utilitzat organismes terrestres com el col·lèmbol *Folsomia candida*, un invertebrat de sòl (Matzke et al., 2007), el cuc de terra *Eisenia foetida* (Luo et al., 2010) o *Caenorhabditis elegans*, un cuc rodó de sòl (Swatloski et al., 2004). En aquest últim cas, els autors suggereixen l'ús de *C. elegans* com a organisme model per a la ràpida

i econòmica determinació dels efectes toxicològics del clorur d'1-alquil-3-metilimidazoli.

Pel que fa a les plantes superiors, Matzke et al. (2008) varen investigar la toxicitat dels líquids iònics derivats de l'imidazoli amb diferents anions per al blat (*Triticum aestivum*) i el morritort (*Lepidium sativum*). L'efecte de la cadena lateral es va confirmar una vegada més, amb models de toxicitat diferents, depenent de l'anió present a la molècula del líquid iònic. Matzke et al. (2009) varen investigar la influència de diferents minerals d'argila i concentracions d'argila sobre la toxicitat de la part aniònica de la molècula dels ILs derivats de l'imidazoli per al blat. Les dades obtingudes van mostrar que bis[(trifluorometil)sulfonil] imida d'1-butil-3-metil-imidazoli va ser el més tòxic, independentment del tipus d'argila afegida al sòl de referència. La toxicitat de diferents ILs amb el mateix catió (clorur, tetrafluoroborat i bisulfat d'1-butil-3-metilimidazoli) depenia principalment del catió i els efectes observats van variar d'acord amb el tipus d'argila afegit i la concentració d'argila en el sòl de referència. L'augment del contingut de l'esmectita va disminuir els efectes inhibitoris d'aquests líquids iònics. Studzinska i Buszewski (2009) van demostrar que els efectes perillosos dels ILs derivats de l'imidazoli estan estretament relacionats amb el contingut de matèria orgànica del sòl. Es va observar que el sòl amb més carboni orgànic pot absorbir més els cations dels ILs, comparat amb el sòl amb poca matèria orgànica; per tant, com més fèrtil sigui el sòl, menor és la probabilitat d'efectes perillosos dels ILs per a les plantes.

Wang i els seus col·laboradors (2009) varen realitzar un estudi sobre l'efecte del tetrafluoroborat d'1-butil-3-metilimidazoli sobre el blat. L'augment de la concentració del líquid iònic en el sòl mostrà un efecte negatiu significatiu tant en la germinació com en la longitud d'arrels i tiges de les plantes de blat. A baixes concentracions, el tetrafluoroborat d'1-butil-3-metilimidazoli no va inhibir, i fins i tot va promoure el creixement de les plàntules de blat. A concentracions altes, aquest líquid iònic va inhibir el creixement de les plàntules de blat de manera significativa, provocant una disminució del contingut de clorofil·la, el que va reduir la fotosíntesi i el creixement de les plantes. En una altre treball d'investigació, els assaigs de fitotoxicitat dels ILs que contenien derivats del (-)-nopil es van dur a

terme en un hivernacle usant ordi (*Hordeum vulgare*), que és una planta monocotiledònia, i rave comú (*Raphanus sativus* L. subvar. *radicula* Pers.) que és una planta dicotiledònia (Bałczewski et al., 2007). Segons els resultats obtinguts, l'augment de la concentració dels ILs va resultar en una disminució sistemàtica en el pes fresc dels brots de cultius i el pes fresc de la planta sencera del cultiu, tant per l'ordi de primavera com pel rave comú. L'ordi era més resistent que el rave i tolerava concentracions més altes dels ILs al sòl.

En un estudi recent sobre els efectes tòxics dels ILs derivats de l'imidazoli sobre l'ordi (Cvjetko Bubalo et al., 2014), on els líquids iònics estudiats tenien diferents anions i longitud de les cadenes alquíliques adossades a l'anell d'imidazoli, una vegada més, els líquids iònics amb cadenes laterals més llargues van demostrar ser més tòxics, en termes de germinació i creixement inicial de l'ordi. La informació novedosa sobre l'alternança de l'estrés oxidatiu i les activitats dels enzims antioxidants que es va trobar a les plantes d'ordi a causa del tractament amb els ILs pot ser útil per avaluar el risc potencial per als humans i per a una millor comprensió dels mecanismes de toxicitat dels ILs a nivell enzimàtic.

Encara que la investigació de la fitotoxicitat dels ILs i del seu efecte en el sòl no ha estat intensiva, les dades disponibles poden donar un impuls important perquè els científics ambientals comencin a tractar més el tema de l'impacte potencial dels ILs sobre les plantes i el sòl.

1.4. Biodegradabilitat dels liquids iònics

1.4.1. Biodegrabilitat en aigua

El potencial de biodegradació dels ILs en medi aquòs s'ha investigat en molts estudis. Wells i Coombe (2006) varen investigar la biodegradabilitat dels compostos d'amoni, imidazoli, fosfoni i piridini mesurant la demanda biològica d'oxigen (DBO) i varen observar que els cations amb cadenes laterals curtes (C_4) no eren biodegradables. Es va observar un fort efecte inhibidor per l'inòcul

utilitzat en l'assaig dels ILs amb cadenes laterals més llarges (C_{12}, C_{16} i C_{18}), fet que indica la toxicitat d'aquests ILs per als microorganismes utilitzats. Docherty i col·laboradors (2007) varen estudiar la biodegradabilitat dels compostos derivats de l'N-metilimidazoli i del 3-metilpiridini substituïts amb cadenes laterals de butil, hexil i octil, i bromur com a anió. Es va trobar una correlació entre la biodegradabilitat i la longitud de la cadena lateral en els tests de desaparició del carboni orgànic dissolt (DOC die-away, segons las seves sigles en anglès Dissolved Organic Carbon) i del seguiment dels canvis en la concentració total de nitrogen dissolt. Un altre líquid iònic, el bromur d'1-octilpiridini, va complir el requisit de l'OCDE per poder ser classificat com fàcilment biodegradable (aconseguint un nivell de biodegradació superior al 60% de la demanda teòrica d'oxigen dins dels 28 dies de prova), mentre que el bromur d'1-hexilpiridini va exhibir una taxa de degradació més baixa. En comparació amb els ILs derivats del piridini, la mineralització dels líquids iònics derivats de l'imidazoli va ser menor. El catió 1-metil-3-octilimidazoli va mostrar una taxa de biodegradació significativa, però no suficientment alta com perquè es pogués classificar com a fàcilment biodegradable. Per als grups catiònics de piridini i imidazoli que porten una cadena lateral de butil, no es va observar cap biodegradació significativa. En un altre estudi, Gathergood et al. (2006) varen trobar que la influència de l'anió era important només en un cas de l'anió octil sulfat, que va resultar ser considerablement més biodegradable que els altres anions habitualment presents en les molècules dels ILs. Amb la introducció d'un grup èster a la cadena lateral del catió 1,3-dialquilimidazoli es van aconseguir valors de biodegradació molt a prop del nivell llindar de l'assaig en recipient tancat (Closed Bottle test).

Stolte et al. (2008) van investigar la biodegradació de diferents compostos derivats de l'imidazoli, del piridini i del 4-dimetilaminopiridini, substituïts amb diverses cadenes laterals alquíliques i varen trobar una biodegradació significativa per als compostos ecotoxicològicament poc favorables, amb cadenes laterals alquíliques llargues (C_6 i C_8). En canvi, en el cas dels ILs amb les cadenes laterals alquíliques curtes ($\leq C_6$) no es va trobar cap mena de biodegradació. La introducció de diferents grups funcionals en la cadena lateral alquílica que proporcionaria una major reactivitat química, no va provocar l'esperada millora en la biodegradació

potencial. Després d'un període d'incubació de 24 dies per al catió 1-metil-3-octilimidazoli van ser identificats diferents productes de transformació amb grups d'hidroxil, carbonil i carboxil. A més, es va identificar l'escurçament de la cadena lateral, indicant la degradació de la cadena lateral octílica mitjançant β-oxidació.

Gathergood i col·laboradors (2004) varen intentar dissenyar, sintetitzar i avaluar ILs biodegradables que contenen grups èster o amida en la cadena lateral alquílica. La introducció d'un grup susceptible d'hidròlisi enzimàtica va millorar en gran mesura la biodegradació, en comparació amb els líquids iònics comunament utilitzats, derivats del dialquilimidazoli (tetrafluoroborat i hexafluorofosfat d'1-butil-3-metilimidazoli). Per a la sèrie de bromur de 3-metil-1-alkiloxicarbonil-metilimidazoli, es va observar la major biodegradació quan la cadena lateral tenia quatre carbonis o més. Els ILs amb amides incorporades van demostrar ser poc biodegradables. També varen tractar d'establir la influència de la part aniònica de la molècula sobre la biodegradabilitat dels líquids iònics. Diferents ILs amb 1-butil-3-metilimidazoli com a catió combinat amb Br^-, $[BF4]^-$, $[PF6]^-$, $[N(CN_2]^-$, $[(CF_3SO_2)_2N]^-$ i octilsulfat com a anions van ser analitzats utilitzant els protocols d'assaig Sturm i Closed Bottle. Cap compost va mostrar un percentatge significatiu de biodegradació amb l'excepció del IL que conté l'anió octilsulfat, que tenia nivells més alts de biodegradabilitat. El mateix grup va publicar un altre treball encaminat cap al descobriment d'ILs biodegradables (Gathergood et al., 2006). L'objectiu de l'estudi era incorporar modificacions estructurals addicionals per tal de millorar la biodegradabilitat. Van incorporar el grup 2-metil en la molècula d'ILs derivats de l'imidazoli per proporcionar un lloc addicional per al metabolisme, partint del fet que el 2-metilimidazoli és significativament més biodegradable que l'imidazoli. La incorporació d'un grup metil en la posició 2 del catió d'imidazoli no va tenir efecte significatiu sobre la biodegradabilitat. L'1-butil-3-metilimidazoli, habitualment utilitzat com a catió, va mostrar nivells insignificants de degradació. Part d'aquest equip va continuar la seva investigació tractant de dissenyar, sintetitzar i avaluar ILs biodegradables, derivats del piridini. Harjani et al. (2008) varen preparar ILs que porten un grup funcional èster a la cadena lateral, utilitzant piridini o àcid nicòtic com a catió. Aquests ILs varen mostrar nivells elevats de biodegradació en condicions aeròbiques i es poden classificar com a "fàcilment biodegradables". Al

contrari, els ILs derivats del piridini, amb cadenes laterals alquíliques van mostrar nivells de biodegradabilitat significativament més baixos en el mateix assaig. En un estudi recent sobre la biodegradabilitat dels 27 cations derivats de pirrolidini, morfolini, piperidini, imidazoli i piridini en condicions aeròbiques, es va trobar que els cinc grups catiònics són "fàcilment o inherentment biodegradables" (Neumann et al., 2014).

El fet que només la presencia de les cadenes laterals llargues en l'estructura dels ILs millorava la biodegradabilitat crea un conflicte d'interessos entre la minimització de la toxicitat i la maximització de la biodegradabilitat. La qüestió de la biodegradabilitat sembla ser un problema en el desenvolupament de líquids iònics ambientalment més segurs.

1.4.2. Biodegradabilitat en sòl

Com en el cas de l'ecotoxicitat terrestre, les dades relatives a la biodegradabilitat en medi terrestre també són escasses. Kumar et al. (2006) varen investigar el destí del tetrafluoroborat d'1-butil-3-metilimidazoli en contacte amb els microorganismes del sòl, microorganismes d'aigües residuals, *Pseudomonas putida* i *Escherichia coli*. Encara que el tetrafluoroborat d'1-butil-3-metilimidazoli es va mostrar recalcitrant en els assajos de Sturm i Closed Bottle tests, com ja s'ha comentat, en aquest estudi es va observar que *P. putida* era capaç de degradar el tetrafluoroborat d'1-butil-3-metilimidazoli després de 15 dies d'incubació.

Modelli i col·laboradors (2008) varen fer el seguiment, per primera vegada, dels processos de biodegradació aeròbica dels líquids iònics en sòl, treballant amb quatre líquids iònics obtinguts a partir dels cations 1-butil-3-metilimidazoli i 1-metoxietil-3-metilimidazoli combinats amb els anions tetrafluoroborat i dicianamida, mitjançant la determinació de la producció total de CO_2 durant més de sis mesos, fent servir el mètode ASTM D 5988-96. Els resultats indiquen que la taxa de biodegradabilitat oscil·la entre 17 i 52% per a l'1-butil-3-metilimidazoli i entre 0,1 i 3,6% per a l'1-metoxietil-3-metilimidazoli, amb anions dicianamida i

tetrafluoroborat respectivament. En ambdós casos la taxa de biodegradació no va superar el 10% en els 28 dies de durada de la prova.

En el primer treball que mostra la biodegradació completa per *Corynebacterium* sp. d'un piridini N-substituït amb àcids orgànics de baix pes molecular com a productes de degradació, Zhang et al. (2010) també varen trobar que l'hexafluorofosfat d'1-butil-3-metilimidazoli no es metabolitza, demostrant una vegada més que els ILs derivats de l'imidazoli no només són més tòxics, sinó també més resistents a la biodegradació, tant en aigua com en sòl.

L'estudi de Deive et al. (2011) es va centrar en la identificació de les soques microbianes amb majors taxes de supervivència en presència d'ILs i que podrien ser potencialment utilitzades en la remediació de sòls. Es van aïllar les soques microbianes supervivents (que contenen fongs i bacteris), es van identificar taxonòmicament, i es va analitzar la degradació dels líquids iònics durant el cultiu. La degradació del catió imidazoli va ser nul·la. Però entre les soques microbianes aïllades es trobaven tant fongs com bacteris que eren capaços de degradar, encara que només parcialment, els anions de cadena curta (etanoat, lactat, etilsulfonat i etilsulfat).

Però no hi ha informació completa o estudi realitzat amb la finalitat d'avaluar l'efecte tòxic dels líquids iònics per a la microbiota del sòl en termes de transformació de carboni i nitrogen.

1.5. Toxicitat enzimàtica i cel·lular

S'han utilitzat diversos assajos de toxicitat enzimàtica i cel·lular per tal de predir l'impacte dels líquids iònics en els éssers humans. L'acetilcolinesterasa (AChE) és un enzim que es troba en gairebé tots els organismes superiors. Arning et al. (2008) varen trobar que l'àtom de nitrogen positivament carregat, un sistema aromàtic àmpliament deslocalitzat, i la lipofília de les cadenes laterals dels grups catiònics són els elements clau per a la inhibició potencial de l'enzim. Es varen

analitzar la influència dels grups catiònics, aniònics i diferents cadenes laterals del catió en el potencial inhibidor dels ILs. Els autors varen concloure que els grups catiònics aromàtics exhibeixen un molt fort potencial inhibidor de l'enzim, en contrast amb els grups catiònics polars i no aromàtics que van mostrar només una molt baixa inhibició de l'activitat enzimàtica. La introducció de grups hidroxils, èters o nitrils en la cadena lateral alquílica redueix el potencial inhibitori. Pràcticament cap dels anions va mostrar efecte negatiu sobre l'AChE. Aquests resultats estan en concordança amb les conclusions dels estudis d'ecotoxicitat comentats anteriorment. Składanowski et al. (2005) varen estudiar la inhibició *in vitro* de l'enzim AMP desaminasa. Els ILs derivats de l'1-butil-3-metilimidazoli inhibien l'activitat de l'AMP desaminasa. Yu i col·laboradors (2008) varen fer un estudi dels enzims antioxidants del fetge de ratolí i es va veure que el bromur d'1-octil-3-metilimidazolio causava danys al fetge del ratolí i d'aquesta manera disminuïa significativament l'activitat de la catalasa.

També s'han dut a terme estudis *in vitro* amb diferents línies de cèl·lules, com ara línies de cèl·lules canceroses de rata (leucèmia promielocítica - IPC-81 i glioma - C6) i línies de cèl·lules humanes (línies cel·lulars de carcinoma de còlon - HT-29 i CaCo-2, cervicals - HeLa i de mama -MCF-7). Els efectes dels líquids iònics sobre la línia cel·lular IPC-81 ha estat estudiat amb freqüència, i els líquids iònics derivats de l'imidazoli amb cadenes laterals alquíliques llargues han demostrat ser més tòxics que els ILs no aromàtics (Ranke et al., 2004, Ranke et al., 2007b, Stolte et al., 2007b i Stasiewicz et al., 2008). La toxicitat d'1-butil-3-metilimidazoli sembla augmentar quan l'halogen va ser substituït per BF_4^- o NTF_2^-, mostrant un efecte tòxic depenent de l'anió. La CE_{50} en 24h per a la línia cel·lular MCF-7 va ser menor per a l'imidazoli que no pas per als ILs basats en el piperidini. L'anió també va afectar la viabilitat de les cèl·lules MCF-7, com en els estudis d'IPC-81 (Kumar et al., 2009). En el cas de la línia cel·lular HeLa, hi va haver una clara influència de la cadena lateral sobre la toxicitat. Els ILs derivats d'amoni quaternari no es van mostrar altament tòxics (Wang et al., 2007). Quant a la línia cel·lular CaCo-2, els ILs derivats de l'1-butil-3-metilimidazoli no van afectar significativament la viabilitat cel·lular, independentment de l'anió triat, però la toxicitat augmentava amb l'augment de la longitud de la cadena alquílica. La introducció d'un grup carboxílic

a l'extrem d'una cadena de deu carbonis va demostrar una reducció considerable de la toxicitat de l'1-decil-3-metilimidazoli, el que no va ocórrer quan el grup carboxílic es va substituir per un èster (Frade et al., 2007 i 2009). Es van obtenir resultats similars per a la línia cel·lular HT-29 (Frade et al., 2007).

1.6. Mètodes *in silico* per predir la toxicitat de líquids iònics

L'objectiu principal del Reglament REACH és garantir un nivell elevat de protecció de la salut humana i el medi ambient. Una de les principals raons per al desenvolupament i l'adopció del Reglament REACH va ser omplir els buits de dades per al gran nombre de substàncies que ja estan en ús. Per a moltes d'aquestes substàncies no hi ha informació adequada sobre els riscos que representen per a la salut humana i el medi ambient. L'ompliment dels buits de dades permetria a la indústria avaluar els perills i els riscos, així com identificar i posar en pràctica les mesures de gestió de riscos que són necessàries per protegir la salut humana i el medi ambient. El Registre CAS (CAS REGISTRY^{SM}) conté gairebé 87 milions de substàncies químiques orgàniques i inorgàniques i s'actualitza diàriament amb aproximadament 15000 substàncies (últim accés el 10 de setembre de 2014, pàgina web del CAS REGISTRY^{SM}), mentre que la base de dades l'ECHA conté 12636 substàncies i 48801 expedients, fets en concordança amb els requisits de REACH (última actualització el 14 d'agost de 2014, pàgina web de l'ECHA). Actualment s'estima que hi ha al voltant de 30000 substàncies químiques que s'utilitzen a la UE i que mai no s'han sotmès als assaigs aprovats a la legislació REACH que requereix que els productes químics existents siguin formalment avaluats i registrats. La creixent preocupació reguladora per protegir la salut humana i el medi ambient indiquen que la situació està canviant. El REACH considera que és responsabilitat de la indústria generar les proves necessàries de seguretat/toxicitat, explicar com es gestionen els riscos en la pràctica i presentar la documentació als reguladors. Per poder avaluar tants productes químics amb assaigs tradicionals es necessitarien dècades, s'utilitzarien milions d'animals d'experimentació i això costaria milers de milions a la indústria. El REACH exigeix que les empreses que utilitzen la mateixa substància química comparteixin les

dades existents, col·laborin en l'avaluació i el registre, i que s'utilitzin mètodes alternatius sempre que sigui possible. Uns dels mètodes alternatius són els mètodes *in silico* (ECHA, 2008).

Els mètodes *in silico* poden ser utilitzats per a la predicció de la toxicitat i així ajuden a evitar les proves costoses que consumeixen molt de temps, ja siguin *in vitro* o *in vivo*. S'han desenvolupat diferents mètodes *in silico* des de finals dels anys setanta, i s'utilitzen principalment en l'àrea de la farmacologia i la formulació de fàrmacs. El terme *in silico* prové del silici, un component dels ordinadors, el que significa que aquests mètodes de predicció es basen en mètodes computacionals, com ara en el cas de relacions estructura-activitat quantitatives (QSAR, segons les seves sigles en anglès Quantitative Structure-Activity Relationship). Els mètodes *in silico* tenen l'avantatge sobre els de laboratori que poden fer prediccions ràpides per a un gran conjunt de compostos utilitzant el processament de dades i programaris específics (Amberg, 2013). Un altre avantatge dels mètodes *in silico* és que poden fer prediccions en base a l'estructura d'un compost, fins i tot abans que hagi estat sintetitzat o utilitzat a una escala més àmplia, que és precisament el cas dels ILs. Per tant, els mètodes *in silico* es poden fer servir en una fase molt primerenca del procés de desenvolupament de ILs, ajudant a descartar els ILs potencialment tòxics.

Els mètodes QSAR es basen en la relació quantitativa entre l'estructura química d'un compost i la seva activitat biològica/toxicològica, amb l'ús de descriptors que es generen a partir de l'estructura molecular. Els mètodes QSAR poden fer prediccions de valors de CE_{50} i així reduir significativament els costos de laboratori i el temps necessari perquè la indústria química pugui complir amb els requeriments que li imposa la legislació REACH. El REACH reconeix que les propietats determinades amb mètodes alternatius són equivalents a les proves realitzades *in vivo* i *in vitro*. I per l'ECHA això és molt important perquè evita proves innecessàries amb l'ús d'animals. En aquest context, l'ECHA recomana l'ús dels mètodes QSAR. En els últims sis anys, l'ECHA ha publicat diversos documents sobre QSAR (ECHA, 2008, 2010 i 2012a).

Els estudis QSAR sobre la toxicitat d'ILs no s'han utilitzat tant com per a altres productes químics (Das i Roy, 2013). Un dels primers estudis que destacava la importància de relacionar l'estructura i la toxicitat dels líquids iònics va ser publicat per Jastorff et al. (2003), on es va presentar una nova estratègia, amb l'objectiu de proporcionar l'avaluació del risc ambiental dels líquids iònics utilitzant una combinació de relacions estructura-activitat (Structure-Activity Relationships - SAR), assaigs toxicològics i ecotoxicològics, i la utilització de models. Els autors consideraven que mitjançant l'ús dels models QSAR i SAR, els costos d'un disseny sostenible d'ILs es reduirien substancialment. Aquesta idea va ser posteriorment desenvolupada pel mateix equip (Stock et al. 2004, Jastorff et al., 2007, Ranke et al., 2007a, Arning et al., 2008), aconseguint un model *in silico* totalment desenvolupat i publicat per Cho et al. (2013) que va permetre la predicció de la hidrofòbia dels cations i de la citotoxicitat dels líquids iònics per a la línia cel·lular leucèmica de rata, *Vibrio fischeri* i *Scenedesmus vacuolatus* basant-se en els potencials d'interacció molecular dels ions. Couling et al. (2006) varen desenvolupar un model de predicció de la toxicitat dels ILs per a *V. fischeri* i *D. magna* basat en descriptors electrònics, espacials, estructurals, termodinàmics i topològics. El model QSAR de contribució de grups, basat en regressió lineal múltiple i proposat per Luis et al. (2007) va ser aplicat amb èxit tres anys després a un conjunt més ampli d'ILs pel mateix grup d'investigadors, però incloïa només dades per al test Microtox® (Luis et al., 2010).

OBJECTIUS

2. OBJECTIUS

L'objectiu de la tesi és avaluar l'(eco)toxicitat i la biodegradabilitat de determinats PILs alifàtics de cadena curta, pertanyents a una nova família derivada d'amines substituïdes i àcids orgànics.

També s'avaluarà l'(eco)toxicitat i la biodegradabilitat d'alguns representants freqüentment utilitzats de la família de líquids iònics apròtics (AILs), amb la finalitat de fer un estudi comparatiu i veure si els líquids iònics de la nova família dels PILs són menys tòxics i tenen menor impacte ambiental que els AILs.

Per tal de determinar l'impacte dels líquids iònics en el medi aquàtic, es realitzaran assaigs d'ecotoxicitat amb els següents organismes diana: bacteri marí *Vibrio fischeri*, alga verda *Pseudokirchneriella subcapitata* i planta aquàtica *Lemna minor*. L'impacte dels líquids iònics en el medi terrestre es determinarà mitjançant la realització d'assaigs d'ecotoxicitat amb plantes terrestres (ceba *Allium cepa*, raigràs anglès *Lolium perenne* i rave *Raphanus sativus*) i amb els microorganismes del sòl que participen en la transformació de carboni i nitrogen. Amb la finalitat d'obtenir una avaluació més detallada de la toxicitat, es realitzaran dues proves de toxicitat addicionals: amb un enzim (per avaluar la inhibició de l'acetilcolinesterasa) i amb les cèl·lules IPC-81 (per avaluar la citotoxicitat). També es realitzaran estudis de biodegradabilitat en aigua i sòl.

Les dades de (eco)toxicitat i l'estructura química dels líquids iònics es relacionaran mitjançant el model QSAR (relació estructura-activitat quantitativa) de contribució de grups amb l'objectiu de predir l'(eco)toxicitat d'altres PILs que no s'han analitzat. Aquest tipus de model també pot proporcionar informació útil per al disseny i la síntesi de líquids iònics amb menor impacte ambiental.

RESULTATS

3. RESULTATS

Els resultats de la tesi s'han publicat en forma d'articles d'investigació en tres revistes científiques diferents i el quart ha estat sotmès i es troba actualment en revisió.

ARTICLE 1

Peric B., Sierra J., Martí E., Garau M.A., Cruañas R., Iglesias M., 2011. Terrestrial ecotoxicity of short aliphatic protic ionic liquids. *Environmental Toxicology and Chemistry Journal*, 30, 2802-2809.

ARTICLE 2

Peric B., Sierra J., Martí E., Cruañas R., Garau M.A., Arning J., Bottin-Weber U., Stolte S., 2013. (Eco)toxicity and biodegradability of short aliphatic protic ionic liquids. *Journal of Hazardous Materials*, 261, 99-105.

ARTICLE 3

Peric B., Sierra J., Martí E., Cruañas R., Garau M.A., 2014. A comparative study of the terrestrial ecotoxicity of selected protic and aprotic ionic liquids. *Chemosphere*, 108, 418-425.

ARTICLE 4

Peric B., Sierra J., Martí E., Cruañas R., Garau M.A., 2014. Quantitative structure-activity relationship (QSAR) prediction of (eco)toxicity of short aliphatic protic ionic liquids. *Ecotoxicology and Environmental Safety* – sotmès, el nombre de manuscrit assignat, en revisió.

3.1. Article 1

ECOTOXICITAT TERRESTRE DE LÍQUIDS IÒNICS PRÒTICS ALIFÀTICS DE CADENA CURTA

Resum

Els líquids iònics (ILs) són un grup de compostos nous i prometedors amb una gran varietat de possibles estructures i usos. En l'actualitat s'estan sintetitzant un gran nombre de nous líquids iònics, però malgrat això la seva ecotoxicitat sovint ha estat poc estudiada, sobretot quan es tracta de la toxicitat terrestre, on la informació disponible és escassa. En aquest article es presenten els primers resultats de l'ecotoxicitat terrestre d'una nova família de líquids iònics pròtics alifàtics (PILs). Es van analitzar tres representants d'aquesta família: formiat de 2-hidroxietanolamina (2-HEAF), propionat de 2-hidroxidietanolamina (2-HDEAPr) i pentanoat de 2-hidroxitrietanolamina (2-HTEAPe). Aquests tres compostos deriven de diferents amines i àcids orgànics, sent el 2-HEAF el més simple de tots els representants d'aquesta nova família, el 2-HDEAPr el "mitjà", i el 2-HTEAPe el més complex en quant a estructura química. Mitjançant l'aplicació d'aquest criteri de selecció, es va establir una primera relació entre l'efecte tòxic i l'estructura dels PILs. Els assaigs realitzats varen incloure l'avaluació dels efectes dels PILs sobre plantes terrestres (*Allium cepa*, *Lolium perenne* i *Raphanus sativus*) i els microorganismes del sòl involucrats en la mineralització del carboni i del nitrogen. Els resultats van mostrar que els PILs investigats no són tòxics per a les tres espècies de plantes assajades, donat que s'obtingueren valors de CE_{50} superiors als 1000 mg kg^{-1} (sobre sòl sec), que és el llindar per a la toxicitat en el cas de plantes terrestres establert pel Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Només el 2-HTEAPe pel cas de *R. sativus* tenia una CE_{50} inferior a aquest llindar, de 826 mg kg^{-1} (sobre sòl sec). Aquests compostos no van resultar ser tòxics per a la microbiota nitrificant del sòl, ja que tots els valors de CE_{50} van ser diversos ordres de magnitud majors que el llindar establert per a la toxicitat de microorganismes del sòl (100 mg kg^{-1} sobre sòl sec), segons el GHS. Des de les corbes de la respiració acumulada del sòl en l'assaig de transformació del carboni es va observar una inhibició de l'activitat microbiana només en el cas

de la concentració més alta assajada per al 2-HDEAPr i el 2-HTEAPe (10.000 mg kg^{-1} sobre sòl sec), fet que indica una possible biodegradabilitat. Per tant, els compostos analitzats no es poden considerar tòxics per la microbiota del sòl i les plantes analitzades, alhora que són potencialment biodegradables en sòl. La toxicitat dels PILs es veu incrementada a mesura que augmenta la complexitat de la seva estructura, per tant, el 2-HTEAPe va resultar ser el més tòxic en tots els assaigs realitzats. Aquestes conclusions corroboren els resultats dels treballs prèviament publicat sobre la toxicitat d'ILs.

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 30, No. 12, pp. 2802–2809, 2011

Printed in the USA
DOI: 10.1002/etc.683

TERRESTRIAL ECOTOXICITY OF SHORT ALIPHATIC PROTIC IONIC LIQUIDS

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(*Submitted* 2 *February* 2011; *Returned for Revision* 14 *March* 2011; *Accepted* 22 *August* 2011)

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

Keywords—Protic ionic liquids Terrestrial ecotoxicity Biodegradability

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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Published online 20 September 2011 in Wiley Online Library (wileyonlinelibrary.com).

[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
2-HEAF	*Allium cepa*	100.00AB	112.14B	99.53AB	91.04AB	98.04AB	80.87A
	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
2-HDEAPr	*A. cepa*	100.00B	75.37AB	73.99AB	76.74AB	81.14B	13.28A
	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
2-HTEAPe	*A. cepa*	100.00B	102.63B	108.56B	92.71B	99.40B	0.00A
	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

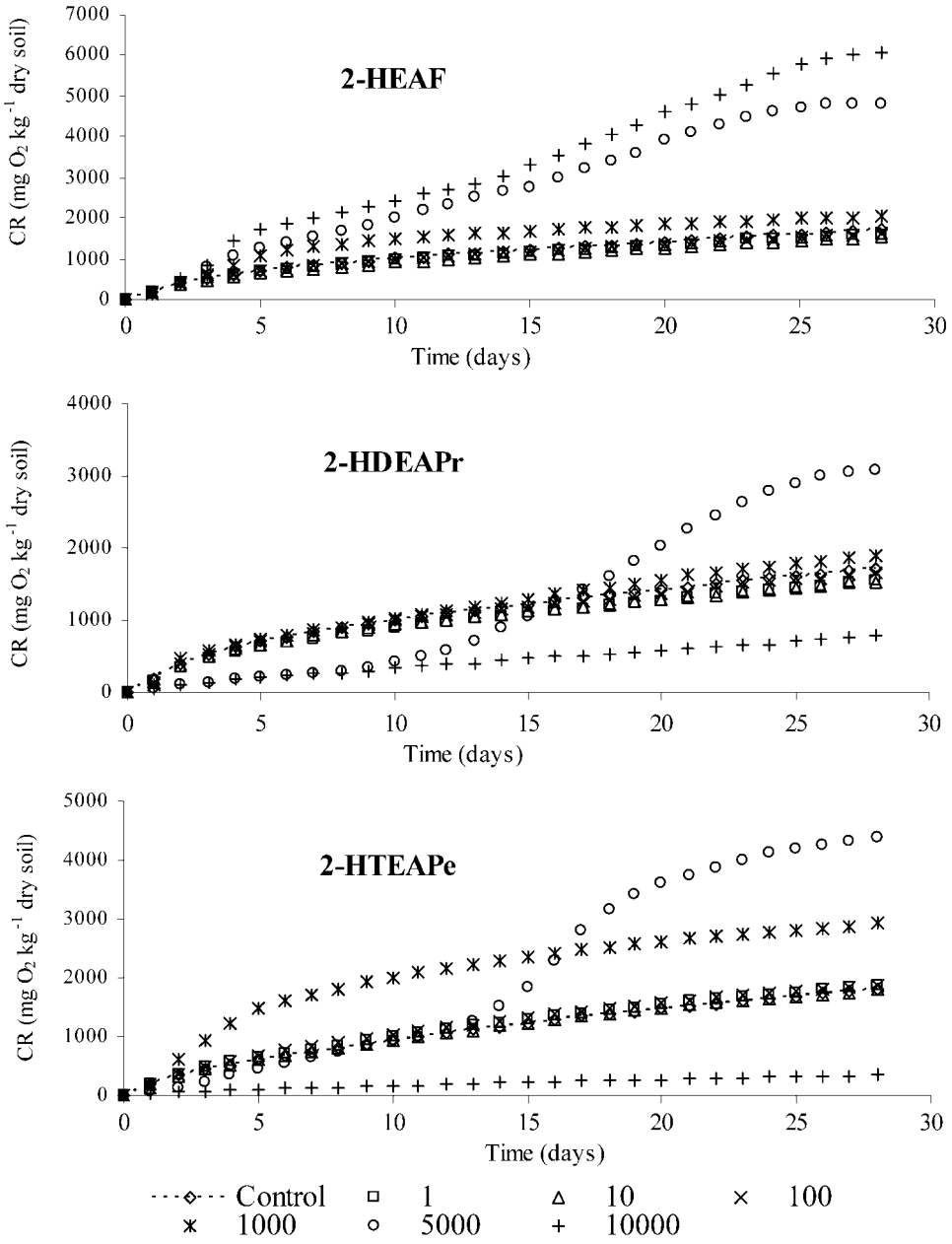

Fig. 1. Cumulative respiration (CR) data expressed as the cumulative oxygen consumed during the 28 d of the respirometric assay for the control and different nominal concentrations (in mg/kg) of the protic ionic liquids treatment groups: 2-hydroxyethanolamine formate (2-HEAF, top panel), 2-hydroxydiethanolamine 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

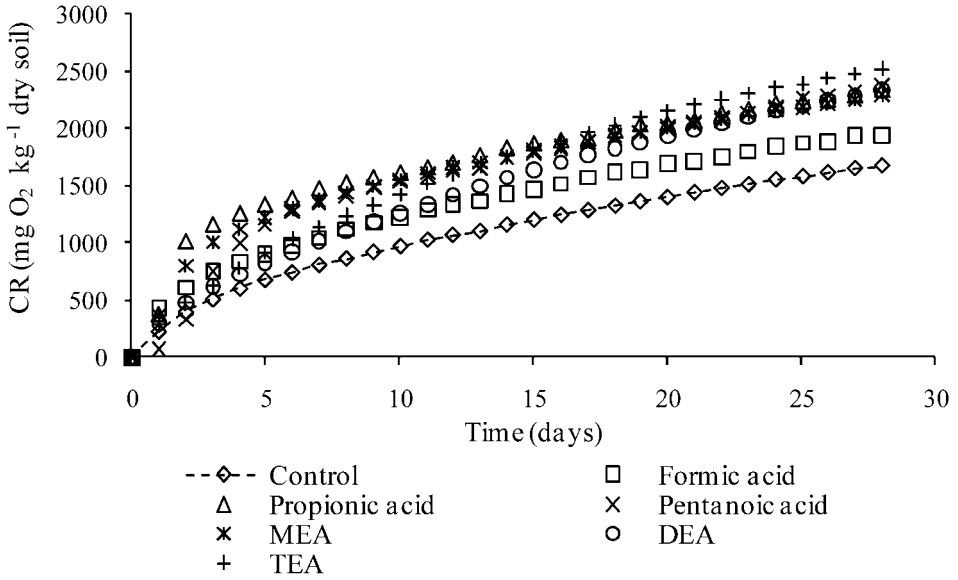

Fig. 2. Cumulative respiration (CR) data expressed as the cumulative oxygen consumed during the 28 d of the respirometric assay for the control and nominal concentrations of formic, propionic, and pentanoic acids and amines monoethanolamine (MEA), diethanolamine (DEA), and triethanolamine (TEA), equivalent 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
2-HEAF	%CR	100.00A	93.21A	89.40A	95.38A	120.07B	280.87C	354.62C
	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
2-HDEAPr	%CR	100.00B	90.22B	89.40B	96.74B	110.60B	179.35C	46.47A
	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
2-HTEAPe	%CR	100.00B	103.61B	99.74B	103.87B	161.27C	242.06D	18.88A
	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.

Acknowledgement—B. Peric thanks the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development (Grant MAEC-AECID II.A). M. Iglesias acknowledges the Fundacion Ibercaja (Programa de Excelencia en Investigacion 2009) for support in developing this research.

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.

3.2. Article 2

(ECO)TOXICITAT I BIODEGRADABILITAT DE LÍQUIDS IÒNICS PRÒTICS ALIFÀTICS DE CADENA CURTA

Resum

Els líquids iònics (ILs) són una família prometedora de compostos i amb una gran varietat d'estructures i usos possibles. Sovint es consideren com a substituts "verds" dels dissolvents orgànics volàtils tradicionals, donat que la seva pressió de vapor és insignificant i perquè tenen altres propietats interessants i bones característiques fisicoquímiques. Però per al compliment d'aquests requisits, han de mostrar baixa petjada ecològica, i no només ser adequats per a una aplicació concreta desitjada. En el present estudi, es van analitzar uns quants representants de dos grups d'ILs: uns formen part d'una nova família de PILs (líquids iònics pròtics), mentre que els altres, AILs (líquids iònics apròtics), s'usen molt en l'actualitat, com ara els clorurs de l'imidazoli i del piridini substituïts. Es van dur a terme assaigs de toxicitat aquàtica amb organismes diana: *Vibrio fischeri, Pseudokirchneriella subcapitata* i *Lemna minor*, i proves de biodegradabilitat en aigua. Per estudiar més a fons la toxicitat d'aquests compostos també es varen fer assaigs d'inhibició enzimàtica (amb l'acetilcolinesterasa, AChE) i d'ecototoxicitat (amb cèl·lules de leucèmia de rata IPC-81). En aquest estudi comparatiu els PILs varen mostrar valors de CE_{50} majors de 100 mg L^{-1} (llindar de toxicitat per a organismes aquàtics, establert pel Sistema Mundialment Harmonitzat de Classificació i Etiquetatge de Productes Químics - GHS) en tots els assajos realitzats, excepte per a tres compostos en el cas de *Lemna minor*. També varen mostrar bones taxes de biodegradabilitat. En canvi els AILs varen tenir valors de CE_{50} diversos ordres de magnitud inferiors als dels PILs en la major part de les proves d'ecotoxicitat aquàtica, i alhora varen manifestar baix potencial de biodegradabilitat. Cap dels PILs analitzats va mostrar inhibició de l'AChE, i o no varen presentar citotoxicitat o va ser molt baixa. En canvi, els AILs varen resultar ser inhibidors de l'AChE i força més citotòxics. A mesura que s'augmenta el nombre de carbonis en la part aniònica de la molècula dels PILs i la longitud de la cadena

alquílica lateral dels AILs, augmenta la toxicitat d'aquests compostos, sent per tant, les estructures més complexes les més tòxiques. Aquestes resultats indiquen que els PILs es podrien considerar com a alternatives ambientalment segures per substituir els AILs més tòxics, especialment si es vol protegir el medi ambient aquàtic.

Journal of Hazardous Materials 261 (2013) 99–105

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

(Eco)toxicity and biodegradability of selected protic and aprotic ionic liquids

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HIGHLIGHTS

- We analyzed (eco)toxicity of selected protic (PILs) and aprotic (AILs) ionic liquids.
- The PILs proved to have by far lower aquatic toxicity than the AILs.
- Complementary toxicity tests also showed the PILs as less toxic.
- The analyzed PILs are potentially biodegradable in water, unlike the AILs.
- The new family of PILs could be an environmentally safer alternative to AILs.

ARTICLE INFO

Article history:
Received 2 May 2013
Received in revised form 25 June 2013
Accepted 28 June 2013
Available online 6 July 2013

Keywords:
Ionic liquids
Toxicity
Aquatic ecosystems
Biodegradability

ABSTRACT

Ionic liquids (ILs) are a promising group of compounds with a large variety of possible structures and uses. They are considered as a potential "green" replacement for traditional volatile organic solvents, but their impact on the environment is often neglected or not studied enough. In the present study, selected representatives of two ILs groups were analyzed: a new family of protic ILs (derived from aliphatic amines and organic acids) and some frequently used aprotic ILs (substituted imidazolium and piridinium chlorides). The aquatic toxicity (test organisms *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Lemna minor*) and biodegradability tests were carried out. The additional tests with enzyme (acetylcholinesterase) and leukemia rat cells (IPC-81) provided more in-depth evaluation of toxicity. In our comparative hazard assessment protic ILs have EC_{50} values >100 mg L^{-1} in all of the tests performed, except in the case of three representatives toward *Lemna minor*. They also show good biodegradability rates. The EC_{50} values for aprotic ILs are various orders of magnitude lower than the ones for protic ILs in most of the tests and they show a lower biodegradability potential. These findings indicate that protic ILs can be considered as environmentally safer alternatives for more toxic ILs and organic solvents.

1. Introduction

In recent years, ionic liquids (ILs) have generated attention for their potential use as environmentally friendly chemicals and as a potential replacement for traditional volatile organic solvents. They are defined as salts consisting only of ions with melting point below 100 °C [1]. The interest in using ILs as solvents in chemical processes is that these compounds are thermally stable, have a high polarity, remain unchanged when mixed with different organic compounds and catalysts, have a very small vapor pressure and are liquid at a wide temperature range [2]. Thanks to their properties, ILs have been utilized in a variety of applications, such as solvents in catalysis [3], extractions [4], polymerization [5], nanotechnology [6], composite molecule dissolution and preparation [7] and renewable resource utilization [8].

Based on their chemical behavior, ILs can be divided in two groups: aprotic (AILs) and protic (PILs) ionic liquids. The structure of AILs is mainly based on bulky organic cations such as imidazolium or pyridinium with long alkyl chain substituents and large variety of anions such as Cl^-, Br^-, BF_4^-, PF_6^-, $N(CN)_2^-$, etc. The aprotic group can be considered as "classic" ILs, whilst the representatives of the protic group are in recent development. The here selected PILs belong to a newly designed family of short aliphatic PILs, with a different structure than the "classic" ILs, that includes compounds

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http://dx.doi.org/10.1016/j.jhazmat.2013.06.070

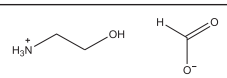
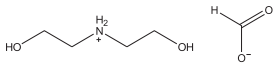
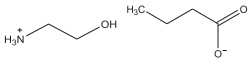
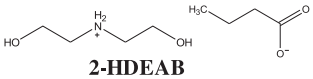
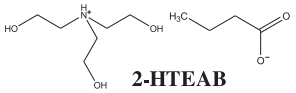
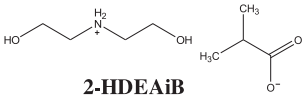
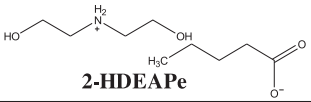
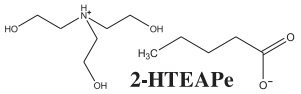
	Monoethanolamine	Diethanolamine	Triethanolamine
Formic acid	 2-HEAF	 2-HDEAF	
Acetic acid		 2-HDEAA	
Propionic acid		 2-HDEAPr	
Butiric acid	 2-HEAB	 2-HDEAB	 2-HTEAB
Isobutiric acid		 2-HDEAiB	
Pentanoic acid		 2-HDEAPe	 2-HTEAPe

Fig. 1. Structures and abbreviations of the analyzed PILs.

based on substituted amines (monoethanolamine, diethanolamine or triethanolamine) as cations and organic acids with different numbers of carbon atoms (formic, propionic, butanoic, isobutanoic and pentanoic acid) as anions. Both cationic and anionic part of the molecule are organic and have a relatively low molecular weight [9]. The potential environmental impact of these new PILs is expected to be smaller than the impact of AILs, due to their simpler structure.

There are numerous possible applications for this new family of PILs. To name but a few: the 2-hydroxyethanolamine formate (2-HEAF) has been found to be able to dissolve many inorganic salts, hydroxylated compounds and some insoluble polymers such as polyaniline and polypyrrole [10]. Also, 2-HEAF together with some other representatives of this family (2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), etc.) have been analyzed for their potential catalytic activity in the aldol condensation reactions [9], for the heterogeneous catalytic hydrogenation of cyclohexene and acetone catalyzed by Pt/Al_2O_3 with methanol, ethanol and propan-1-ol [11]. Other PILs, also derived from 2-hydroxyethylammonium and organic acids have been studied in order to develop a new process for absorption of CO_2 [12]. Also, the catalytic activity in the aldol condensation processes of the PILs derived from mono, di and triethanolamine and penthanoic acid has been studied, with successful results [13].

However, apart from good technical performance, current European Union environmental legislation including REACH (Regulation concerning registration, evaluation, authorization and restriction of chemicals) [14] makes insistent demands for safety of chemicals and, among others, ecotoxicological as well as biodegradation data are required. As the group of PILs is fairly new in their use and still in development, it is very important to assess their hazard potential before they can be safely used.

The aim of the present study is therefore an initial hazard assessment of the new PILs and a comparative analysis with classical AILs. For our investigations we applied tests to determine aquatic toxicity (with bacteria *Vibrio fischeri*, green algae *Pseudokirchneriella subcapitata* and higher aquatic plant *Lemna minor*) and the ready biodegradability in water. We used additional test systems with an enzyme (acetylcholinesterase inhibition) and isolated leukemia IPC-81 cells from rats (cytotoxicity), which have been proven to be useful for determining the acute toxicological hazards of ILs [15,16].

2. Experimental

2.1. Ionic liquids

The PILs analyzed in the present study are: 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxyethanolamine butanoate (2-HEAB), 2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine propionate (2-HDEAPr), 2-hydroxydiethanolamine butanoate (2-HDEAB), 2-hydroxydiethanolamine isobutanoate (2-HDEAiB), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), 2-hydroxytriethanolamine butanoat (2-HTEAB) and 2-hydroxytriethanolamine pentanoate (2-HTEAPe) and some of the most frequently used AILs [17–19]: 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-methyl-3-octylimidazolium chloride ([OMIM]Cl) and N-butylpyridinium chloride ([BPy]Cl). The structures of analyzed PILs are shown in the Fig. 1 and the AILs in the Fig. 2.

The PILs were obtained through synthesis starting from the amine compounds (monoethanolamine, diethanolamine and triethanolamine) and the corresponding acids (formic, acetic, n-propionic, n-butyric, isobutyric and n-pentanoic acid) as described in detail by Iglesias et al. [20]. To confirm the products

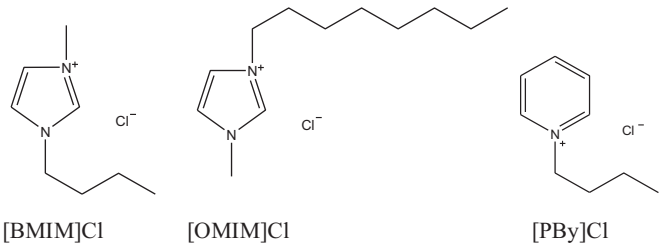


Fig. 2. Structures and abbreviations of the analyzed AILs.

structures ^{1}H NMR were performed [21,22]. The obtained NMR spectrums confirmed the synthesized structure.

The AILs were obtained commercially from Sigma–Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

2.2. Inhibition of bacterial luminescence (Microtox®) test

The toxicity of ionic liquids was determined by a bioassay according to the ISO 11348-3 procedure for Microtox® Basic test [23] based on the decrease of luminescence of the marine bacteria *V. fischeri* (strain NRRL B-11177) after exposure to a toxic substance. Isotonic solutions of the ionic liquids were prepared using 2% NaCl as solvent. The prepared concentrations of ILs were 0.5% or 1%. Starting from these concentrations, four serial dilutions in 1:2 ratio were prepared in triplicate, along with the control samples that contained no ILs. The reduction of light emission was measured at least two times after 15 min of contact between bacteria previously reconstituted with 2% NaCl and serial dilutions of ILs, at 15 °C. Bacterial luminescence is directly linked with cellular respiration, so a decrease in luminescence corresponds to bacterial toxicity. The equipment used was the Microtox® M500 Analyzer (SDIX, Newark, DE, USA, 2008).

2.3. Algal growth inhibition test

The conventional algal chronic toxicity assay was done according to the procedures set out in Organization for Economic Cooperation and Development (OECD) guidelines 201 [24]. For this assay, the unicellular limnic green algae *P. subcapitata* (strain 61.81, SAG, Culture Collection of Algae, Universität Göttingen, Germany) was used. The stock culture was grown under conditions recommended in OECD guideline 201 and diluted to a cell density of 5×10^4 cells mL^{-1}. The nominal concentrations of ILs used in the test were: 0.001, 0.01, 0.1, 1, 10, 100, 1000 and 5000 mg L^{-1}. The test was done with quadruplicates of each concentration of PILs and six control samples, two of which were stored in the refrigerator (4 °C) to prevent growth and have a referral from the initial concentration of cells and the other four were incubated under the same conditions as the treated samples. The incubation was carried out in a chamber for 72 h at 22 °C temperature and white light with intensity of 1000 lux. During the three days, regular agitation was done to promote gas exchange but preventing cell lysis. The cell count was done using the Coulter cell counter (Beckman, Nürnberg, Germany).

2.4. Growth inhibition test with duckweed

The growth inhibition assay with *Lemna minor* (duckweed) was performed as described in detail by Drost et al. [25]. The *L. minor* plants (Department of Agricultural Science, The Royal Veterinary and Agricultural University, Taastrup, Denmark) were grown in open Erlenmeyer flasks in sterilized Steinberg medium, in an illuminated climate chamber with a constant temperature of 25 ± 2 °C. The assays were conducted in 6-well titer plates. The nominal concentrations of ILs used in the test were between 0.1 and 5000 mg L^{-1}. All ILs were tested at least twice, with a minimum of 6 controls, containing only pure Steinberg medium, for each test. The test started with 18 fronds, 3 in each well. The resulting growth inhibition was determined after 7 days of incubation, basing on different end points like the number of fronds or frond area calculated in relation to the controls. The growth rate was determined on the basis of the counted fronds; for the detection of the frond area (mm^2) a Scanalyzer from Lemnatec GmbH (Würselen, Germany) was used.

2.5. Acetylcholinesterase inhibition assay

The assay was performed according to Ellman et al. [26] as modified for use with microtiter plates [27]. The inhibition of acetylcholinesterase (AChE) was measured using a colorimetric assay based on the reduction of the dye 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) by the enzymatically formed thiocholine moiety from the AChE substrate acetylthiocholine iodide. A dilution series (1:1) of the ILs in phosphate buffer (0.02 M, pH 8.0) containing maximum 1% methanol was prepared directly in the wells of a 96-well microtiter plate. The start concentration was 5000 mg L^{-1}. DTNB (2 mM, 0.185 mg mL^{-1} $NaHCO_3$ in phosphate buffer pH 8.0) and the enzyme (0.2 U mL^{-1}, 0.25 mg mL^{-1} bovine serum albumin in phosphate buffer pH 8.0) were added to each well. The reaction was started by the addition of acetylthiocholine iodide (2 mM in phosphate buffer). The final test concentrations were 0.5 mM of DTNB and acetylthiocholine iodide and 0.05 U mL^{-1} acetylcholinesterase, respectively. Each plate contained blanks (no enzyme) and controls (no toxicant). Enzyme kinetics were measured at 405 nm at 30 s intervals in a microplate-reader (MRX Dynatech) for 5 min with on at least two different 96-well plates. The enzyme activity was expressed as the slope of optical density (in OD min^{-1}) from a linear regression.

2.6. Cell viability assay with IPC-81 cells

The cytotoxicity of ILs was determined using the WST-1 reagent. The assay was described in detail in Ranke et al. [16]. Briefly, promyelocytic leukemia cells from the rat IPC-81 cell line were incubated for 4 h in 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent. Each plate contained blanks (no cells) and controls (no toxicant). The cell viability assays were carried out in a 1:1 dilution series, starting from 5000 mg L^{-1} of aqueous solution of ILs. Each dose-response curve was recorded for at least 9 parallel dilution series on three different 96-well plates.

2.7. Biodegradability in water

The determination of potential biodegradability of ILs in water was done according to OECD guideline 301 [28] in manometric respirometers (Velp® Scientifica, Usmate, Italy) which allow the determination of the sample oxygen consumption. The ILs were added at concentration of 100 mg L^{-1} in 250 mL of the mineral nutrient solution and inoculated with a mixture of microorganisms from the sludge of an urban biological treatment plant. All the experiments were done at least in triplicate, including the control sample where no ILs were added. During 28 days, at a constant temperature of 22 ± 2 °C, the amount of oxygen consumed (Biochemical oxygen demand – BOD) was determined. The percentage of biodegradation was obtained by dividing BOD values with ThOD (Theoretical Oxygen Demand) and multiplying by 100 for each ionic liquid. Theoretical oxygen demand is the total amount of oxygen required to oxidize a chemical completely, it is a calculated value, expressed as mg oxygen required per mg of the test compound. After 0, 5 and 28 days of the incubation during the biodegradation test, the samples containing PILs were analyzed by ion chromatography in a Dionex DX300 (Sunnyvale, CA, USA), with a Hamilton PRP-X200 cationic column (Reno, NV, USA, I.D. 4.1 mm, 250 mm long) and electrical conductivity detector without ion suppression. The eluent used was 2 mM nitric acid at 1 mL min^{-1}. In the case of the AILs the samples were analyzed via HPLC [29]. The chromatography analysis was done in order to determine possible decreases in cationic moiety concentration of ILs during the course of the test, as a consequence of eventual primary degradation process.

OECD guideline 301 defines primary biodegradation as the alteration of the chemical structure of a substance, brought about by biological action, resulting in the loss of a specific property of that substance. The percentage of primary degradation of each sample was calculated referring to the initial concentration.

2.8. Statistical analysis

Dose-response relationship was assessed, and the effective concentrations 50 (EC_{50}) with their 95% confidence intervals were calculated for each compound from suitable regression models (Gompertz, hormetic, or logistic) using Statistica 6.0 (Stat Soft Inc., Tulsa, OK, USA) and the drfit package (version 0.05-92) for the R language and environment for statistical computing (R Development Core Team, 2005). The choice of the model was based on the best fit to the data. In the case of Microtox® test the log-lineal model was used to obtain the dose-response curve and the corresponding EC_{50} value (MicrotoxOmni™ Software for Windows, Azur Environmental, Carlsbad, CA, USA, 1999). The parameter EC_{50} was selected because it is used in the environmental regulation and most of the literature. Spearman's coefficient of correlation was calculated using PASW Statistics 17.0.1 program (IBM Corporation, NY, USA).

3. Results and discussion

3.1. Toxicity tests

3.1.1. Aquatic ecotoxicity tests

For all ILs tested dose-response curves were obtained and EC_{50} values were calculated in mg L^{-1} and mmol L^{-1} (Table 1).

In the case of the test with marine bacteria *V. fischeri* the EC_{50} values for the analyzed PILs were between 1.69 and 15.13 mmol L^{-1} (350–2239 mg L^{-1}), with 2-HDEAPe being the most toxic and 2-HEAB the least toxic compound. The two compounds with the lowest EC_{50}s (2-HDEAPe and 2-HTEAPe) have the same pentanoic anionic part, which is indicating the influence of the anion on toxicity. The EC_{50}s corresponding to AILs were up to several orders of magnitude lower, being between 0.002 and 1.72 mmol L^{-1} (0.5–295 mg L^{-1}), with [OMIM]Cl being the most toxic one. The obtained EC_{50} values for the PILs in test of inhibition of the growth of *P. subcapitata* range between 0.70 and 22.93 mmol L^{-1} (104–2453 mg L^{-1}). The highest as well as the lowest toxicity was found for the 2-HEA cation showing again the strong influence of the anionic moiety formiate vs. butyrate. In the case of AILs, the obtained EC_{50} are several orders of magnitude lower, between 0.005 and 0.17 mmol L^{-1} (1.21–30 mg L^{-1}), with [OMIM]Cl once again being the most toxic one. As far as the growth inhibition test of duckweed (*L. minor*) is concerned, three of the analyzed PILs showed some toxic effect on duckweed. The 2-HDEAB, 2-HEAB and 2-HDEAiB have EC_{50} values of 0.17 (33 mg L^{-1}), 0.40 (59 mg L^{-1}), and 0.41 (79 mg L^{-1}) mmol L^{-1} respectively. The AILs showed to be more toxic toward *L. minor* than the most of the PILs, with [OMIM]Cl having the EC_{50} of 0.005 mmol L^{-1} (1.15 mg L^{-1}), the lowest of all analyzed ILs.

L. minor has proved to be more sensitive to the presence of PILs than *P. subcapitata* or *V. fischeri*. In the case of algae and bacteria the cell membrane is a target site and entry route for the contaminants, so the increased lipid solubility can be related to the higher toxicity of a lipophilic contaminant [30] as in the case of the investigated AILs. The nutrient uptake from soluble phase in the case of *L. minor* is via its fronds (mainly) and roots [31], and being an aquatic plant there is an increased exposure area during a prolonged period of time that can also lead to higher sensitivity. The analyzed PILs tend to be more soluble and more hydrophilic than the analyzed AILs. Other authors have also proven that duckweed was more sensitive

Table 1
Results of dose-response curves in mg L^{-1} and mmol L^{-1} for the aquatic ecotoxicity tests.

Ionic liquid	MW (mg mol^{-1})	*Vibrio fischeri*				*Pseudokirchneriella subcapitata*				*Lemina minor*			
		EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval	EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval	EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval
2-HEAF	107	700	225–920	6.54	2.10–8.60	2453	1584–3801	22.93	14.80–35.52	288	234–380	2.70	2.19–3.55
2-HEAB	148	2239	1400–3500	15.13	9.46–23.65	104	23–482	0.70	0.16–3.26	59	44–81	0.40	0.29–0.55
2-HDEAF	151	800	455–1950	5.30	3.01–12.91	976	615–1548	6.46	4.07–10.25	525	398–631	3.48	2.64–4.18
2-HDEAA	165	1750	1330–2600	10.61	8.06–15.76	870	630–1201	5.27	3.82–7.28	631	550–708	3.82	3.33–4.29
2-HDEAPr	179	650	250–1650	3.63	1.40–9.22	2569	1478–4365	14.35	8.26–24.38	209	229–363	1.17	1.28–2.03
2-HDEAB	193	800	300–1100	4.15	1.55–5.70	294	151–574	1.52	0.78–2.97	33	13–60	0.17	0.07–0.31
2-HDEAiB	193	850	450–1540	4.40	2.33–7.98	1275	709–2290	6.61	3.67–11.86	79	60–68	0.41	0.31–0.35
2-HDEAPe	207	350	75–525	1.69	0.36–2.54	574	129–1965	2.77	0.62–9.49	155	148–158	0.75	0.71–0.77
2-HTEAB	237	501	195–1500	2.11	0.82–6.32	1287	630–2690	5.43	2.66–11.35	178	145–219	0.75	0.61–0.92
2-HTEAPe	251	461	257–828	1.84	1.02–3.30	757	138–1952	3.02	0.55–7.78	525	490–562	2.09	1.95–2.24
[BMIM]Cl	175	287	178–559	1.64	1.02–3.19	14	7.00–27	0.08	0.04–0.15	48.98	42–59	0.28	0.24–0.34
[OMIM]Cl	231	0.50	0.29–0.88	0.002	0.001–0.004	1.21	1.10–1.35	0.005	0.004–0.006	1.15	0.95–1.35	0.005	0.004–0.006
[BPy]Cl	172	295	255–336	1.72	1.48–1.95	30	22–47	0.17	0.13–0.27	51.29	44–60	0.30	0.25–0.36

$p < 0.05$; MW, molecular weight.

than algae and bacteria [32,33] and that the compounds with a higher lipophilicity affect more the algae than the duckweed [34].

According to the Globally Harmonized System of Classification and Labelling of Chemicals [35,36], most of the PILs can be classified as non-toxic for aquatic organisms, because their EC_{50}s are higher than 100 mg L^{-1}. Only 2-HDEAB, 2-HEAB, 2-HDEAiB fit into the category of harmful for the aquatic environment (category Acute 3) with their EC_{50} between 10 and 100 mg L^{-1} in the case of *L. minor*. All of the AILs can be placed into some of the acute toxicity categories according to their EC_{50} values. In the case of the Microtox test [OMIM]Cl can be classified as very toxic for aquatic organisms, belonging to the category Acute 1 (EC_{50} < 1 mg L^{-1}). The rest of the analyzed AILs cannot be placed in none of the categories of substances considered as dangerous for the aquatic environment, because their EC_{50} are all higher than 100 mg L^{-1}. In the test of inhibition of the growth of *P. subcapitata* [OMIM]Cl can be classified as toxic (category Acute 2, EC_{50} between 1 and 10 mg L^{-1}) and [BMIM]Cl and [BPy]Cl as harmful for aquatic organisms (category Acute 3). According to the results of the inhibition of growth of *L. minor*, [OMIM]Cl can be placed into the category of very toxic (category Acute 1) and [BMIM]Cl and [BPy]Cl into harmful for the aquatic environment (category Acute 3).

The higher toxicity of [OMIM]Cl compared to the other two representatives of AILs is supported by other authors' findings, where the increase of toxicity is provoked by the elongation of the alkyl side chain [37,38]. The higher toxicity of the AILs compared with the PILs analyzed in this study is also related to the structural differences, the AILs have a voluminous heterocyclic cation with alkyl side chains in their structure, whilst the PILs have simpler structure with smaller, highly hydrophilic molecule. The differences in toxicity among the PILs can also be seen in case of the more complex structure, when the anionic part has 4 or 5 carbons (butyric, isobutyric and pentanoic acid). The ionic liquids with short alkyl chains should be always be preferred if technically possible [39] and the introduction of the hydrophilic functional groups in the side chain reduces the toxicity of the ionic liquid [40]. These facts also lead to a decrease of toxicity of the PILs compared to the AILs.

3.1.2. Cytotoxicity and inhibition of acetylcholinesterase

The PILs and AILs were also assayed with respect to their inhibitory activity toward enzyme acetylcholinesterase (AChE) and their cellular toxicity toward IPC-81 rat promyelocytic leukemia cell line. These test systems yielded reproducible measurements of the acute toxicity of ILs, and many data are available for comparison [41]. The obtained EC_{50} values are shown in Table 2.

EC_{50} data for [BMIM]Cl, [OMIM]Cl and [BPy]Cl are taken from The UFT/Merck Ionic Liquids Biological Effects Database [41]

In the test of inhibition of acetylcholinesterase, the PILs showed a very low inhibitory potential with EC_{50}s ranging from 1.27 to 59.02 mmol L^{-1}. The AILs show a moderate inhibition potential [41], with EC_{50} values between 0.04 and 0.08 mmol L^{-1}. The EC_{50} of AILs were once again several orders of magnitude lower than the ones found for PILs. The [OMIM]Cl is the most toxic one of the three AILs.

Regarding cytotoxicity toward IPC-81, the EC_{50} values for the analyzed PILs ranged between 1.76 and 30.95 mmol L^{-1}, while the AILs had EC_{50} between 0.1 and 5 mmol L^{-1}. One of the PILs, 2-hydroxydiethanolamine formate, showed no inhibitory activity. The AILs proved to be more toxic than the PILs, with [OMIM]Cl being the most toxic one, as in the previously described aquatic toxicity tests. Their EC_{50} indicate moderate cytotoxicity [41,42].

3.2. Biodegradability

The biodegradation results are shown in Table 3. There are two types of data: the percentage of biodegradation after 5, 14 and 28 days according to OECD 301 manometric respirometry test procedure and the primary biodegradation of the ILs molecule determinated by means of ionic chromatography (IC).

According to OECD 301 guidelines, the compounds which reach a biodegradation level higher than 60% of ThOD during a 10-day window within 28 days of testing can be considered as "readily biodegradable". PILs show much better biodegradation rates than AIL in these tests. After 28 days of the test, the biodegradation rates of most of the PILs are higher than 60%. Three of the tested PILs do not fit into "readily biodegradable" criteria: 2-HDEAF, 2-HTEAB and 2-HTEAPe. The results of 59 and 57% for the 2-HTEAB and 2-HTEAPe respectively are very close to the 60% limit, so they can be considered as potentially readily biodegradable. But the results of 13% for the 2-HDEAF indicates that this PIL has a low biodegradation rate. The levels of biodegradation are usually dependent on compound's stability and toxicity. The analyzed AILs have proven to be resistant to water biodegradation, with the % of biodegradability being around 1%.

According to the chromatographic quantification results, after 5 days of incubation in water during the biodegradability test, the cationic moiety of the 2-HEAF, 2-HEAB, 2-HDEAF and 2-HDEAPr cannot be detected in the water samples, whereas for the rest of the PILs the corresponding cationic moiety remains at available concentrations between 30 (for 2-HDEAiB) and 84% (for 2-HDEAPe). After 28 days of incubation, only the cationic moiety of 2-HDEAPe was detectable in the extracts, which indicates that all but one of the PILs were completely degraded after 28 days. The 2-HDEAPe was not completely metabolized by the activated sludge microbial community, remaining at available concentration of 38%. The primary degradation of 2-HEAF, 2-HEAB, 2-HDEAF and 2-HDEAPr was complete within 5 days, while the other PILs molecules (except in the case of 2-HDEAPe) are completely mineralized during the period between the fifth and the twenty-eighth day. In a study performed by Stolte et al. [29], imidazolium and pyridinium ILs with short alkyl side chains ([BMIM]Cl and [BPy]Cl) were highly resistant to mineralization during the incubation period. A complete primary biodegradation could be detected for the [OMIM]Cl after 28 days.

3.3. Correlation analysis of the toxicity and biodegradability data

The Spearman's correlation coefficients were determined in order to get more information on potential toxic modes of action of the analyzed PILs. The correlation between the structure and the toxic effects and potential biodegradability of the PILs was investigated. For this analysis the molecular weight of PILs, the number of C-atoms in the cationic (amine) and anionic (acid) part of the molecule and EC_{50} values were used. All statistically relevant results of the correlation analysis are presented in the Table 4.

In the case of the Microtox test, there is an increase of toxicity with the increase of the molecular weight and elongation of alkyl chains of the anionic part of the molecule, and with the complexity of the cationic part. These three findings indicate that the complexity of PILs structure has a positive influence on the potential toxicity in the case of the Microtox test and also in the test of the inhibition of acetylcholinestherase, where the same correlations were found. A positive influence of the elongation of the carboxyl chain in the anionic part of the PILs molecule on their toxicity has also been found in the case of the tests performed on green algae, duckweed and IPC-81 cells. In these three tests, the acid part of the PILs molecule seems to have more influence on the toxicity then the amine part. These findings confirmed other authors conclusions,

Table 2
Results of dose-response curves in mg L^{-1} and mmol L^{-1}.

Ionic liquid	AChE				IPC-81			
	EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval	EC_{50} (mg L^{-1})	Confidence interval	EC_{50} (mmol L^{-1})	Confidence interval
2-HEAF	3467	3090–3890	32.41	29.88–36.36	3311	2089–7943	30.95	19.53–74.24
2-HEAB	7079	6166–8128	47.83	41.66–54.92	269	232–295	1.82	1.57–1.99
2-HDEAF	8912	7413–10965	59.02	49.09–72.61	>5000	nd	>5000	nd
2-HDEAA	2089	1950–2291	12.66	11.82–13.88	1778	1349–2344	10.77	8.18–14.21
2-HDEAPr	2399	2239–2570	13.40	12.51–14.36	1047	912–1202	5.85	5.10–6.72
2-HDEAB	2344	2188–2570	12.15	11.34–13.32	339	302–372	1.76	1.56–1.93
2-HDEAiB	1995	1820–2138	10.34	9.43–11.08	575	468–708	2.98	2.42–3.67
2-HDEAPe	1862	1738–1995	9.00	8.40–9.64	1096	977–1259	5.30	4.72–6.08
2-HTEAB	302	288–324	1.27	1.22–1.37	589	468–741	2.48	1.97–3.13
2-HTEAPe	427	398–447	1.70	1.59–1.78	851	741–977	3.39	2.95–3.89
[BMIM]Cl	14.40	13.10–15.70	0.08	0.07–0.09	626	530–751	3.58	3.03–4.29
[OMIM]Cl	9.10	8.47–9.27	0.04	0.03–0.04	24	21–27	0.10	0.09–0.12
[BPy]Cl	8.59	8.30–8.89	0.05	0.04–0.05	862	781–960	5.01	4.54–5.58

$p < 0.05$.

Table 3
Percentage of biodegradation of the ILs after 5, 14 and 28 days and the percentage of primary biodegradation after 5 and 28 days analyzed by ionic chromatography and HPLC (results for AILs are taken from Stolte at al. [29]).

Ionic liquid	Ready biodegradability (% biodegradation)			Chromatography -IC and HPLC (% primary biodegradation)	
	5 days	14 days	28 days	5 days	28 days
2-HEAF	11	61	86	100	100
2-HEAB	64	69	95	100	100
2-HDEAF	4	4	13	100	100
2-HDEAA	21	46	69	65	100
2-HDEAPr	45	62	68	100	100
2-HDEAB	47	66	78	21	100
2-HDEAiB	46	71	79	70	100
2-HDEAPe	38	57	69	16	62
2-HTEAB	28	32	59	34	100
2-HTEAPe	23	38	57	33	100
[BMIM]Cl	0.23	0.66	1.17	0	0
[OMIM]Cl	0.29	0.35	1.33	31	100
[BPy]Cl	0.31	0.46	0.61	0	0

Table 4
Spearman's correlation coefficient for different correlation pairs.

	EC_{50} (mmol L^{-1})					% biodegradation	
	Microtox	Green algae	*L. minor*	AChE	IPC-81	5 days	28 days
MW	−0.863**	ns	ns	−0.924**	ns	ns	ns
Amine	−0.716**	ns	ns	−0.771**	ns	ns	−0.773**
Acid	−0.661*	−0.617*	−0.574*	−0.736**	−0.661*	0.567*	ns

ns, not significant; MW, molecular weight.
* Level of significance $p < 0.05$.
** Level of significance $p < 0.01$.

i.e. that the toxicity strongly depends on the side chain elongation in the case of AILs [33,43,44].

The correlation analysis for the biodegradation results shows that the complexity of an anionic part of the molecule (acid) is directly correlated with BOD_5, whilst there is an inverse correlation between the increase of the complexity of the cationic part of the molecule (amine) and BOD_{28} values. This means that during the first 5 days of biodegradation in water, it is the increase of the number of carbons in the anionic part of the molecule that causes higher biodegradation rate, but in the later stages of the biodegradation process the complexity of the cationic part of the molecule causes lower rates of biodegradability, which is in concordance with a previously established inverse correlation in the case of Microtox, meaning that the PILs are more toxic to microbial communities if they have a more complex structure.

The correlation analysis performed by other authors [33] showed that there is correlation between lipophilicity and toxicity for bacteria and algae in the case of AILs. In addition, a correlation between an increasing chain length of the side chains connected to the cationic head groups and an enhanced inhibitory potential on the enzyme acetylcholinestherase was found [39]. In the case of the rat leukemia cell line, the same group found a correlation between a HPLC derived lipophilicity parameter and the observed cytotoxicity [42].

4. Conclusions

The PILs analyzed in the present study have shown no toxicity in the performed aquatic toxicity tests, with EC_{50} values above 100 mg L^{-1}, with the exception of three PILs in the case of duckweed growth inhibition test. *L. minor* was found to be the most sensitive species in the case of the aquatic toxicity tests for the PILs, but not for the AILs, probably due to the hydrophilicity of the PILs. The [OMIM]Cl proved to be the most toxic in all of the aquatic

toxicity tests, and the other two representatives of AILs proved to be harmful for the aquatic organisms. In the case of cytotoxicity and inhibition of acetylcholinesterase tests, none of the analyzed ionic liquids showed very high toxicity. There is a clear difference in EC_{50} values between the two groups of ILs, with the EC_{50} for AILs being various orders of magnitude lower than the EC_{50} for PILs in most of the tests performed. From the results we can deduce that, in general, the analyzed AILs with more complex molecular structure have a greater tendency to cause toxic effect in the aquatic organisms tested than the PILs with the smaller molecule and simpler structure. Based on the biodegradation test results and ulterior chromatographic quantification, the analyzed PILs are potentially biodegradable in water, whilst the AILs have shown practically no biodegradation. The complexity of the ILs molecules has a positive impact on the toxicity. The initial comparative hazard assessment showed that PILs are, in terms of toxicity as well as in terms of biodegradability, much favorable than the here tested AILs. This fact, together with their low production cost, simple synthesis and possible applications, suggests that they have a potential "greener" profile among other ionic liquids and a good prospect for a wider use.

Acknowledgments

B. Peric would like to thank the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development (Grant MAEC-AECID). The authors would like to thank Màrius Mumbrú for his technical help with the ionic chromatography and Andrea Böschen for her advices and positive spirit during the Acetylcholinesterase inhibition assay.

References

[1] R.D. Rogers, K.R. Seddon, Ionic Liquids as Green Solvents: Progress and Prospects, ACS Symposium Series, American Chemical Society, Washington D.C, 2003, 856.
[2] J.O. Valderrama, R.E. Rojas, Critical properties of ionic liquids, Ind. Eng. Chem. Res. 48 (2009) 6890–6900, Revisited.
[3] Z.C. Zhang, Catalysis in ionic liquids, Adv. Catal. 49 (2006) 153–237.
[4] M.L. Dietz, Ionic liquids as extraction solvents: where do we stand? Sep. Sci. Technol. 41 (2006) 2047–2063.
[5] P. Kubisa, Application of ionic liquids as solvents for polymerization processes, Prog. Polym. Sci. 29 (2004) 3–12.
[6] M. Antonietti, D. Kuang, B. Smarsly, Y. Zhou, Ionic liquids for the convenient synthesis of functional nanoparticles and other inorganic nanostructures, Angew. Chem. Int. Ed. 43 (2004) 4988–4992.
[7] M.B. Turner, S.K. Spear, J.D. Holbrey, R.D. Rogers, Production of bioactive cellulose films reconstituted from ionic liquids, Biomacromolecules 5 (2004) 1379–1384.
[8] S. Zhu, Y. Wu, Q. Chen, Z. Yu, C. Wang, S. Jin, Y. Ding, G. Wu, Dissolution of cellulose with ionic liquids and its application: a mini-review, Green Chem. 8 (2006) 325–327.
[9] I. Cota, R. Gonzalez-Olmos, M. Iglesias, F. Medina, New short aliphatic chain ionic liquids: synthesis, physical properties, and catalytic activity in aldol condensations, J. Phys. Chem. B 111 (2007) 12468–12477.
[10] N. Bicak, A new ionic liquid: 2-hydroxy ethylammonium formate, J. Mol. Liq. 116 (2005) 15–18.
[11] M. Khodadadi-Moghaddam, A. Habibi-Yangjeh, M.R. Gholami, Kinetic study of heterogeneous catalytic hydrogenation of cyclohexene to cyclohexane in ionic liquid–alcohols mixtures, Appl. Catal. A 341 (2008) 58–64.
[12] X. Yuan, S. Zhang, J. Liu, X. Lu, Solubilities of CO_2 in hydroxyl ammonium ionic liquids at elevated pressures, Fluid Phase Equilib. 257 (2007) 195–200.
[13] M. Khodadadi-Moghaddam, A. Habibi-Yangjeh, M.R. Gholami, 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 (2009) 11–16.
[14] European Union, 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 (2006) 1–856.
[15] J. Arning, S. Stolte, A. Boeschen, F. Stock, W.R. Pitner, U. Welz-Biermann, B. Jastorff, J. Ranke, Qualitative and quantitative structure activity relationships for the inhibitory effects of cationic head groups, functionalised side chains and anions of ionic liquids on acetylcholinesterase, Green Chem. 10 (2008) 47–58.
[16] J. Ranke, K. Mölter, F. Stock, U. Bottin-Weber, J. Poczobutt, J. Hoffmann, B. Ondruschka, J. Filser, B. Jastorff, Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays, Ecotoxicol. Environ. Saf. 58 (2004) 396–404.
[17] National Toxicology Program (NTP) and National Institute of Environmental Health Sciences (NIEHS), Review of Toxicological Literature for Ionic Liquids, prepared by Integrated Laboratory Systems Inc., Research Triangle Park, NC, 2004.
[18] A. Mohammad, D. Inamuddin (Eds.), Green Solvents II. Properties and Applications of Ionic Liquids, 2012. Springer.
[19] P. Domínguez de María (Ed.), Ionic Liquids in Biotransformations and Organocatalysis: Solvents and Beyond, John Wiley & Sons, Inc., 2012.
[20] M. Iglesias, R. Gonzalez-Olmos, I. Cota, F. Medina, Brønsted ionic liquids: study of physico-chemical properties and catalytic activity in aldol condensations, Chem. Eng. J. 16 (2010) 2802–2808.
[21] V.H. Álvarez, N. Dosil, R. Gonzalez-Cabaleiro, S. Mattedi, M. Martin-Pastor, M. Iglesias, J.M. Navaza, Brønsted ionic liquids for sustainable processes: synthesis and physical properties, J. Chem. Eng. Data 55 (2010) 625–632.
[22] B. Peric, E. Martí, J. Sierra, R. Cruañas, M. Iglesias, M.A. Garau, Terrestrial ecotoxicity of short aliphatic ionic liquids, Environ. Toxicol. Chem. 30 (2011) 2802–2809.
[23] ISO guideline 11348-1, Water quality. Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test), Part 1: Method using freshly prepared bacteria, Geneva, 2007.
[24] OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems Test No. 201: Freshwater Alga and *Cyanobacteria*, Growth Inhibition Test, Paris, France, 2011.
[25] W. Drost, M. Matzke, T. Backhaus, Heavy metal toxicity to *Lemna minor*: studies on the time dependence of growth inhibition and the recovery after exposure, Chemosphere 67 (2007) 36–43.
[26] G.L. Ellman, K.D. Courtney, V.A. Andres Jr., R.M. Featherstone, A new coloromet-ric determination of acetylcholinesterase activity, Biochem. Pharm. 7 (1961) 88–95.
[27] T.C. Fisher, M. Crane, A. Callaghan, An optimized microtiter plate assay to detect acetylcholinesterase activity in individual *Chironomus riparius* Meigen, Environ. Toxicol. Chem. 19 (2000) 1749–1752.
[28] OECD Guidelines for the Testing of Chemicals, Guideline 301: Ready biodegradability, Paris, France, 1992.
[29] S. Stolte, S. Abdulkarim, J. Arning, A.K. Blomeyer-Nienstedt, U. Bottin-Weber, M. Matzke, J. Ranke, B. Jastorff, J. Thöming, Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazolium chloride and electrochemical wastewater treatment of poorly biodegradable compounds, Green Chem. 10 (2008) 214–224.
[30] N. Cedergreen, J.C. Streibig, The toxicity of herbicides to non-target aquatic plants and algae: Assessment of predictive factors and hazard, Pest Manage. Sci. 61 (2005) 1152–1160.
[31] N. Cedergreen, T.V. Madsen, Light regulation of root and leaf NO_3^- uptake and reduction in the floating macrophyte *Lemna minor*, New Phytol. 161 (2003) 449–457.
[32] M. Mohammad, T. Kishimoto, K. Itoh, K. Suyama, H. Yamamoto, Comparative sensitivity of *Pseudokirchneriella subcapitata* vs. *Lemna* sp. to eight sulfonylurea herbicides, Bull. Environ. Contam. Toxicol. 75 (2005) 866–872.
[33] S. Stolte, M. Matzke, J. Arning, A. Böschen, W.R. Pitner, U. Welz-Biermann, B. Jastorff, J. Ranke, Effects of different head groups and functionalised side chains on the aquatic toxicity of ionic liquids, Green Chem. 9 (2007) 1170–1179.
[34] J.F. Fairchild, D.S. Ruessler, P.S. Haverland, A.R. Carlson, Comparative sensitivity of *Selenastrum capricornutum* and *Lemna minor* to sixteen herbicides, Arch. Environ. Contam. Toxicol. 32 (1997) 353–357.
[35] UN/SCEGHS/12/IN.F.5, Sub-Committee of Experts on the Globally Harmonized System of classification and labelling of chemicals (GHS), United Nations, New York and Geneva, 2007.
[36] Globally Harmonized System of classification and labelling of chemicals (GHS), 4th revised edn, United Nations, New York and Geneva, 2011.
[37] M. Stasiewicz, E. Mulkiewicz, R. Tomczak-Wandzel, J. Kumirska, E. Siedlecka, M. Gołębiowski, J. Gajdus, P. Stepnowski, Assessing toxicity and biodegradation of novel, environmentally benign ionic liquids (1-alkoxymethyl-3-hydroxypyridinium chloride, saccharinate and acesulfamates) on cellular and molecular level, Ecotoxicol. Environ. Saf. 71 (2008) 157–165.
[38] S.P.M. Ventura, C.S. Marques, A.A. Rosatella, C.A.M. Afonso, F. Gonçalves, J.A.P. Coutinho, Toxicity assessment of various ionic liquid families towards *Vibrio fischeri* marine bacteria, Ecotoxicol. Environ. Saf. 76 (2012) 162–168.
[39] F. Stock, J. Hoffmann, J. Ranke, R. Störmann, B. Ondruschka, B. Jastorff, Effects of ionic liquids on the acetylcholinesterase – a structure-activity relationship consideration, Green Chem. 6 (2004) 286–290.
[40] C. Samorì, Introduction of oxygenated side chain into imidazolium ionic liquids: evaluation of the effects at different biological organization levels, Ecotoxicol. Environ. Saf. 73 (2010) 1456–1464.
[41] The UFT/Merck Ionic Liquids Biological Effects Database: http://www.ileco.uft.uni-bremen.de/ (last accessed on 30.11.12).
[42] S. Stolte, J. Arning, U.Bottin-Weber, A. Müller, W.R. Pitner, U. Welz-Biermann, B. Jastorff, J. Ranke, Effects of different head groups and functionalised side chains on the cytotoxicity of ionic liquids, Green Chem. 9 (2007) 760–767.
[43] P. Luis, I. Ortiz, R. Aldaco, A. Irabien, A novel group contribution method in a developnment of a QSAR for predicting the toxicity (*Vibrio fischeri* EC_{50}) of ionic liquids, Ecotoxicol. Environ. Saf. 67 (2006) 423–429.
[44] T.P.T. Pham, C.W. Cho, Y.S. Yun, Environmental fate and toxicity of ionic liquids: a review, Water Res. 44 (2010) 252–272.

3.3. Article 3

ESTUDI COMPARATIU DE L'ECOTOXICITAT TERRESTRE D'ALGUNS LÍQUIDS IÒNICS PRÒTICS I APRÒTICS

Resum

Els líquids iònics (ILs) han estat considerats com a potencialment "verds" i els estudis de l'obtenció de noves estructures d'ILs amb aplicacions interessants supera notablement en nombres els estudis sobre l' impacte mediambiental d'ILs. Això és particularment evident en l'àrea d'ecotoxicitat terrestre, ja que hi ha molt pocs treballs exhaustius publicats, sobretot quan es tracta d'avaluar els efectes dels PILs i AILs sobre els microorganismes del sòl que intervenen en la transformació del carboni i del nitrogen. En aquest article es presenta un estudi comparatiu de l'ecotoxicitat terrestre de dos grups d'ILs: deu representants d'una nova família de PILs (derivats d'amines alifàtiques i àcids orgànics) i alguns AILs d'ús freqüent (clorurs de l'imidazoli i del piridini substituïts). Es va avaluar la toxicitat d'aquests líquids iònics per a tres espècies de plantes terrestres (*Allium cepa*, *Lolium perenne* i *Raphanus sativus*) i per als microorganismes del sòl que participen en la transformació del carboni i el nitrogen. Els AILs seleccionats van ser més tòxics que els PILs, amb valors de CE_{50} inferiors en diversos ordres de magnitud. Tots els AILs varen mostrar toxicitat per *A. cepa*, i un d'ells va resultar ser tòxic per a les tres plantes assajades. En el cas dels PILs, només en cas d'un es va obtenir la CE_{50} inferior a 1000 mg kg^{-1} per a totes les plantes analitzades, que és el valor llindar de toxicitat per a les plantes terrestres, segons el Sistema Mundialment Harmonitzat de Classificació i Etiquetatge de Productes Químics (GHS), mentre que tres dels PILs van ser classificats com a nocius només per a una planta. Pel que fa als assajos de mineralització de carboni i nitrogen els ILs analitzats van mostrar valors CE_{50} superiors als llindars de toxicitat establerts pel GHS, sobretot en el cas dels PILs. La forma de les corbes de respiració del sòl pel cas dels PILs indica que estimulen l'activitat microbiana, per tant sembla que poden ser potencialment biodegradables en sòl. D'altra banda els AILs varen mostrar una activitat respiratòria inferior a la de la mostra control, fet que pot indicar que es tracta de compostos més resistents a la biodegradació. Els líquids iònics més complexos

d'ambdós grups varen resultar ser els més tòxics. Les conclusions de l'estudi indiquen que els PILs poden ser considerats com a menys tòxics i més segurs per al medi terrestre que els AILs.

Chemosphere 108 (2014) 418–425

Contents lists available at ScienceDirect

Chemosphere

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

A comparative study of the terrestrial ecotoxicity of selected protic and aprotic ionic liquids

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HIGHLIGHTS

- Toxicity of selected ionic liquids (ILs) towards terrestrial organisms was analyzed.
- The aprotic ILs have lower EC_{50} than the protic ILs in all of the performed tests.
- The analyzed protic ILs are potentially biodegradable in soil, unlike the aprotic ILs.
- The new family of protic ILs is environmentally more favourable than the aprotic ILs.

ARTICLE INFO

Article history:
Received 10 November 2013
Received in revised form 1 February 2014
Accepted 6 February 2014
Available online 13 March 2014

Keywords:
Protic ionic liquids
Aprotic ionic liquids
Terrestrial ecotoxicity
Phytotoxicity
Carbon and nitrogen transformation

ABSTRACT

Ionic liquids (ILs) are a fairly new and very promising group of compounds with a vast variety of possible structures and uses. They are considered to be potentially "green", but their impact on the environment tends to be neglected or not studied enough, especially when it comes to terrestrial ecotoxicity, where there are very few studies performed to date. This work presents a comparative study of the terrestrial ecotoxicity of selected representatives of two ILs groups: a new family of protic ILs (derived from aliphatic amines and organic acids) and some frequently used aprotic ILs (substituted imidazolium and piridinium chlorides). Toxicity of the ILs towards three terrestrial plant species (*Allium cepa*, *Lolium perenne* and *Raphanus sativus*) and soil microorganisms involved in carbon and nitrogen transformation was analyzed. Protic ILs have shown no toxic effect in most of the tests performed. The EC_{50} values for aprotic ILs are various orders of magnitude lower than the ones for protic ILs in all of the tests. The most toxic ILs are the most complex ones in both of the analyzed groups. Protic ILs seem to have a potential for biodegradation in soil, while aprotic ILs exhibit inhibitory effects towards the carbon transforming microbiota. These findings indicate that protic ILs can be considered as less toxic and safer for the terrestrial environment than the aprotic ILs.

1. Introduction

During the last decades ionic liquids (ILs) have generated a lot of interest as new promising group of compounds. They are composed entirely of ions, liquid over a wide range of temperatures and practically non-volatile. The number of combinations of ions that form ILs is estimated to be as high as 10^{18}, so they have the potential to be tailored to suite the desired application. Their use spreads from chemical laboratories to various industrial processes, mostly being used as a reaction medium in a wide variety of chemical transformations, separations and extractions that until recently could only be carried out in organic solvents (Han and Row, 2010; Olivier-Bourbigou et al., 2010; Mohammad Ali, 2012). New possible applications of ILs are being constantly discovered (Kokorin, 2011).

One of many possible classifications of ILs is dividing them into two groups: aprotic (AILs) and protic (PILs) ionic liquids. The first group has been in use for more than a decade and the second one is still in development. A new family of PILs with simple and lineal cations and anions has been designed and they could have a potentially smaller environmental impact than the bulky and heterocyclic "classic" ILs which have been mainly in use up to date. The new PILs are composed of polysubstituted amines (mono-, di- and triethanolamine) as cations, and aliphatic organic acids (formic, acetic, propionic, butyric, isobutyric and pentanoic acid) as anions (Cota et al., 2007). The AILs analyzed in this study are one of the most frequently used and are derived from imidazolium and pyridinium (Masten, 2004; Werner et al., 2010; Domínguez de María, 2012; Mohammad Ali, 2012).

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http://dx.doi.org/10.1016/j.chemosphere.2014.02.043
0045-6535/

There are many possible applications for this new family of PILs. The 2-hydroxyethanolamine formate (2-HEAF) has been found to be able to dissolve many inorganic salts, hydroxylated compounds and some insoluble polymers such as polyaniline and polypyrrole (Bicak, 2005). The representatives of this family have been analyzed for their potential catalytic activity in the aldol condensation reactions (Iglesias et al., 2010) and for the heterogeneous catalytic hydrogenation of cyclohexene and acetone catalyzed by Pt/Al_2O_3 with methanol, ethanol and propan-1-ol (Khodadadi-Moghaddam et al., 2009). Other PILs, also derived from 2-hydroxyethylammonium and organic acids, have been studied in order to develop a new process for absorption of CO_2 (Yuan et al., 2007).

Ionic liquids are usually described as "green", but they are chemical products, and as such have to fulfill the current European chemical legislation for Registration, Evaluation, Authorisation and Restriction of Chemical Substances (REACH) (European Union, 2006). A very important aspect that has to be studied in sufficient depth is the potential negative environmental impact of ILs. This feature is often neglected, because the usual focus is to obtain new compounds with desired characteristics, sometimes without taking into account the environmental hazards. It is true that due to their extremely low vapour pressure ILs cannot be found in the atmosphere, but they can be soluble in water, like in case of the ILs presented in this study, so they could be found in water and soil as part of industrial and laboratory effluents. And as the new family of PILs is still under development and fairly new in their use, it is very important to assess their hazard potential, both in terms of aquatic (Peric et al., 2013) and terrestrial ecotoxicity.

The information on terrestrial ecotoxicity and biodegradability of ILs in literature is scarce. There are some articles published on the sorption and transport of ILs in soil (Studzińska et al., 2008, 2009; Matzke et al., 2008a; Mrozik et al., 2012) where the authors showed that the ILs cations can adsorb onto different types of soils and sediments and that hydrophobic ILs with long side chains adsorb much more strongly than the ILs with short and hydroxylated side chains, so their minimal retention could result in contamination of soils and consequently ground waters. The study of Deive et al. (2011) focused on identification of the microbial strains with higher survival rates towards ILs which could be potentially used in soil remediation. The potential biodegradability of ILs in water has been frequently studied (Stolte et al., 2008; Harjani et al., 2009; Coleman and Gathergood, 2010; Pham et al., 2010) with different conclusions, from zero biodegradation to potential biodegradability, depending on the hydrophylicity and hydrophobicity of the ILs molecule. But, there is a very limited number of publications on their biodegradation in soil (Modelli et al., 2008; Zhang et al., 2010), where the authors analyzed four ILs, three of which are derived from imidazolium and one from pyridinium, and found limited biodegradation after two months or complete lack of biodegradation. The studies published on the phytotoxicity of ILs have been mainly focused on the aquatic plants (Zhu et al., 2009; Pham et al., 2010) with certain degree of toxicity of ILs for *Lemna minor*, which depends on the cation present in the molecule, and on the length of the side chains. But there is few data available on the ecotoxicological effects of ionic liquids on terrestrial plants, with data published on toxicity of ILs (mainly derived from imidazolium) to wheat (Matzke et al., 2007; Wang et al., 2009), garden cress (Jastorff et al., 2005; Matzke et al., 2007), barley and radish (Bałczewski et al., 2007) and watercress (Studzińska and Buszewski, 2009) where the toxicity was found to be increasing with the decrease of the hydrophobicity of the ILs molecule. In their two studies Matzke et al. (2008b, 2009) analyzed the relation between the toxic effects of imidazolium based ionic liquids (depending on their anions and side chains) on wheat and cress and the content of different clay minerals and clay concentrations in soil, and found that the toxicity of the ILs was mainly dominated by the cation and that higher clay content lowered the toxicity, whilst the addition of clay minerals caused higher toxic effects in comparison to the control soil sample. To our knowledge, there is no complete information or study done in order to evaluate the toxic effect of ILs on the soil microbiota in terms of carbon and nitrogen transformation.

The present study aimed to evaluate and compare the terrestrial ecotoxicity of the representatives of a new family of PILs and frequently used AILs 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).

2. Materials and methods

2.1. Ionic liquids

The PILs analyzed in the present study are: 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxyethanolamine butanoate (2-HEAB), 2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine propionate (2-HDEAPr), 2-hydroxydiethanolamine butanoate (2-HDEAB), 2-hydroxydiethanolamine isobutanoate (2-HDEAiB), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), 2-hydroxytriethanolamine butanoate (2-HTEAB) and 2-hydroxytriethanolamine pentanoate (2-HTEAPe). The analyzed AILs are: 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-methyl-3-octylimidazolium chloride ([OMIM]Cl) and N-butylpyridinium chloride ([BPy]Cl).

The PILs were synthesized and characterized by Dr. Miguel Iglesias's group at the University of Santiago de Compostela (Spain) as described in detail by Cota et al., 2007; Álvarez et al., 2010; Iglesias et al., 2010; Peric et al., 2011. The AILs were obtained commercially from Sigma–Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

The structures of analyzed PILs are shown in Fig. S1 and the structures of AILs in Fig. S2 of Supplementary material.

2.2. Soil samples

Soil samples (air dried and 2 mm-sieved) were obtained from the superficial layer (A horizon) of natural pine wood forest soil sampled in Vilassar de Mar, Barcelona (UTM: 31T 444376E 4596459N). The soil corresponds to a Haplic arenosol (FAO, 1998) granitic origin and sandy texture (74% of sand). Its pH value is 6.3 and it has 1.2% of oxidizable carbon and around 3% 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) guideline for testing soil microorganisms involved in carbon transformation process (OECD, 2000b).

2.3. Terrestrial plants test: Seedling emergence and seedling growth test

The seedling emergence and seedling growth test was performed with the seeds of two monocotyledonae plants: *Allium cepa* (onion) and *Lolium perenne* (grass) and one dicotyledonae *Raphanus sativus* (radish). These plant species were chosen based on the criteria for selection of test species and the list of species historically used in plant testing, included in the OECD 208 Terrestrial plant test guideline (OECD, 2006). The seeds were put in 20 mL plastic pots, with four replicates of 5 seeds, in 15 g of soil for each application rate and control sample. Aqueous solutions of the ILs were added to the soil at concentrations of 1, 10, 100, 1000, and 5000 mg kg^{-1}, including the control samples where no ILs were added to the soil. The final water content of all samples was the

equivalent to 60% of the soil water holding capacity, and the water lost during the assay was periodically restored. The plant germination and growth assay lasted 14–21 d after the emergence of 50% of the seedlings in the control group, and was performed according to OECD 208 guideline (OECD, 2006). Once the assay was concluded the shoot length was measured. The morphological changes of the plants were also noted, such as chlorosis.

2.4. Soil microorganisms: Carbon transformation test

In the carbon transformation test, 50 g of soil sample was used, adjusting the water content to the 60% of the soil water holding capacity. All the experiments were done at least in triplicate. The soil was treated with the following concentrations of ILs: 1, 10, 100, 1000, 5000, and 10000 mg kg^{-1}, including the control samples where no ILs were added to the soil. These samples were incubated in manometric respirometers, which allow the determination of the sample oxygen consumption (Oxitop® OC 110, WTW GmbH, Weilheim, Germany). The samples were kept at 25 °C in the darkness, in an incubator equipped with a thermostat for 28 d. The oxygen consumption was periodically registered. Cumulative respiration (CR) was determined by the cumulative oxygen consumption at the end of the incubation period. Once the incubation was completed, substrate induced respiration (SIR) was determined according to OECD 217 carbon transformation test guideline (OECD, 2000b). This former test was done by adding an aqueous solution equivalent to 4000 mg glucose per kg of soil to the incubated samples and the determination of 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 the respiration was stable. The respiratory activation quotient (Q_R) was calculated dividing BR by SIR (ISO, 2002).

2.5. Soil microorganisms: Nitrogen transformation test

The nitrogen transformation test was done according to the OECD 216 guideline (OECD, 2000a). The soil was treated with the following concentrations of ILs: 10, 100, 1000, 10000, and 20000 mg kg^{-1}, including the control samples where no ILs were added to the soil. The test was done with the 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 (US EPA, 1971).

2.6. Statistical analysis

In order to know the statistical significance of the differences between treated soil samples and controls an analysis of variance (ANOVA) test was done followed by post hoc Duncan test ($p < 0.05$). Based on the results obtained by the ANOVA test, a Lowest Observable Adverse Effect Concentration (LOAEC) for the ionic liquids has been determined after the carbon transformation test. Dose–response relationship was assessed, and the effective concentrations 10, 20 and 50 (EC_{10}, EC_{20} and EC_{50}) with their 95% confidence intervals were calculated for each compound from suitable regression models (Gompertz, hormetic, or logistic) using Statistica 6.0 (Stat Soft Inc., 1984–2001). The choice of the model was based on the best fit to the data. The EC_{50} was selected because it is the parameter used in the environmental regulations and most of the available literature. It can be considered that EC_{20}, which represents 20% level of negative effect, is a significant alteration, taking into account that this work was done in controlled laboratory experimental conditions (Isnard et al., 2001). In the case of EC_{10}, the lowest bound of its confidence interval was found to be close to No Observed Effect Concentration (NOEC) (Fulladosa et al., 2005), so it can be considered as a safe concentration. Spearman and Pearson's coefficient of correlation were calculated using IBM® SPSS® Statistics 20 program (IBM Corporation, NY, USA).

3. Results and discussion

3.1. Terrestrial plants test: Seedling emergence and seedling growth test

The values of EC_{50} (mg kg^{-1}) and the confidence interval of dose–response curves for the analyzed ILs are shown in Table 1.

In the case of *A. cepa* EC_{50} values for the analyzed PILs are between 655 and 7793 mg kg^{-1}, with 2-HDEAPe being the most toxic and 2-HDEAF the least toxic compound. The AILs showed to be more toxic towards *A. cepa* than the PILs, with considerably lower EC_{50} values, ranging between 150 and 930 mg kg^{-1}. The [OMIM]Cl is the most toxic one, with the lowest EC_{50} of all analyzed ILs for the three tested plants. The EC_{50} values obtained for the PILs in the test with *L. perenne* range between 503 and 7166 mg kg^{-1}, with 2-HDEAPe and 2-HEAB being the most toxic ones, and 2-HEAF and 2-HDEAF the least toxic ones. In the case of AILs, the obtained EC_{50} are in general lower, being between 561 and 2890 mg kg^{-1}, with [OMIM]Cl once again being the most toxic one. As far as the inhibition of growth of *R. sativus* is concerned, the EC_{50} values vary between 422 and 6430 mg kg^{-1}, with 2-HDEAPe being the most toxic one and 2-HDEAF the least toxic compound, as in the case of *A. cepa*. The EC_{50} values corresponding to AILs are lower, being between 371 and 3742 mg kg^{-1}, with [OMIM]Cl as the most toxic one. *L. perenne* and *R. sativus* have proven to be some more sensitive to the presence of PILs than *A. cepa*. In contrast, for the AILs the most sensitive species is *A. cepa*.

It can be observed that the most toxic of all the analyzed ILs is the [OMIM]Cl, with EC_{50} values far below 1000 mg kg^{-1} in case of all of the three plants. According to the Classification criteria for substances hazardous to plants (soil exposure) of the Globally Harmonized System of Classification and Labelling of Chemicals (United Nations, 2006) this AIL can be classified as toxic for the terrestrial environment in the Acute 3 category of this classification ($100 < EC_{50} \leqslant 1000$ mg kg^{-1}). Two of the PILs show some toxic effect and belong to the Acute 3 category, 2-HDEAPe for the *A. cepa*, 2-HEAB and 2-HDEAPe for *L. perenne* and 2-HDEAPe and 2-HTEAPe for *R. sativus*. The most toxic of the analyzed PILs is 2-HDEAPe, with EC_{50} values below 1000 mg kg^{-1} for the three plants. It can also be classified into the Acute 3 category. The other two representatives of AILs can also be classified into the Acute 3 category in the case of *A. cepa*, but with a significant difference in EC_{50} values from the [OMIM]Cl, being 930 and 588 mg kg^{-1} for [BMIM]Cl and [BPy]Cl respectively. The analyzed PILs are less toxic than the AILs. The comparison of the results from this study with those obtained by other authors for ILs (Jastorff et al., 2005; Bałczewski et al., 2007; Matzke et al., 2007, 2008b, 2009; Wang et al., 2009; Pham et al., 2010) shows that the analyzed PILs are less toxic than the AILs in general, including the ones analyzed in this study. Even though the plant species used in the test were not the same as those used by other authors, the values of EC_{50} for the AILs generally have lower EC_{50} values than the ones obtained for the PILs tested during the present study. Increased toxicity of [OMIM]Cl in comparison with the other two representatives of AILs is consistent with the findings of other authors that indicate that the elongation of the alkyl side chains leads to increased toxicity (Ranke et al., 2004; Matzke et al., 2008b; Pham et al., 2010).

When it comes to EC_{20} values, in the case of PILs most of them are above 1000 mg kg^{-1}, with the values ranging between 272 and 4374 mg kg^{-1}, whilst the EC_{10} values range between 87 and 3580 mg kg^{-1}. If we consider EC_{20} as the concentration where

Table 1
Results of dose–response curves in mg kg^{-1} for the seedling emergence and growth and nitrogen transformation tests, $p < 0.05$.

	Allium cepa		*Lolium perenne*		*Raphanus sativus*		Nitrogen transformation	
	EC_{50}	Confidence interval	EC_{50}	Confidence interval	EC_{50}	Confidence interval	EC_{50}	Confidence interval
2-HEAF	6887	3805–12459	7166	6878–14257	3383	2464–4645	10014	4865–14157
2-HEAB	2012	437–4446	680	458–1009	1729	362–2495	7485	7082–7773
2-HDEAF	7793	1797–9136	3949	2317–6730	6430	3698–10179	8018	7500–8571
2-HDEAA	6415	4260–8316	996	692–1433	1584	508–4931	8272	7863–8704
2-HDEAPr	3891	2468–6025	3163	1951–5158	2128	1714–2640	8454	3973–9704
2-HDEAB	4174	2226–5247	1180	845–1648	3646	2761–4815	7014	6236–7888
2-HDEAiB	2313	1517–3528	1780	368–8584	2166	1847–2541	6487	6135–6858
2-HDEAPe	655	243–1067	503	294–1002	422	241–739	6639	6201–7108
2-HTEAB	1568	1149–2140	1261	995–1597	2013	1262–3210	6358	5983–6756
2-HTEAPe	1428	422–3655	2326	1930–2568	826	605–1128	6201	3763–8184
BMIM[Cl]	930	707–1223	1317	1120–1548	3742	3288–4258	555	426–723
OMIM[Cl]	150	84–362	561	367–857	371	316–435	321	216–321
BPy[Cl]	588	274–1216	2890	2458–3399	2774	2068–3721	663	566–775

Results for 2-HEAF, 2-HDEAPr and 2-HTEAPe are taken from Peric et al. (2011).

the first adverse effect can be spotted, and EC_{10} as a "safe" concentration, the analyzed PILs can be considered as low toxic and fairly environmentally safe. On the other hand, the analyzed AILs have all but two values for EC_{20} below 1000 mg kg^{-1}, and the EC_{10} are all but two below 500 mg kg^{-1}. The values of EC_{10} and EC_{20} and the confidence interval of dose–response curves for the analyzed ILs are shown in Supplementary material Table S1.

The Spearman's correlation coefficients were determined in order to get more information on toxicity of the analyzed PILs. The correlation between the complexity of the PILs molecule and their toxicity was investigated. For this analysis the molecular weight of PILs, the number of C-atoms in the cationic (amine) and anionic (acid) part of the molecule and EC_{50} values were used. The correlation analysis has shown that the elongation of the anionic part of the molecule causes higher toxicity in the case of the analyzed plants, with Pearson correlation coefficient being −0.955 ($p < 0.01$) for the *A. cepa*, −0.715 ($p < 0.05$) for *L. perenne* and −0.713 ($p < 0.05$) for *R. sativus*. There is also a negative correlation between the increase of the molecular weight and the obtained EC_{50} values for *A. cepa* and *L. perenne*, with PILs with higher molecular weight (the most complex ones) being the most toxic ones ($r = -0.716$, $p < 0.01$ and $r = -0.583$, $p < 0.05$ respectively). These findings are in concordance with the correlation analysis results for the aquatic plant *Lemna minor* (Peric et al., 2013), where the number of carbons in the anionic part of the molecule proved to be in negative correlation with EC_{50} values.

Apart from being more toxic than the PILs in the terms of lower EC_{50}, the AILs also caused chlorosis in the case of *R. sativus* and *L. perene* at concentrations $\geqslant$100 mg kg^{-1} (Fig. 1). A lower synthesis of chlorophyll is manifested as chlorosis, and it can be caused by nutrient deficiency (especially iron), higher soil salinity, damaged or compressed roots, atmospheric pollutants and toxic action of contaminants in the soil (Pessarakli, 2011). The electrical conductivity corresponding to the analyzed AILs was 0.879 dS m^{-1} as a maximum value, so the increased soil salinity can be discarded as a potential cause of chlorosis. The observed toxic effect of AILs could be due to root damage or blockage of the nutrient transport into the plant.

3.2. Soil microorganisms: Carbon transformation test

The cumulated respiration is graphically presented in the form of the respiration curves of the oxygen consumed during the 28 d of the carbon transformation test. The respiration curves obtained for the ILs can be divided into 4 groups according to their shape and distribution, and we have chosen one of each group to present the results of the cumulative respiration. The graph for 2-HDEAA is

Fig. 1. Chlorosis of the leaves of *Raphanus sativus* caused by [BMIM]Cl. (For interpretation to colours in figure, the reader is referred to the web version of this paper.)

representative for two more PILs: 2-HEAF and 2-HDEAF. The graph for 2-HEAB also represents 2-HDEAiB and 2-HTEAB. The 2-HDEAB graph also corresponds to 2-HDEAPr, 2-HDEAPe and 2-HTEAPe. And finally, the graph presented for [OMIM]Cl also corresponds to [BMIM]Cl and [BPy]Cl. The graphs in Fig. 2 show the cumulative respiration (CR) curves corresponding to control and the concentrations 1, 10, 100, 1000, 5000, and 10000 mg kg^{-1} for the representative ionic liquids. The graphs for 2-HEAF, 2-HDEAPr and 2-HTEAPe are published in Peric et al., 2011 and the graphs for the rest of the ILs can be found in Supplementary material, Figs. S3–S7.

In the case of 2-HEAF, 2-HDEAF and 2-HDEAA, none of the assayed concentrations produced values of accumulated oxygen below the control. This indicates that these ionic liquids show no toxicity towards soil microbiota, reflected as uninterrupted respiratory activity of the soil. The case of 2-HEAB, 2-HDEAiB and 2-HTEAB is similar in a sense that at the final point of the test, after 28 d, all of the values of the accumulated oxygen are above the control. The curves for the lowest concentrations (1, 10, and 100 mg kg^{-1}) show no difference when compared to the control, while the curve for the concentration of 1000 mg kg^{-1} of PILs shows a very strong response of the soil microbiota from the very start of the assay, with final accumulated oxygen values being between 123% and 135% of the control sample at the final point of the assay. In the case of higher concentrations (5000 and 10000 mg kg^{-1}) there is an initial inhibitory effect on the soil

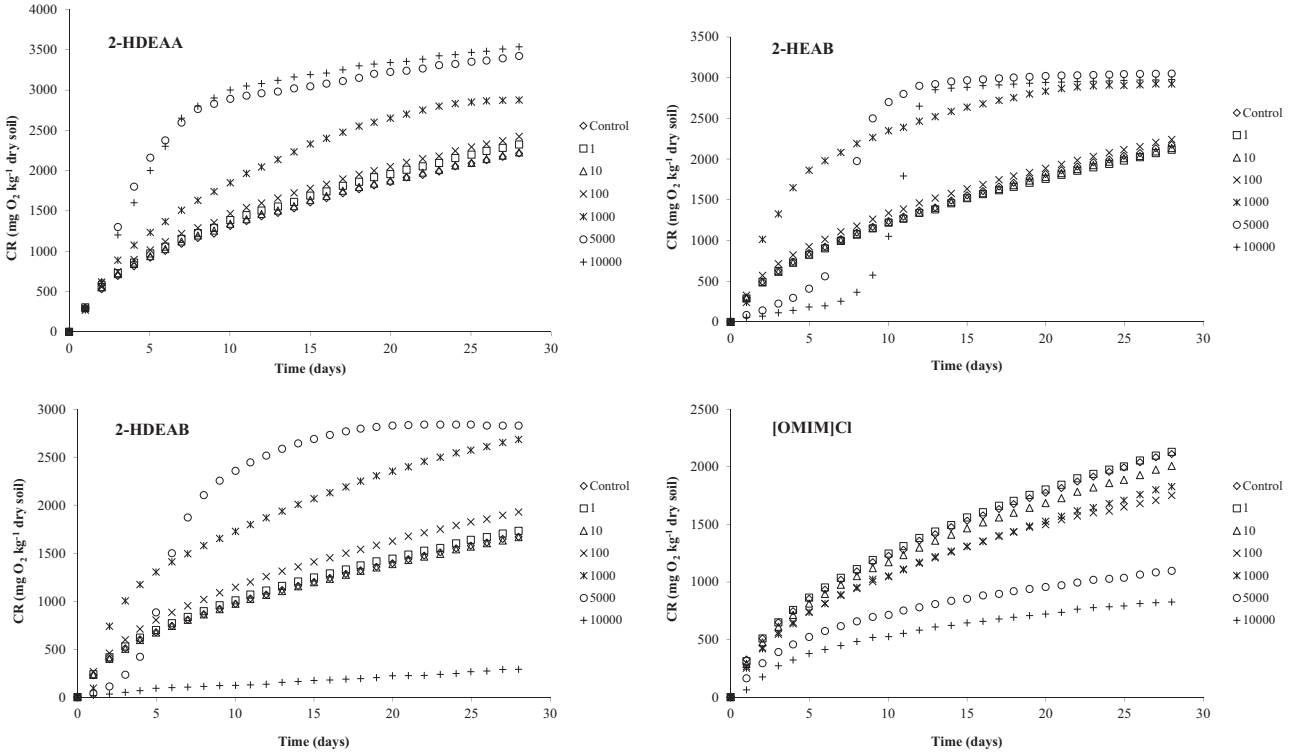

Fig. 2. Values of the cumulative respiration expressed as the cumulative oxygen consumed during 28 d of the respirometric assay for the control and different concentrations in mg kg^{-1} of the selected ILs. The values correspond to the averages of at least three replicates. The standard deviation is between 8 and 170 mg kg^{-1} for 2-HDEAA, between 8 and 78 mg kg^{-1} for 2-HEAB, between 8 and 53 mg kg^{-1} for 2-HDEAB and between 14 and 162 mg kg^{-1} for [OMIM]Cl after 28 d.

microbiota which manages to recuperate and start to respirate, with final percentage of the accumulating oxygen being between 132% and 142% of the value of the control. The third pattern of the PILs effect on the soil microbiota (the case of 2-HDEAPr, 2-HDEAB, 2-HDEAPe and 2-HTEAPe) implies little or no difference between the lowest concentrations (1, 10, and 100 mg kg^{-1}) and the control, and also the initial inhibition at the concentration of PILs 5000 mg kg^{-1}. But in the case of the highest concentration (10 000 mg kg^{-1}), the inhibition of the respiratory activity occurs throughout the whole assay, thus indicating toxicity for soil microbiota at this concentration. In the case of temporary inhibition and posterior recovery of the respiratory activity, the levels of the accumulated oxygen are higher than in the case of the control sample, which could be due to the degradation of labile organic matter coming from the microorganisms affected by the initial toxicity and/or due to the biodegradation of the analyzed ILs.

The AILs have a different behaviour. All of the curves are at the same level or lower than the control. There is no difference between the control samples and the lower concentrations (1, 10, and 100 mg kg^{-1}) in case of the [BMIM]Cl, but there is an inhibitory effect starting at 1000 mg kg^{-1}, which indicates that this compound shows toxicity towards the microorganisms involved in the C mineralization cycle. The inhibition of the respiratory activity in the case of [OMIM]Cl and [BPy]Cl starts at 100 mg kg^{-1} (Fig. 2). In general, the analyzed AILs have proven to be more toxic for the soil microbiota than the analyzed PILs.

Table 2 shows the results for the CR, BR, SIR and Q_R for the analyzed ILs.

The CR reflects the overall soil state (microbiota and nutrient availability) and the results show that at the lower concentrations of PILs (1, 10 and 100 mg kg^{-1}) there is no difference with the control samples. A significant ($p < 0.05$) increase of CR can be observed at 1000 and 5000 mg kg^{-1}. Only in the case of the highest concentration of 2-HDEAPr, 2-HDEAB, 2-HDEAPe and 2-HTEAPe (10 000 mg kg^{-1}) the inhibition of respiration occurs. When it comes to the AILs, significantly lower values of CR compared to the control can be seen starting at the concentration of 100 mg kg^{-1} in the case of [OMIM]Cl and [BPy]Cl and 1000 mg kg^{-1} in the case of [BMIM]Cl.

Basal respiration rate (BR) indicates the current biological activity which remains constant after the soil respiration is stabilized (once the labile carbon source is exhausted and the microbiota is adapted to the conditions of the incubation). This depends on the microbiota and nutritional state of the soil. Taking into account the fact that the same soil was used in all of the experiments, the nutritional state is the same for all of the samples, and the variable is the microbiota state, the results for the BR can indicate if the analyzed ILs cause inhibition or damage to the soil microbiota. According to the results obtained in the present study, 2-HDEAB and 2-HTEAB show significant decrease in BR values starting from 5000 mg kg^{-1}. The 2-HDEAPe and 2-HTEAPe show significant decrease at the highest concentration (10 000 mg kg^{-1}). In some cases there are low BR values when there is no inhibition of respiration (in concentrations of 5000 mg kg^{-1} for 2-HDEAB, and 5000 mg kg^{-1} and 10 000 mg kg^{-1} in the case of 2-HTEAB) or the BR value cannot be calculated because the stimulation of respiration is too high, like in the concentration of 1000, 5000 and 10 000 mg kg^{-1} for 2-HEAB, 5000 and 10 000 mg kg^{-1} for 2-HDEAiB, and 10 000 mg kg^{-1} in the case of 2-HDEAA. A significant increase of the BR is noted in the case of the concentrations of 5000 and 10 000 mg kg^{-1} for 2-HEAF, 1000, 5000 and 10 000 mg kg^{-1}, for 2-HDEAF, 5000 mg kg^{-1} for 2-HDEAPr, 1000 mg kg^{-1} for 2-HDEAiB, and 5000 mg kg^{-1} for 2-HTEAPe. All of the analyzed AILs have shown a stronger inhibition of BR than the PILs. A significant inhibition starts at 100 mg kg^{-1} in the case of [BPy]Cl and at 1000 mg kg^{-1} in the case of [BMIM]Cl and [OMIM]Cl.

The SIR is proportional to the active microbial biomass and is often used as an indicator of this parameter. Only the highest

Table 2
Average rate of cumulative respiration (CR) during the incubation period expressed as the % relative to the control soil; BR (mg O_2 h^{-1} kg^{-1} dry soil), SIR (mg O_2 h^{-1} kg^{-1} dry soil) and Q_R. The letters a, b and c indicate different homogeneous groups determined by Duncan test ($p < 0.05$) within rows.

	Control	Ionic liquid concentration					
		1	10	100	1000	5000	10000
2-HEAF							
% CR	100 a	93 a	89 a	95 a	120 b	281c	355 c
BR	1.35 a	1.05 a	1.24 a	1.40 a	1.14 a	2.78 b	6.92 c
SIR	11.43 a	10.00 a	9.11 a	11.79 a	10.71 a	15.00 b	28.57 c
Q_R	0.12	0.11	0.14	0.12	0.11	0.19	0.24
2-HEAB							
% CR	100 a	97 a	98 a	103 a	129 b	134 b	132 b
BR	1.79 b	1.81 b	1.75 b	1.75 b	nd	nd	nd
SIR	7.35 a	7.16 a	7.23 a	7.59 a	10.14 b	9.78 b	10.03 b
Q_R	0.24	0.25	0.24	0.23	nd	nd	nd
2-HDEAF							
% CR	100 a	104 a	101 a	105 a	110 a	108 a	110 a
BR	2.02 a	2.10 a	1.98 a	1.98 a	3.89 c	2.49 b	2.57 b
SIR	8.48 a	10.07 ab	9.54 ab	10.60 ab	13.25 ab	15.90 bc	20.14 c
Q_R	0.24	0.21	0.21	0.19	0.29	0.16	0.13
2-HDEAA							
% CR	100 a	105 a	101 a	109 a	130 b	158 c	156 c
BR	1.87 a	1.91 a	1.79 a	2.02 a	1.52 a	1.54 a	nd
SIR	7.72 a	10.90 b	12.26b	12.90 b	14.08 c	15.89 d	23.61 e
Q_R	0.24	0.17	0.15	0.16	0.11	0.10	nd
2-HDEAPr							
% CR	100 b	90 b	89 b	97 b	111 b	179 c	46 a
BR	1.40 a	1.32 a	1.09 a	1.20 a	1.63 a	3.77 b	1.22 a
SIR	12.57 b	10.84 b	11.27 b	12.70 b	20.29 c	25.86 c	2.48 a
Q_R	0.11	0.12	0.10	0.09	0.08	0.15	0.49
2-HDEAB							
% CR	100 b	104 b	100 b	115 c	161 d	163 d	18 a
BR	1.36 b	1.48 b	1.46 b	1.48 b	1.56 b	0.42 a	0.44 a
SIR	8.59 b	8.91 b	8.51 b	10.01 c	14.91 d	19.90 e	1.26 a
Q_R	0.16	0.14	0.09	0.05	0.04	0.02	0.35
2-HDEAiB							
% CR	100 a	100 a	105 a	104 a	135 b	142 b	133 b
BR	1.56 a	1.56 a	1.59 a	1.36 a	2.02 b	nd	nd
SIR	7.86 a	8.57 a	8.57 a	10.36 a	22.50 b	30.00 b	8.29 a
Q_R	0.20	0.18	0.19	0.13	0.10	nd	nd
2-HDEAPe							
% CR	100 a	106 a	113 a	114 a	133 b	138 b	18 c
BR	1.71 b	1.79 b	1.79 b	1.71 b	2.68 c	1.32 b	0.35 a
SIR	8.56 b	7.00 b	8.94 b	7.78 b	12.44 b	31.50 c	0.39 a
Q_R	0.20	0.26	0.20	0.22	0.22	0.04	0.90
2-HTEAB							
% CR	100 a	104 a	98 a	101 a	123 b	136 b	133 b
BR	1.79 b	1.79 b	1.59 b	1.63 b	1.51 b	0.19 a	0.16 a
SIR	7.51 a	7.76 a	8.51 a	16.27 b	17.52 b	22.03 b	17.52 b
Q_R	0.24	0.23	0.19	0.10	0.09	0.01	0.01
2-HTEAPe							
% CR	100 b	104 b	100 b	104 b	161 c	242 d	19 a
BR	1.71 b	1.55 b	1.59 b	1.48 b	1.42 b	3.18 c	0.36 a
SIR	10.60 b	10.28 b	10.93 b	10.93 b	24.43 c	34.70 d	0.63 a
Q_R	0.16	0.15	0.15	0.14	0.06	0.09	0.58
[BMIM]Cl							
% CR	100 c	94 bc	104 c	99 bc	86 b	69 a	63 a
BR	1.63 bc	1.44 abc	1.87 c	1.71 bc	1.48 abc	1.24 a	1.05 a
SIR	5.83 a	5.42 a	7.08 ab	7.92 ab	10.83 c	9.17 ab	8.75 ab
Q_R	0.28	0.27	0.26	0.22	0.14	0.14	0.12
[OMIM]Cl							
% CR	100 d	101 d	95 d	87 c	83 c	52 b	39 a
BR	1.63 c	1.59 c	1.56 bc	1.52 bc	1.24 b	0.66 a	0.41 a
SIR	6.54 ab	7.69 ab	8.57 ab	9.17 ab	10.00 b	6.35 a	5.77 a
Q_R	0.25	0.21	0.18	0.16	0.12	0.11	0.07
[BPy]Cl							
% CR	100 c	98 c	100 c	84 b	78 b	65 a	54 a
BR	2.28 c	2.43 c	2.36 c	1.79 b	1.30 a	1.22 a	0.98 a
SIR	9.73 b	9.44 b	9.16 b	9.75 b	9.73 b	8.02 ab	6.89 a
Q_R	0.23	0.26	0.26	0.18	0.13	0.15	0.13

Results for 2-HEAF, 2-HDEAPr and 2-HTEAPe are taken from Peric et al. (2011).
% CR = percentage of cumulative respiration, BR = basal respiration rate, SIR = substrate induced respiration, Q_R = respiratory activation quotient, nd = not determined.

concentration of 2-HDEAPr, 2-HDEAPe or 2-HTEAPe (10000 mg kg^{-1}) shows a substantial decrease in SIR values. The higher concentrations of PILs (1000, 5000 and 10000 mg kg^{-1}) generally produce an increase in the SIR values. The AILs show a moderate inhibition of SIR values at highest concentrations.

The increase of the CR values, found in the case of most of the PILs could indicate that these compounds are biodegraded by soil microorganisms, as confirmed by a preliminary biodegradation study (Peric et al., 2011). In contrast, the AILs have all of the CR values lower than the control, which can indicate that they do not have the same degradation potential as the PILs and that they inhibit the activity of soil microorganisms.

According to the ISO standards for the determination of abundance and activity of soil microflora using respiration curves, the values of Q_R which are higher than 0.30 indicate polluted soil, especially in the case of heavy metals (ISO, 2002). For the organic compounds this ratio can be altered if the compounds are susceptible to biodegradation. When it comes to PILs, only in the case of the highest concentrations (10000 mg kg^{-1}) of 2-HDEAPr, 2-HDEAB, 2-HDEAPe and 2-HTEAPe this effect can be noted. In some cases the calculated Q_R was below 0.30, even though there was a visible toxic effect the carbon transforming microbiota. As Simek et al. (2013) also concluded in their study, the meaning of the Q_R is not always fully clear, especially because of the complex physiological status of microbial communities in individual soil horizons, so we will not speculate about the meaning of these lower than expected values in the case of the AILs analyzed in our study.

Based on the significant differences in % CR, the LOAEC values for the tested PILs are high, being 10000 mg kg^{-1} for 2-HDEAB, 2-HDEAPr, 2-HDEAPe and 2-HTEAPe, and above 10000 mg kg^{-1} for 2-HEAF, 2-HEAB, 2-HDEAF, 2-HDEAA, 2-HDEAiB and 2-HTEAB. The tested AILs have shown lower values of LOAEC, of 100 mg kg^{-1} for [OMIM]Cl and [BPy]Cl and 1000 mg kg^{-1} for [BMIM]Cl. The AILs do not show a high inhibition potential for carbon mineralization processes, but have one order of magnitude lower values of LOAEC compared to the PILs.

The correlation analysis between the structure of PILs (cationic and anionic moiety size) and their toxic action in the case of the carbon transformation test shows that there is an inverse correlation between the increase of the number of carbons in the acid part of the PILs molecule and the values for % CR, BR and SIR (correlation coefficients r are −0.911, −0.621 and −0.565 respectively, with the level of significance $p < 0.05$). The same type of correlation was found for the molecular weight ($r = -0.721$ for CR, −0.761 for BR, $p < 0.01$ and $r = -0.588$ for SIR, $p < 0.05$), while the increase of complexity of the cationic part of the PILs molecules causes the decrease of BR values ($r = -0.670$, $p < 0.05$).

3.3. Soil microorganisms: Nitrogen transformation test

The values of EC_{50} (in mg kg^{-1}) calculated from the dose–response curves in the nitrogen transformation test can be seen in Table 1.

The EC_{50} values for the analyzed PILs are very high, ranging between 6201 and 10014 mg kg^{-1}. There is no toxic effect of the analyzed ILs on nitrogen transformation processes according to the Globally Harmonized System of classification and labelling of chemicals (EC_{50} > 100 mg kg^{-1}) (United Nations, 2006), but a clear difference in EC_{50} values between the two analyzed families was found. The values of EC_{50} for AILs are between 10 and 30 times lower than the EC_{50} values for PILs, with [OMIM]Cl being the most complex and the most toxic one.

The EC_{10} and EC_{20} values are also very high, being far over 100 mg kg^{-1} in the case of all of the analyzed PILs. The lowest value of EC_{10} obtained for the 2-HTEAPe is one order of magnitude higher than the Globally Harmonized System benchmark for chronic terrestrial toxicity (100 mg kg^{-1}). The rest of the EC_x values are almost all higher than 5000 mg kg^{-1}, the fact that clearly confirms that PILs have no toxic effect on the nitrifying microbiota. The AILs also have values of EC_{10} and EC_{20} which are higher than 100 mg kg^{-1}. And once again, there is a clear difference in the order of magnitude between the EC_{10} and EC_{20} values for PILs and AILs, being the ones for AILs 12–30 times lower than the ones for PILs. The values of EC_{10} and EC_{20} and the confidence interval of dose–response curves for the analyzed ILs are shown in Supplementary material Table S1.

The correlation analysis showed that there is a negative correlation between the increase of complexity of the amine part of the molecule (from mono-, over di-, and onto triethanolamine) and the EC_{50} values of the analyzed PILs, with values for the Pearson correlation coefficient being $r = -0.683$ ($p < 0.05$). The same correlation was found in the case of the elongation of the carbon alkyl chain in the anion moiety (the acid part of the molecule) and the increase of the molecular weight and the EC_{50} values obtained in the nitrogen transformation test, with r coefficient values being −0.850 and −0.878 respectively, with $p < 0.01$. This means that the more complex is the PILs molecule, there is a higher toxicity on the nitrifying microbiota.

4. Conclusions

Comparing the terrestrial ecotoxicity results for the representatives of two groups of ionic liquids, it can be seen that the analyzed AILs are more toxic than the new group of PILs. All of the AILs proved to be toxic for the onion, and the [OMIM]Cl for the radish and grass as well. In the case of PILs, only 2-HDEAPe can be classified as toxic for the three analyzed plants, with three other being classified as toxic for only one plant species. The shape of the soil respiration curves for PILs indicate that they stimulate the microbiota's activity, which can indicate that they are potentially biodegradable in soil. However, the AILs seem to be more resistant to biodegradation, as their respiration curves present inhibition of the microbiota's activity. None of the analyzed ionic liquids show inhibition of the nitrifying microbiota. According to the correlation analysis it can be observed that the molecular weight and the size of the acid (anionic part of the molecule) have more influence on the PILs toxicity than the amine (cationic part of the molecule). More complex ILs are the most toxic ones, with [OMIM]Cl being the most toxic in all of the tests performed. This comparative study indicates that ILs with simpler structure and with short alkyl side chains show lower toxicity and should be considered as a path to follow in the future synthesis of new ionic liquids.

Acknowledgments

B. Peric would like to thank the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development (Grant MAEC-AECID).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.02.043.

References

Álvarez, V.H., Iglesias, M., Dosil, N., Gonzalez-Cabaleiro, R., Martin-Pastor, M., Mattedi, S., Navaza, J.M., 2010. Brønsted ionic liquids for sustainable processes: synthesis and physical properties. J. Chem. Eng. Data 55, 625–632.

Bałczewski, P., Bachowska, B., Białas, T., Biczak, R., Wieczorek, W.M., Balińska, A., 2007. Synthesis and phytotoxicity of new ionic liquids incorporating chiral cations and/or chiral anions. J. Agric. Food Chem. 55, 1881–1892.

Bicak, N., 2005. A new ionic liquid: 2-hydroxy ethylammonium formate. J. Mol. Liq. 116, 15–18.
Coleman, D., Gathergood, N., 2010. Biodegradation studies of ionic liquids. Chem. Soc. Rev. 39, 600–637.
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.
Deive, F.J., Rodríguez, A., Varela, A., Rodrígues, C., Leitão, M.C., Houbraken, J.A.M.P., Pereiro, A.B., Longo, M.A., Sanromán, M.Á., Samson, R.A., Rebelo, L.P.N., Silva Pereira, C., 2011. Impact of ionic liquids on extreme microbial biotypes from soil. Green Chem. 13, 687–696.
Domínguez de María, P. (Ed.), 2012. Ionic Liquids in Biotransformations and Organocatalysis: Solvents and Beyond. John Wiley & Sons Inc., USA.
European Union, 2006. Regulation 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.
FAO (Food and Agriculture Organization), United Nations Educational, Scientific and Cultural Organization, 1998. World Reference Base for Soil Resources, Rome, Italy.
Fulladosa, E., Murat, J.C., 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.
Han, D., Row, K.H., 2010. Recent applications of ionic liquids in separation technology. Molecules 15, 2405–2426.
Harjani, J.R., Singer, R.D., Garcia, M.T., Scammells, P.J., 2009. Biodegradable pyridinium ionic liquids: design, synthesis and evaluation. Green Chem. 11, 83–90.
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. 16, 2802–2808.
Isnard, P., Flammarion, P., Roman, G., Babut, M., Bastien, P., Bintein, S., Esserméant, L., Férard, J.F., Schmitt, S., Saouter, E., Saroli, M., Thiébaud, H., Tomassone, R., Vindimian, E., 2001. Statistical analysis of regulatory ecotoxicity tests. Chemosphere 45, 659–669.
ISO, 2002. ISO guideline 17155: Soil Quality. Determination of Abundance and Activity of Soil Microflora Using Respiration Curves. International Organization for Standardization, Geneva, Switzerland.
Jastorff, B., Mölter, K., Behrend, P., Bottin-Weber, U., Filser, J., Heimers, A., Ondruschka, B., Ranke, J., Schaefer, M., Schröder, H., Stark, A., Stepnowski, P., Stock, F., Störmann, R., Stolte, S., Welz-Biermann, U., Ziegert, S., Thöming, J., 2005. Progress in evaluation of risk potential of ionic liquids-basis for an eco-design of sustainable products. Green Chem. 7, 362–372.
Khodadadi-Moghaddam, M., Habibi-Yangjeh, A., Gholami, M.R., 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.
Kokorin, A. (Ed.), 2011. Ionic Liquids: Applications and Perspectives. InTechOpen, New York.
Masten, S.A., 2004. Review of Toxicological Literature for Ionic Liquids. Integrated Laboratory Systems Inc., Research Triangle Park, NC, USA.
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 1-alkyl-3-methylimidazolium ionic liquids observed in an (eco) toxicological test battery. Green Chem. 9, 1198–1207.
Matzke, M., Thiele, T., Müller, A., Filser, J., 2008a. Sorption and desorption of imidazolium based ionic liquids in different soil types. Chemosphere 74, 568–574.
Matzke, M., Stolte, S., Arning, J., Uebers, U., Filser, J., 2008b. Imidazolium based ionic liquids in soils: effects of the side chain length on wheat (*Triticum aestivum*) and cress (*Lepidium sativum*) as affected by different clays and organic matter. Green Chem. 10, 584–591.
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.
Modelli, A., Sali, A., Galletti, P., Samorì, C., 2008. Biodegradation of oxygenated and non-oxygenated imidazolium-based ionic liquids in soil. Chemosphere 73, 1322–1327.
Mohammad Ali, I. (Ed.), 2012. Green Solvents II: Properties and Applications of Ionic Liquids. Springer, New York, USA.
Mrozik, W., Kotłowska, A., Kamysz, W., Stepnowski, P., 2012. Sorption of ionic liquids onto soils: experimental and chemometric studies. Chemosphere 88, 1202–1207.
OECD, 2000a. Guideline for Testing of Chemicals 216, Soil Microorganisms: Nitrogen Transformation Test. Organization for Economic Cooperation and Development, Paris, France.
OECD, 2000b. Guideline for Testing of Chemicals 217, Soil Microorganisms: Carbon Transformation Test. Organization for Economic Cooperation and Development, Paris, France.
OECD, 2006. Guideline for Testing of Chemicals 208, Terrestial Plants Test: Seedling Emergence and Seedling Growth Test. Organization for Economic Cooperation and Development, Paris, France.
Olivier-Bourbigou, H., Magna, L., Morvan, D., 2010. Ionic liquids and catalysis: recent progress from knowledge to applications. Appl. Catal. A 373, 1–53.
Peric, B., Martí, E., Sierra, J., Cruañas, R., Iglesias, M., Garau, M.A., 2011. Terrestrial ecotoxicity of short aliphatic protic ionic liquids. Environ. Toxicol. Chem. 30, 2802–2809.
Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M.A., Arning, J., Bottin-Weber, U., Stolte, S., 2013. (Eco)toxicity and biodegradability of short aliphatic protic ionic liquids. J. Hazard. Mater. 261, 99–105.
Pessarakli, M. (Ed.), 2011. Handbook of Plant and Crop Stress, third ed. CRC Press, Taylor & Francis Group, Boca Raton, FL, USA.
Pham, T.P.T., Cho, C.W., Yun, Y.S., 2010. Environmental fate and toxicity of ionic liquids: a review. Water Res. 44, 252–272.
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.
Simek, M., Virtanen, S., Simojoki, A., Chroňáková, A., Elhottová, D., Krištůfek, V., Yli-Halla, M., 2013. The microbial communities and potential greenhouse gas production in boreal acid sulphate, non-acid sulphate, and reedy sulphidic soils. Sci. Total Environ. 466–467, 663–672.
Stolte, S., Abdulkarim, S., Arning, J., Blomeyer-Nienstedt, A.K., Bottin-Weber, U., Matzke, M., Ranke, J., Jastorff, B., Thöming, J., 2008. Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazolium chloride and electrochemical wastewater treatment of poorly biodegradable compounds. Green Chem. 10, 214–224.
Studzińska, S., Buszewski, B., 2009. Study of toxicity of imidazolium ionic liquids to watercress (*Lepidium sativum* L.). Anal. Bioanal. Chem. 393, 983–990.
Studzińska, S., Sprynskyy, M., Buszewski, B., 2008. Study of sorption kinetics of some ionic liquids on different soil types. Chemosphere 71, 2121–2128.
Studzińska, S., Kowalkowski, T., Buszewski, B., 2009. Study of ionic liquid cations transport in soil. J. Hazard. Mater. 168, 1542–1547.
United Nations, 2006. Globally Harmonized System of classification and labeling of chemicals. UN/SCEGHS/12/INF5. New York, USA.
US EPA, 1971. Brucine Method for Determination of Nitrates. Washington, DC, USA.
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.
Werner, S., Haumann, M., Wasserscheid, P., 2010. Ionic liquids in chemical engineering. Annu. Rev. Chem. Biomol. Eng. 1, 203–230.
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.
Zhang, C., Wang, H., Malhotra, S.V., Dodge, C.J., Francis, A.J., 2010. Biodegradation of pyridinium-based ionic liquids by an axenic culture of soil *Corynebacteria*. Green Chem. 12, 851–858.
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.

3.4. Article 4

PREDICCIÓ DE L'(ECO)TOXICITAT DE LÍQUIDS IÒNICS PROTICS ALIFÀTICS DE CADENA CURTA MITJANÇANT LA RELACIÓ ESTRUCTURA-ACTIVITAT QUANTITATIVA (QSAR)

Resum

El nombre de combinacions entre cations i anions que poden formar líquids iònics (ILs) és pràcticament infinita i els ILs poden ser dissenyats a mida per a una aplicació concreta desitjada. Sens dubte, en l'actualitat es té molt poca informació sobre el possible impacte ambiental d'aquests compostos. És evident que no és factible analitzar la toxicitat d'un nombre tan elevat de compostos. L'aplicació de mètodes *in silico,* com el QSAR, podria accelerar el procés d'avaluació toxicològica i estimar la toxicitat potencial dels ILs abans de ser comercialitzats. L'elecció d'un model QSAR apropiat pot permetre la predicció fiable de la toxicitat, evitant experiments innecessaris amb animals. Aquest fet ha estat recolzat per l'Agència Europea de Productes Químics (ECHA) i pel CE Reglament REACH. En aquest estudi, es va utilitzar un model de contribució de grup QSAR per tal de predir l' (eco)toxicitat de líquids iònics pròtics i apròtics (PIL i AILs). Alguns compostos d'una nova família dels PILs alifàtics de cadena curta i AILs van ser prèviament estudiats per avaluar la seva (eco)toxicitat, tant a l'aigua com en sòls. Es van comparar els valors d'CE_{50} determinats experimentalment i mitjançant el model QSAR de cinc bioassaigs (Microtox®, *Pseudokirchneriella subcapitata*, *Lemna minor*, inhibició de l'acetilcolinesterasa i citotoxicitat en cèl.lules IPC-81) i es varen obtenir coeficients de correlació elevats. A més, es va fer la predicció de les CE_{50} per a aquest conjunt de bioassajos de vuit representants de la nova família dels PILs, els quals no se'ls havia estudiat la toxicitat en l'actualitat. El model QSAR aplicat en aquest estudi pot permetre la selecció d'ILs potencialment menys tòxics entre els ja existents (com en el cas dels líquids iònics apròtics), però també pot ser molt útil per al desenvolupament de nous líquids iònics "més verds" respectuosos amb el medi ambient (com per exemple, els ILs pròtics alifàtics de cadena curta).

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Quantitative structure-activity relationship (QSAR) prediction of (eco)toxicity of the short aliphatic protic ionic liquids

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Highlights

• The EC_{50} for different protic and aprotic ILs in 5 (eco)toxicity tests were compiled.
• Group contribution QSAR model was used to predict EC_{50} values for ionic liquids (ILs).
• The experimental and predicted EC_{50} values are well correlated.
• The EC_{50} values for eight previously untested protic ILs were predicted.

Abstract

Ionic liquids (ILs) are considered as a group of very promising compounds due to their excellent properties (practical non-volatility, high thermal stability and very good and diverse solving capacity). The ILs have a good prospect of replacing the traditional organic solvents in vast variety of applications. However, the complete information on their environmental impact is still not available. There is also an enormous number of possible combinations of anions and cations which can form ILs, the fact that requires a method allowing to predict the toxicity of existing and potential ILs. In this study, a group contribution QSAR model was used in order to predict the (eco)toxicity of protic and aprotic ILs. The representatives of a new family of short aliphatic protic ILs have been previously investigated on their (eco)toxicity, both in water and soil, as well as some selected aprotic ILs. A QSAR prediction of the EC_{50} was performed and compared with the experimentally determined EC_{50} in five (eco)toxicity tests (Microtox®, *Pseudokirchneriella subcapitata* and *Lemna minor* growth inhibition test, and Acetylcholinestherase inhibition and Cell viability assay with IPC-81 cells), obtaining high correlation coefficients. Additionally, a prediction of EC_{50} for these (eco)toxicity tests was made for the eight representatives of the new family of protic ILs, whose toxicity has not been determined to date. The QSAR model applied in this study can allow the selection of potentially less toxic ILs amongst the existing ones (as in the case of aprotic ILs), but it can also be very helpful in directing the synthesis efforts toward developing new "greener" ILs respectful with the environment (e.g. short aliphatic protic ILs).

Keywords: Ionic liquids; QSAR modelling; Group contribution; Aquatic ecotoxicity; Cytotoxicity; Acetylcholinesterase inhibition

1. Introduction

Ionic liquids (ILs) have gained a lot of attention in the past decades because of their properties, such as: negligible vapor pressure, high thermal stability and very high and versatile solving capacity. These properties make them interesting for many applications, usually aiming to replace volatile organic and toxic solvents, which have been traditionally used in laboratories and industries up to now (Petkovic et al., 2011). Even though ILs are practically non-volatile and thus cannot get into atmosphere and cause air pollution, many of them are soluble in water and can represent a danger for the environment if released with industrial and laboratory effluents. There are many studies that demonstrate the aquatic toxicity of the ILs (Pham et al., 2010). The main focus has been on so called aprotic ionic liquids (AILs), namely the ones derived from imidazolium and pyridinium. The protic ionic liquids (PILs) have come into the focus of the investigators only recently and they show a good potential, both in their applications and "greenness". It is considered that the possible number of ILs is about 10^{18}, and around one million can be easily synthesized in the laboratory. Although dramatic reductions in air pollution would result from the use of these new compounds, water pollution may increase because many ILs are water soluble and would inevitably be released into wastewater, groundwater and aquatic environments (Docherty et al., 2007). The ILs are chemical products, and that means that the requirements of a new European Union regulation on chemical substances REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) apply to ILs as well (EU, 2006).

It is very important to discover the hazardous potential of the ILs before putting them into use, and taking into account such a large potential number of ILs, it is important to save up time and money and possibly do a pre-application screening in order to determine the potential negative effect of the ILs on the environment. As the in depth studies of water and terrestrial ecotoxicity of ILs are either incomplete or missing, the prediction of the environmental impact of the new and existing, but not yet tested ILs is a necessity for the widespread application of the ILs. *In silico* methods such as QSAR (Quantitative Structure-Activity Relationship) can be used for the prediction of toxicity and thus reduce the costly and time consuming toxicity testing, either *in vitro* or *in vivo*. The use of the QSAR modelling for the prediction of toxicity is recognized by REACH regulation, stating that properties determined with the use of *in silico* methods are equivalent to laboratory testing. The QSAR modelling, as its name indicates, provides toxicity data based on the quantitative relationship between a chemical structure and its biological and/or toxicological activity, using the chemical descriptors generated from the molecular structure. These descriptors are statistically analyzed in order to develop a model that would describe the desired activity e.g. toxicity (Amberg, 2013). The QSAR

studies on toxicity of ILs have been far less frequent compared to other chemicals (Das and Roy, 2013).

A new family of short aliphatic PILs has been thoroughly investigated on their (eco)toxicity and biodegradability, both in water and soil, together with comparative studies regarding the most frequently used AILs (Peric et al., 2013, Peric et al., 2014). The aim of this study was to apply the QSAR model described by Luis et al. (2007) on the EC_{50} values of ILs obtained in different studies and thus be able to predict the EC_{50} of the PILs that have not been tested yet. A prediction of EC_{50} was done for five (eco)toxicity tests (Microtox® test with bacteria *Vibrio fischeri*, green algae *Pseudokirchneriella subcapitata* and aquatic plant *Lemna minor* growth inhibition test, Acetylcholinestherase inhibition and Cell viability assay with IPC-81 cells).

2. Material and methods

In order to perform the QSAR modelling, a database of the EC_{50} values of ILs for different (eco)toxicity tests was built. The experimentally obtained EC_{50} values for thirteen ILs, of which ten are PILs and three are AILs, are taken from Peric et al. (2013 and 2014). The remaining forty-two EC_{50} values were taken from the literature (Table 1). The structures of PILs and AILs included in this study are shown in Fig. 1 and Fig. 2 respectively. The Fig. 3 shows cations and anions of different ILs whose EC_{50} values were taken from the literature and were also used in the process of QSAR modelling. These ILs are derived from disubstituted imidazolium, pyridinium and pyrrolidinium and were selected in order to have a larger database of EC_{50} values. The PILs belong to a family of short aliphatic ionic liquids: 2-hydroxyethanolamine formate (2-HEAF), 2-hydroxyethanolamine butanoate (2-HEAB), 2-hydroxydiethanolamine formate (2-HDEAF), 2-hydroxydiethanolamine acetate (2-HDEAA), 2-hydroxydiethanolamine propionate (2-HDEAPr), 2-hydroxydiethanolamine butanoate (2-HDEAB), 2-hydroxydiethanolamine isobutanoate (2-HDEAiB), 2-hydroxydiethanolamine pentanoate (2-HDEAPe), 2-hydroxytriethanolamine butanoate (2-HTEAB) and 2-hydroxytriethanolamine pentanoate (2-HTEAPe). The experimentally analyzed AILs are: 1-butyl-3-methylimidazolium chloride ($[C_4MIM][Cl]$), 1-methyl-3-octylimidazolium chloride ($[C_8MIM][Cl]$) and N-butylpyridinium chloride ($[C_4Py][Cl]$). Using the QSAR model, a prediction of EC_{50} values for the remaining eight ILs from the new family of PILs was made: 2-hydroxyethanolamine acetate (2-HEAA), 2-hydroxyethanolamine propionate (2-HEAPr), 2-hydroxyethanolamine isobutanoate (2-HEAiB), 2-hydroxyethanolamine pentanoate (2-HEAPe), 2-hydroxytriethanolamine formate (2-HTEAF), 2-hydroxytriethanolamine acetate (2-HTEAA) and 2-hydroxytriethanolamine propionate (2-HTEAPr), 2-hydroxytriethanolamine isobutanoate (2-HTEAiB).

Fig. 1.

Fig. 2.

Fig. 3.

3. Results and discussion

The QSAR model was based on the group contribution method, proposed by Luis et al. (2007) where molecular properties can be viewed as a sum of the contributions of its atoms and/or fragments. Every ILs has been divided into basic fragments/groups, which can contribute to toxicity. The groups were encoded in a Boolean manner (0/1), meaning that they are equal to 1 if the group is present in the ILs molecule and 0 if not. A detailed overview of all of the molecular descriptors can be found in the Table 1 and Table 2. Toxicity data has been expressed on molar basis in order to be comparable between structurally different ILs. The EC_{50} values were converted into a logarithmic scale and the toxicity was expressed as the calculated dimensionless ecotoxicity (Y*), with values between 0 and 1 according to the following formula:

$$Y^* = \frac{\log EC_{50\_max} - \log EC_{50}}{\log EC_{50\ max} - \log EC_{50\ min}}$$

where log EC_{50}_max and log EC_{50}_min are the highest and the lowest EC_{50} value in the EC_{50} database, respectively. The log EC_{50} is the logarithm of the experimentally obtained EC_{50} value. The predicted dimensionless ecotoxicity used in this model represents the sum of the contributions of all of the groups present in the ILs molecule, as presented in the following equation:

$$Y^* = \sum_i a_i \cdot A_i + \sum_j c_j \cdot C_j + \sum_k s_k \cdot S_k$$

The structure of the ILs is described by three main groups: Anions (A), Cations (C) and Substitutions (S) of the cation. The A_i and C_j are variables that have value of 1 o 0, depending if the anion or cation are present or not in the molecule. For example, the value will be 1 if pyridinium is present in the ILs. On the other hand, the S_k has the value of the number of C atoms in the alkyl chains (R_1 and R_2). The a_i, c_j and s_k are the coefficients of regression representing the contribution of each group to ILs toxicity. Table 1 shows the group descriptors used in the QSAR modelling, the experimentally obtained value for the log EC_{50} in μmol L^{-1} for the ILs in the inhibition of the *Vibrio fischeri* luminescence assay (Microtox®) and the calculated dimensionless toxicity (Y*). The structure of the ILs has been described based on the main contributions of anions, cations and the side alkyl chains. Anions include the ones who appear in the AILs molecule: chloride (Cl^-), bromide (Br^-), tetrafluoroborate (BF_4^-), hexafluorophosphate

(PF_6^-), dicyanamide $N(CN_2)_2^-$, bis-trifluoromethan- sulfonimide $N(CF_3SO_2)_2^-$; and anions belonging to the PILs molecule: formate, acetate, propionate, butanoate, isobutanoate and pentanoate. The anions were grouped into four main descriptors, based on the similar contribution: A_1 (Cl^- and Br^-), A_2 (BF_4^- and PF_6^-), A_3 ($N(CN_2)_2^-$ and $N(CF_3SO_2)_2^-$) and A_4 (number of C atoms in the PILs anions). The AILs cations are imidazolium, pyridinium, and pyrrolidinium (Im, Py, and Pyr, respectively) and the PILs cations are monoethanolamine, diethanolamine or triethanolamine (Amine). Alkyl side chain substituents of the heterocyclic ring, from propyl to decyl (R_1) and the methyl substituent (R_2) were also considered.

Table 1

The data set of 55 EC_{50} values was fitted to the QSAR model by multiple linear regression using IBM SPSS Statistics 20 software. The calculated dimensionless toxicity obtained from experimental EC_{50} data and the predicted dimensionless ecotoxicity obtained after QSAR modelling were plotted and the data plot graph is shown in the Fig. 4.

Fig. 4.

A good fitting was achieved, with n=55, R^2=0.9184 and 10 descriptors. The distribution of residuals is presented in the Table 3. Residuals were calculated as the differences between the calculated dimensionless ecotoxicity and the one predicted by the QSAR method (taking into account the absolute values).

The distribution of the residuals and the Figure 4 indicate that there are practically no outliners. The Table 3 shows that 84% of the residuals are lower than 0.10. Both the most and the least toxic ILs were well predicted by the model, with the residuals being lower than 0.10. This means that there is a deviation of ±10% in the log EC_{50} ($\mu mol\ L^{-1}$) calculated applying the equation:

$$\log EC_{50}\ (\mu mol\ L^{-1}) = 4.55 - (4.55+0.18) \cdot Y^*$$

This equation was also used to predict the EC_{50} values for the eight PILs whose toxicity has not yet been tested.

All of the molecular descriptors are presented and explained in the Table 2, together with the corresponding group contributions and confidence intervals for the QSAR model. The anions were grouped in four groups in order to avoid the overfitting that may be caused by an excess of descriptors. The analysis of the contributions of each of the descriptors (Table 1) provides information on the influence of the structural groups on the ILs toxicity. The anions of the AILs have a negative contribution to toxicity. The contributions of anions show very small differences between three groups of AILs anions. The contribution of the cations is positive, which means that the presence of

either of these cations in the AILs molecules leads to an increase in toxicity. Many studies confirmed that the influence of cations on ILs toxicity is stronger than the one of anions (Ranke et al., 2004; Matzke et al., 2007; Stolte et al., 2007). Imidazolium, pyridinium and pyrrolidinium cation contribute around 27%, 17% and 3% to the toxicity, respectively. The imidazolium's higher contribution to toxicity and lower toxicity of pyrrolidinium cation compared to imidazolium and piridinium has been also found by other authors (Stolte et al., 2007; Pham et al., 2008, Latała et al., 2009). On the other hand, the cations and anions of the PILs do not exhibit a high overall influence on the ILs toxicity, if they are analyzed in the present set of ILs. This coincides with the fact that the PILs showed much lower toxicity than the AILs in the Microtox® test. The amines have a stronger influence on toxicity towards *Vibrio fischeri* in the Microtox® test as the contribution of the amine is five times higher than the one for the acid part of the PILs molecule. These results are in agreement with those reported by Peric et al. (2013) where a higher correlation between the complexity of the cationic moiety and toxicity was found.

The contributions for the number of carbon atoms in long alkyl side chains indicates that with every carbon added to the side chain, an increase of about 13% in toxicity of AILs is produced. The presence of the short chain also has a positive influence on toxicity. These two results are in agreement with other authors findings that the substitution of the cation ring and the increase of the side alkyl chain length leads to an increase of toxicity (Ranke et al., 2004; Wells and Coombe, 2006, Pham et al., 2010; Radošević et al., 2013).

Table 2

In summary, the here presented QSAR model has 10 descriptors and could allow the prediction of the (eco)toxicity of existing and potential ILs, resulting from the possible combinations of 11 types of anions, 6 types of cations and 8 types of different substituents of the cationic moiety.

Using this QSAR model, EC_{50} values for ILs that have not been experimentally analyzed can be predicted. This was done for the 8 representatives of the new family of short aliphatic PILs. The results can be found in the Table 5.

Having achieved a good fitting with the Microtox® test results, the same QSAR model was used for four more tests in order to predict the ILs (eco)toxicity: aquatic ecotoxicity (toxic effect on algae and aquatic plant *Lemna minor*), inhibition of acetylcholinesterase (AChE) and cytotoxicity (IPC-81 cells). The fitting plots for the calculated and predicted Y* can be seen in the Fig. 5.

Fig. 5.

In case of these four (eco)toxicity tests a good fitting was also achieved, with R^2 being 0.866 for the Test of inhibition of algal growth (n=32), 0.901 for the *Lemna minor* growth inhibition test (n=24), 0.9918 for the Acetylcholinestherase inhibition test (n=61) and 0.9799 for the Cytotoxicity test with IPC-81 cell line (n=45).

The distribution of residuals for the QSAR modelling for the four (eco)toxicity tests is presented in the Table 3.

Table 3

The group contributions and confidence intervals are shown in the Table 4. All but one (eco)toxicity test (Acetylcholinestherase inhibition) shows negative, very low or no contribution of the anions to the toxicity, meaning that the selected anions do not play a key role in AILs toxicity, which, as previously mentioned, coincides with the literature findings. The contribution of the cations is the strongest in the case of the Acetylcholinesterase inhibition test, both for AILs and PILs. There is also a positive influence of anions for this test. This can be explained with the different toxicity mode of action between the Acetylcholinesterase inhibition test and the other three tests. While in Algal and *Lemna minor* growth inhibition test and Cell viability assay with IPC-81 the toxicity is based on the membrane disruption, the Acetylcholinesterase inhibition test involves binding to the enzyme's active center. As initially found by Stock et al. (2004), acetylcholinesterase can be inhibited by ILs containing a cation with positively charged nitrogen and certain lipophilicity. The study of Arning et al. (2008) revealed that the pyridinium derived ILs show stronger inhibition than the imidazolium ones, which has also been confirmed in this study, with the contribution to toxicity of pyridinium being two times higher than the one for imidazolium. The anions do not show inhibitory effect on the enzyme activity with only exception of the fluoride and fluoride containing anions (Matzke et al., 2007), also confirmed by the results of this study. We also found a stronger correlation of PILs toxicity with cationic moiety in our previous study (Peric et al., 2013), as presented in the Table 4.

Table 4

Pyrrolidinium cation showed the lowest contribution to the ILs toxicity, as found by Stolte et al. (2007). The statistical models applied by Roy et al. (2014) also demonstrated that aromatic ILs are more toxic than non-aromatic and aliphatic ones.

Also in case of these four tests, the cations and anions of the PILs do not exhibit a high overall influence on the ILs toxicity, if they are analyzed in the present set of ILs. Also in case of these four tests, the PILs showed much lower toxicity than the AILs in all of the (eco)toxicity tests. The amines have a stronger influence on toxicity towards acetylcholinesterase, and the acids in the anionic moiety have a stronger contribution to the toxicity towards *P. subcapitata*, *L. minor* and IPC-81. These results are also in agreement with those reported by Peric et al. (2013) where good correlation between the

acid moieties and toxicity was found for these tests. It can be seen that both PILs cation and anion contribute to toxicity in the Test of *Lemna*'s growth inhibition, which coincides with our findings that *L. minor* is the most sensitive species in the case of the aquatic ecotoxicity tests (Peric et al., 2013).

Once again, the results for group contribution indicate that there is an increasing toxicity with an increase in the alkyl chain length. The influence of the long side alkyl chains is predominant, as the short alkyl chains show negative, very low o zero influence in the AILs toxicity. This influence also coincides with the strong relationship between the (eco)toxicity of ILs and its hydrophobic character described in the literature (Ranke et al., 2007, Stolte et al., 2007, Cvjetko Bubalo et el., 2014), meaning that longer the alkyl chain, the higher the hydrophobic character of the ionic liquid, the fact that facilitates the ILs molecule's penetration through cell membranes and thus potentiates their toxicity. A correlation between an increasing chain length of the side chains connected to the cationic head groups and an enhanced inhibitory potential of the ILs was found in other author's studies (Stock et al., 2004, Arning et al., 2008) and confirmed in this study.

As in the case of Microtox®, from the QSAR model, the EC_{50} values for the eight representatives of the new family of short aliphatic PILs that have not been experimentally obtained were predicted. The results for the four toxicity tests (Algal and *Lemna minor* growth inhibition test, and Acetylcholinestherase inhibition and Cell viability assay with IPC-81 cells) can be found in the Table 5. The model proposed by Luis et al. (2007), although initially designed for the AILs, seems to be adequate for the PILs too.

Table 5

4. Conclusions

The model based on group contribution used in this work allows the prediction of the EC_{50} values of aprotic and protic ILs. A good correlation between the experimental and predicted EC_{50} data was obtained and the EC_{50} values for eight new, until the date untested PILs, were predicted. This model can also help to determine the positive or negative influence of different structural groups on the (eco)toxicity and in that way "guide" the synthesis of the new ILs toward more sustainable ones. Literature findings of the predominant influence of cations on toxicity and the increase of toxicity with the elongation of the side alkyl chains in AILs molecule were confirmed by the results of this study. In the case of new PILs, both anionic and cationic part of the molecule have influence on (eco)toxicity, and it is the elongation of the acid in the anionic moiety which has more influence on the overall toxicity in three of the five (eco)toxicity tests (algae, aquatic plants and cytotoxicity). The QSAR models can estimate not only the toxicity of the existing ILs, but also offer the ability to predict the effects of new ILs

that could be synthesized in order to suite the desired application. *In silico* models are also a lot quicker than the standard laboratory toxicity tests and thus can save up time and money. This kind of QSAR model can be of useful for the designers of ILs, because it allows them to evaluate the toxicity of an ILs with the desired application potential.

Acknowledgments

B. Peric would like to thank the Spanish Ministry of Foreign Affairs and Cooperation and the Spanish Agency for International Cooperation for Development (Grant MAEC-AECID).

The authors would like to thank Francesc Fàbrega for his advice on the initial QSAR modelling and Dr. Jesús Gómez for his valuable suggestions and comments.

References:

Amberg, A., 2013. *In silico* methods, in: Vogel, H.G., Maas, J., Hock, F.J., Mayer, D. (Eds.), Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays. Springer, Berlin Heidelberg, pp. 1273–1296.

Arning, J., Stolte, S., Böschen, A., Stock, F., Pitner, W.-R., Welz-Biermann, U., Jastorff, B., Ranke, J., 2008. Qualitative and quantitative structure activity relationships for the inhibitory effects of cationic head groups, functionalized side chains and anions of ionic liquids on acetylcholinesterase. Green Chem. 10, 47–58.

Couling, D.J., Bernot, R.J., Docherty, K.M., Dixon, J.K., Maginn, E.J., 2006. Assessing the factors responsible for ionic liquid toxicity to aquatic organisms via quantitative structure-property relationship modeling. Green Chem. 8, 82–90.

Cvjetko Bubalo, M., Radošević, K. Radojčić Redovniković, I., Halambek, J., Gaurina Srček, V, 2014. A brief overview of the potential environmental hazards of ionic liquids. Ecotoxicol. Environ. Saf. 99, 1–12.

Das, R.N., Roy, K., 2013. Advances in QSPR/QSTR models of ionic liquids for the design of greener solvents of the future. Mol. Divers. 17, 151–96.

Docherty, K.M., Dixon, J.K., Kulpa, C.F., 2007. Biodegradability of imidazolium and pyridinium ionic liquids by an activated sludge microbial community. Biodegradation. 18, 481–493.

Docherty, K.M., Kulpa, C.F., 2005. Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. Green Chem. 7, 185–189.

European Union, Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). 2006. Off. J. Eur. Union. 396, 1–856.

Garcia, M.T., Gathergood, N., Scammells, P.J., 2005. Biodegradable ionic liquids. Part II: effect of the anion and toxicology. Green Chem. 7, 9–14.

Latała, A., Nędzi, M., Stepnowski, P., 2009. Toxicity of imidazolium and pyridinium based ionic liquids towards algae. *Chlorella vulgaris*, *Oocystis submarina* (green algae) and *Cyclotella meneghiniana*, *Skeletonema marinoi* (diatoms). Green Chem. 11, 580–588.

Luis, P., Ortiz, I., Aldaco, R., Irabien, A., 2007. A novel group contribution method in the development of a QSAR for predicting the toxicity (*Vibrio fischeri* EC50) of ionic liquids. Ecotoxicol. Environ. Saf. 67, 423–429.

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 1-alkyl-3-methylimidazolium ionic liquids observed in an (eco)toxicological test battery. Green Chem. 9, 1198–1207.

Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M.A., Arning J., Bottin-Weber U., Stolte S., 2013. (Eco)toxicity and biodegradability of short aliphatic protic ionic liquids. J. Hazard. Mater. 261, 99–105.

Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M.A., 2014. A comparative study of the terrestrial ecotoxicity of selected protic and aprotic ionic liquids. Chemosphere. 108, 418–425.

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

Pham, T.P.T., Cho, C.-W., Min, J., Yun, Y.-S., 2008. Alkyl-chain length effects of imidazolium and pyridinium ionic liquids on photosynthetic response of *Pseudokirchneriella subcapitata*. J. Biosci. Bioeng 105, 425–428.

Pham, T.P.T., Cho, C.W., Yun, Y.S., 2010. Environmental fate and toxicity of ionic liquids: a review. Water Res. 44, 252–272.

Radošević, K., Cvjetko, M., Kopjar, N., Novak, R., Dumić, J., Gaurina Srček, V., 2013. *In vitro* cytotoxicity assessment of imidazolium ionic liquids: biological effects in fish Channel Catfish Ovary (CCO) cell line. Ecotoxicol. Environ. Saf. 92, 112–118.

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 length in acute *Vibrio fischeri* and WST-1 cell viability assays. Ecotoxicol. Environ. Saf. 58, 396–404.

Ranke, J., Müller, A., Bottin-Weber, U., Stock, F., Stolte, S., Arning, J., Störman, R., Jastroff, B., 2007. Lipophilicity parameters for ionic liquid cations and their correlation to in vitro cytotoxicity. Ecotoxicol. Environ. Saf. 67, 430–438.

Ranke, J., Stolte, S., Störmann, R., Arning, J., Jastorff, B., 2007. Design of sustainable chemical products – the example of ionic liquids. Chem. Rev. 107, 2183–2206

Roy K., Das R.N., Popelier P.L.A, 2014. Quantitative structure–activity relationship for toxicity of ionic liquids to Daphnia magna: Aromaticity vs. lipophilicity. Chemosphere, 112, 120–127.

Stock, F., Hoffmann, J., Ranke, J., Störmann, R., Ondruschka, B., Jastorff, B., 2004. Effects of ionic liquids on the acetylcholinesterase – a structure-activity relationship consideration. Green Chem. 6, 286–290.

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

Wells, A.S., Coombe, V.T., 2006. On the freshwater ecotoxicity and biodegradation properties of some common ionic liquids. Org. Pro. Res. Dev. 10, 794–798.

Figure captions

Fig. 1. Structures and abbreviations of the studied PILs.

Fig. 2. Structures and abbreviations of the analyzed AILs.

Fig. 3. Structures of AILs used in QSAR modelling.

Fig. 4. Calculated and predicted dimensionless ecotoxicity data plot for Microtox® test.

Fig. 5. Calculated and predicted dimensionless ecotoxicity data plot for Algal and *Lemna minor* growth inhibition and Cell viability assay with IPC-81 cells.

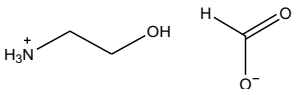
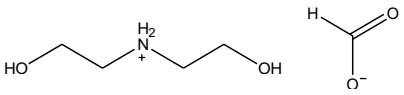
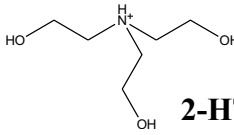
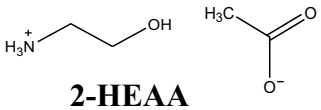
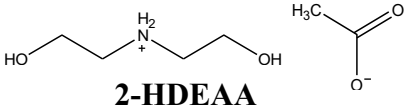
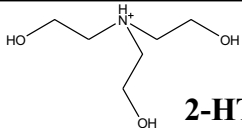
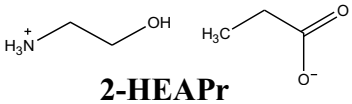
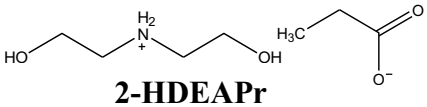
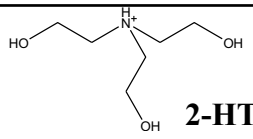
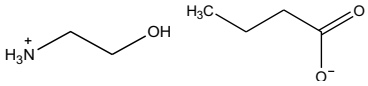
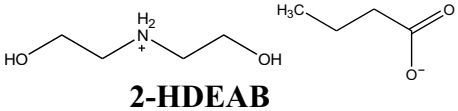
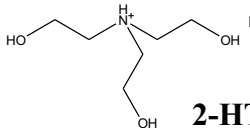
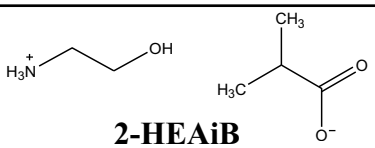
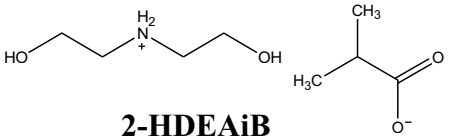
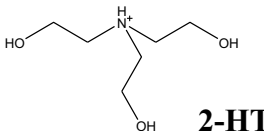
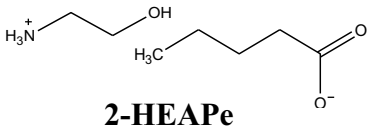
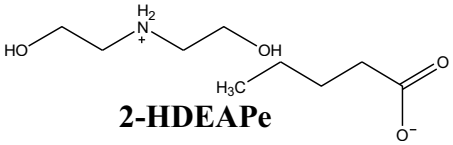
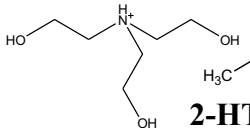
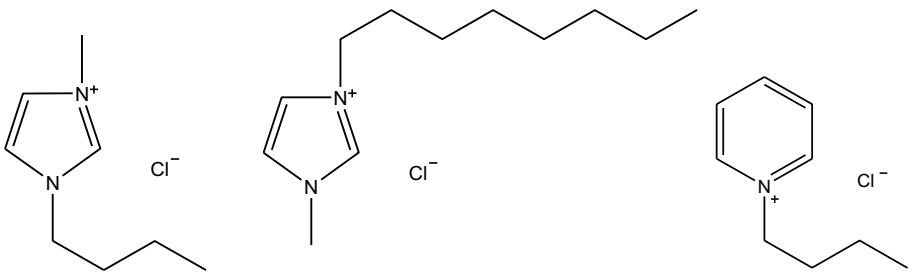
	Monoethanolamine	Diethanolamine	Triethanol
Formic acid	 **2-HEAF**	 **2-HDEAF**	 **2-H**
Acetic acid	 **2-HEAA**	 **2-HDEAA**	 **2-H**
Propionic acid	 **2-HEAPr**	 **2-HDEAPr**	 **2-HT**
Butiric acid	 **2-HEAB**	 **2-HDEAB**	 **2-H**
Isobutiric acid	 **2-HEAiB**	 **2-HDEAiB**	 **2-HT**
Pentanoic acid	 **2-HEAPe**	 **2-HDEAPe**	 **2-HT**

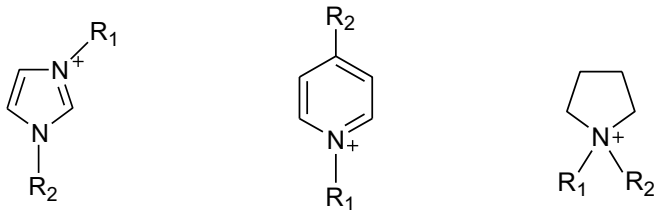
Fig. 1.



[C_4MIM][Cl] [C_8MIM][Cl] [C_4Py][Cl]

Fig. 2.

Disubstituted cations



Imidazolium (Im) Pyridinium (Py) Pyrrolidinium (Pyr)

Anions

Cl^-, Br^-, BF_4^-, PF_6^-, $(CN)_2N^-$, $(CF_3SO_2)_2N^-$

Fig. 3.

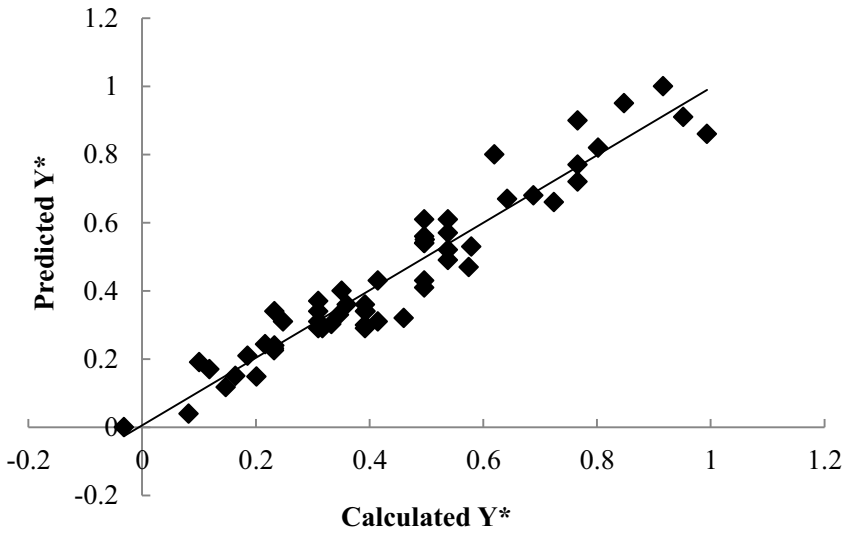


Fig. 4.

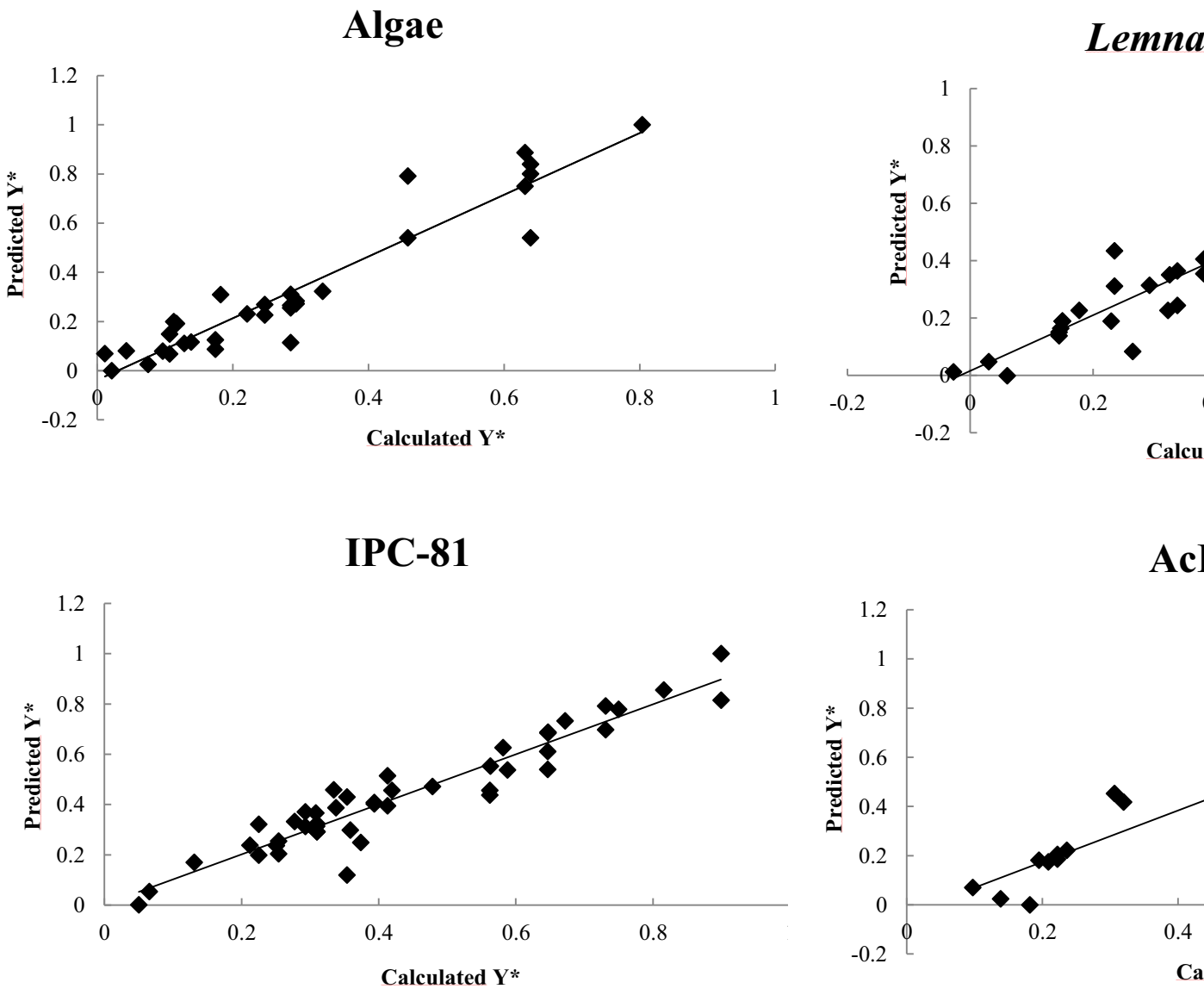

Algae
Predicted Y*
Calculated Y*
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.2
0.4
0.6
0.8
1
Lemna
Predicted Y*
Calcu
-0.2
0
0.2
IPC-81
Predicted Y*
Calculated Y*
Acl
Predicted Y*
Ca

Fig. 5.

Table 1
Ionic liquid toxicities in μmol L^{-1} ($logEC_{50}$) for the Microtox® test, calculated dimensionless ecotoxicity (Y*)

No.	Compound	log EC_{50} (μmol L^{-1})	Y*	A_1	A_2	A_3	A_4	Im	Py
1	$[C_2MIM][Cl]$	4.55	0.00	1	0	0	0	1	0
2	$[C_2MIM][Cl]$	4.33	0.05	1	0	0	0	1	0
3	$[C_3MIM][BF_4]$	3.94	0.13	0	1	0	0	1	0
4	$[C_3MIM][PF_6]$	3.60	0.20	0	1	0	0	1	0
5	$[C_4MIM][Cl]$	3.71	0.18	1	0	0	0	1	0
6	$[C_4MIM][Cl]$	2.95	0.34	1	0	0	0	1	0
7	$[C_4MIM][Cl]$	3.34	0.26	1	0	0	0	1	0
8	$[C_4MIM][Cl]$	3.21	0.28	1	0	0	0	1	0
9	$[C_4MIM][Br]$	3.35	0.25	1	0	0	0	1	0
10	$[C_4MIM][Br]$	3.27	0.27	1	0	0	0	1	0
11	$[C_4MIM][Br]$	3.07	0.31	1	0	0	0	1	0
12	$[C_4MIM][BF_4]$	3.55	0.21	0	1	0	0	1	0
13	$[C_4MIM][BF_4]$	3.10	0.31	0	1	0	0	1	0
14	$[C_4MIM][(CF_3SO_2)_2N]$	3.39	0.25	0	0	1	0	1	0
15	$[C_4MIM][(CN)_2N]$	3.67	0.19	0	0	1	0	1	0
16	$[C_5MIM][BF_4]$	3.18	0.29	0	1	0	0	1	0
17	$[C_6MIM][Cl]$	1.94	0.55	1	0	0	0	1	0
18	$[C_6MIM][Cl]$	2.32	0.47	1	0	0	0	1	0
19	$[C_6MIM][Cl]$	2.18	0.50	1	0	0	0	1	0
20	$[C_6MIM][Cl]$	2.91	0.34	1	0	0	0	1	0
21	$[C_6MIM][Br]$	1.42	0.66	1	0	0	0	1	0
22	$[C_6MIM][BF_4]$	3.15	0.30	0	1	0	0	1	0

23	$[C_6MIM][PF_6]$	2.17	0.50	0	1	0	0	1	0
24	$[C_6MIM][PF_6]$	3.07	0.31	0	1	0	0	1	0
25	$[C_8MIM][Cl]$	1.19	0.71	1	0	0	0	1	0
26	$[C_8MIM][Cl]$	1.01	0.75	1	0	0	0	1	0
27	$[C_8MIM][Cl]$	0.30	0.90	1	0	0	0	1	0
28	$[C_8MIM][BF_4]$	1.41	0.66	0	1	0	0	1	0
29	$[C_8MIM][PF_6]$	0.95	0.76	0	1	0	0	1	0
30	$[C_{10}MIM][Cl]$	0.5	0.86	1	0	0	0	1	0
31	$[C_{10}MIM][BF_4]$	-0.18	1.00	0	1	0	0	1	0
32	$[C_4Py][Cl]$	3.41	0.24	1	0	0	0	0	1
33	$[C_4Py][Cl]$	2.64	0.40	1	0	0	0	0	1
34	$[C_4Py][Cl]$	3.24	0.28	1	0	0	0	0	1
35	$[C_4Py][Br]$	3.40	0.24	1	0	0	0	0	1
36	$[C_4Py][Br]$	2.73	0.38	1	0	0	0	0	1
37	$[C_4Py][(CN)_2N]$	2.61	0.41	0	0	1	0	0	1
38	$[C_4MPy][Br]$	2.12	0.51	1	0	0	0	0	1
39	$[C_4MPy][Br]$	2.75	0.38	1	0	0	0	0	1
40	$[C_4MPy][(CN)_2N]$	1.99	0.54	0	0	1	0	0	1
41	$[C_6MPy][Cl]$	1.44	0.66	1	0	0	0	0	1
42	$[C_6MPy][Br]$	1.48	0.65	1	0	0	0	0	1
43	$[C_4MPyr][(CF_3SO_2)_2N]$	2.54	0.42	0	0	1	0	0	0
44	$[C_6MPyr][Cl]$	2.99	0.33	1	0	0	0	0	0
45	$[C_8MPyr][Br]$	0.25	0.91	1	0	0	0	0	0
46	2-HEAF	3.82	0.16	0	0	0	1	0	0
47	2-HEAB	4.18	0.08	0	0	0	4	0	0
48	2-HDEAF	3.72	0.17	0	0	0	1	0	0
49	2-HDEAA	4.03	0.11	0	0	0	2	0	0
50	2-HDEAPr	3.56	0.21	0	0	0	3	0	0

51	2-HDEAB	3.62	0.20	0	0	0	4	0	0
52	2-HDEAiB	3.64	0.19	0	0	0	4	0	0
53	2-HDEAPe	3.23	0.28	0	0	0	5	0	0
54	2-HTEAB	3.32	0.26	0	0	0	4	0	0
55	2-HTEAPe	3.26	0.27	0	0	0	5	0	0

References: 5, 15, 16, 22, 32, 35, 37 and 39 from Couling et al., (2006); 6, 9, 21, 33, 36, 38, 40 and 45 from D
13, 18, 25 and 29 from Garcia et al. (2005); 1, 17, 41, 42 and 44 from Luis et al. (2007); 2, 14, 26 and 43 fron
19, 20, 23, 24, 28, 30 and 31 from Ranke et al., (2007); 8, 27, 34 and 46-55 from Peric et al., 2013.

Table 2
Significance and contribution of the descriptors used in the QSAR modelling.

Group	Molecular descriptor	Comments	Contribution
Anion	A_1	Influence of anions: chloride (Cl^-), bromide (Br^-). Value is 1 if it exists and 0 if not.	-0.269
	A_2	Influence of anions: hexafluorophosphate (PF_6^-), tetrafluoroborate ($BF4^-$). Value is 1 if it exists and 0 if not.	-0.300
	A_3	Influence of anions: dicyanamide $N(CN_2)_2^-$, bis-trifluoromethan- sulfonimide $N(CF_3SO_2)_2^-$. Value is 1 if it exists and 0 if not.	-0.251
	A_4	Influence of number of carbons in PILs anion. Value of A_4: 1 to 5	0.016
Cation	Im - Imidazolium	Influence of imidazolium cation. Value = 1 if it exists and 0 if not.	0.274
	Py - Pyridinium	Influence of pyridinium cation. Value = 1 if it exists and 0 if not.	0.175

	Pyr - Pyrrolidinium	Influence of pyrrolidinium cation. Value = 1 if it exists and 0 if not.	0.035
	Amine	Influence of amine. Value = 1, 2 or 3 in function of the complexity of the PILs cation	0.085
Cation subsitution	R_1	Influence of number of carbons in long chains of the AILs molecule. Value of R_1: 0 to 10	0.132
	R_2	Influence of number of carbons in short chains of the AILs molecule. Value of R_1: 1 if methyl group exists and 0 if not.	0.112

Table 3

Distribution of residuals between the calculated and predicted dimensionless (eco)toxicity for Microtox® t
inhibition test, Acetylcholinesterase inhibition and Cell viability assay with IPC-81 cells.

	Microtox		Algae		*Lemna minor*		AChE		IPC-81	
Range	Residuals	%	Residuals	%	Residuals	%	Residuals	%	Residuals	%
<0,10	46	84	23	72	20	83	53	87	41	91
0,10-0,20	8	14	4	12	4	17	8	13	3	7
>0,20	1	2	5	16	0	0	0	0	1	2
<0,28	55	100	32	100	24	100	61	100	45	100

Table 4
The group contributions to calculated dimensionless ecotoxicity.

		Algae			*Lemna minor*			AChE		
Group	Molecular descriptor	Contribution	Confidence interval (95%)		Contribution	Confidence interval (95%)		Contribution	Confidence inte (95%)	
Anion	A_1	-0.098	-0.225	0.029	-0.244	-0.357	-0.131	0.023	-0.031	0.0
	A_2	-0.091	-0.233	0.041	-0.231	-0.349	-0.113	0.019	-0.037	0.0
	A_3	-0.051	-0.182	0.080	-0.419	-0.541	-0.297	0.000	0.000	0.0
	A_4	0.032	-0.048	0.111	0.087	0.003	0.174	0.014	-0.020	0.0
Cation	Im	0.075	-0.064	0.214	0.005	-0.023	0.033	0.361	0.312	0.4
	Py	0.000	0.000	0.000	0.048	-0.061	0.157	0.699	0.629	0.7
	Pyr	0.000	0.000	0.000	0.085	0.035	0.140	0.000	0.000	0.0
	Amine	-0.010	-0.146	0.126	-0.057	-0.206	0.092	0.084	0.027	0.1
Substitution	R_1	0.086	0.048	0.124	0.141	0.057	0.224	0.018	0.010	0.0
	R_2	-0.036	-0.187	0.114	0.000	0.000	0.000	0.000	0.000	0.0

Table 5

The predicted values of log EC_{50} (μmol L^{-1}) for Microtox® test, Algal and *Lemna minor* growth inhibition t and Cell viability assay with IPC-81 cells for eight representatives of the new family of PILs.

	Microtox	Algae	*L. minor*	AChE	IPC-81
2-HEAA	4.19	3.94	3.22	4.36	4.12
2-HEAPr	4.11	3.68	2.94	4.31	3.81
2-HEAiB	4.03	3.43	2.67	4.26	3.49
2-HEAPe	3.96	3.18	2.40	4.21	3.17
2-HTEAF	4.26	4.19	3.49	4.41	4.44
2-HTEAA	3.35	4.10	3.57	3.74	4.25
2-HTEAPr	3.27	3.85	3.30	3.69	3.93
2-HTEAiB	3.19	3.59	3.03	3.64	3.61

DISCUSSIÓ

4. DISCUSSIÓ

En aquesta tesi es va determinar la toxicitat aquàtica, terrestre i la toxicitat cel·lular, juntament amb el potencial de biodegradabilitat en aigua i en sòl de determinats PILs i AILs. Per a tots els ILs estudiats es van obtenir corbes dosi-resposta i es van calcular els valors de CE_{50}. La classificació del perill potencial dels ILs es va realitzar d'acord amb el Globally Harmonized System of Classification and Labelling of Chemicals (GHS, United Nations, 2006 i 2013), tant per al medi aquàtic com terrestre. Els criteris de classificació de substàncies perilloses d'acord amb el GHS, tant per al medi aquàtic com per al terrestre, es poden trobar a la Taula 4. Per als assaigs d'inhibició de l'acetilcolinesterasa i la citotoxicitat IPC-81 , els líquids iònics assajats es classifiquen d'acord amb la base de dades de UFT/Merck Ionic Liquids Biological Database (base de dades UFT/Merck). Els criteris de classificació es presenten a la Taula 5.

Taula 4. Criteris de classificació de substàncies perilloses per al medi ambient aquàtic i terrestre d'acord amb el Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Categoria de perill (GHS 2013)	**CE_{50} (mg L^{-1})**	**Comunicació del perill**
Medi aquàtic		
Aguda 1	≤ 1	Molt tòxic per a organismes aquàtics
Aguda 2	>1 però ≤ 10	Tòxic
Aguda 3	>10 però ≤ 100	Nociu
	> 100	No tòxic
Categoria de perill (GHS 2006)	**CE_{50} (mg kg^{-1})**	**Comunicació del perill**
Medi terrestre		
Compostos perillosos per als microorganismes del sòl		
Crònica 1	≤ 1	Molt tòxic per a organismes terrestres
Crònica 2	>1 però ≤ 10	Tòxic
Crònica 3	>10 però ≤ 100	Nociu
	> 100	No tòxic
Compostos perillosos per als microorganismes del sòl: invertebrats i plantes (exposició mitjançant sòl)		
Aguda 1	≤ 10	Molt tòxic per a organismes terrestres
Aguda 2	>10 però ≤ 100	Tòxic
Aguda 3	>100 però ≤ 1000	Nociu
	> 1000	No tòxic

Taula 5. Classificació de la toxicitat en l'assaig d'inhibició d'acetilcolinesterasa i l'assaig de toxicitat per a la línia cel·lular IPC-81 d'acord amb la base de dades de UFT/Merck Ionic Liquids Biological Database.

CE_{50} (mmol L^{-1})	
Inhibició d'acetilcolinesterasa	
< 0,01	potencial inhibitori molt alt
0,01 - 0,1	potencial inhibitori alt
0,1 - 1	potencial inhibitori moderat
> 1	potencial inhibitori baix
Citotoxicitat per a IPC-81	
< 0,001	citotoxicitat molt alta
0,001 - 0,1	citotoxicitat alta
0,1 - 5	citotoxicitat moderada
> 5	citotoxicitat baixa

En la taula 6 es presenta una visió general dels efectes dels líquids iònics analitzats en diferents assaigs d'(eco) toxicitat i la seva biodegradabilitat.

Taula 6. Efectes dels líquids iònics analitzats en diferents assaigs d'(eco)toxicitat i biodegradabilitat.

	GHS (2013) medi aquàtic			Directriu OECD 301	GHS (2006) medi terrestre					UFT Merck ILs base de dades biològiques	
	V.fischeri	*P. subcapitata*	*L. minor*	Biodegradabilitat en aigua	*A. cepa*	*L. perenne*	*R. sativus*	Microorganismes del sòl	Corbes de respiració del sòl	AChE	IPC-81
2-HEAF	no tòxic	no tòxic	no tòxic	fàcilment biodegradable	no tòxic	no tòxic	no tòxic	no tòxic	Tipus1	sense inhibició	citotoxicitat baixa
2-HEAB	no tòxic	no tòxic	Aguda 3	fàcilment biodegradable	no tòxic	Aguda 3	no tòxic	no tòxic	Tipus 2	sense inhibició	citotoxicitat moderada
2-HDEAF	no tòxic	no tòxic	no tòxic	fàcilment biodegradable	no tòxic	no tòxic	no tòxic	no tòxic	Tipus1	sense inhibició	no citotoxic
2-HDEAA	no tòxic	no tòxic	no tòxic	fàcilment biodegradable	no tòxic	Aguda 3	no tòxic	no tòxic	Tipus1	sense inhibició	citotoxicitat baixa
2-HDEAPr	no tòxic	no tòxic	no tòxic	fàcilment biodegradable	no tòxic	no tòxic	no tòxic	no tòxic	Tipus 3	sense inhibició	citotoxicitat baixa
2-HDEAB	no tòxic	no tòxic	Aguda 3	fàcilment biodegradable	no tòxic	no tòxic	no tòxic	no tòxic	Tipus 3	sense inhibició	citotoxicitat moderada
2-HDEAiB	no tòxic	no tòxic	Aguda 3	fàcilment biodegradable	no tòxic	no tòxic	no tòxic	no tòxic	Tipus 2	sense inhibició	citotoxicitat moderada
2-HDEAPe	no tòxic	no tòxic	no tòxic	fàcilment biodegradable	Aguda 3	Aguda 3	Aguda 3	no tòxic	Tipus 3	potencial inhibitori baix	citotoxicitat baixa
2-HTEAB	no tòxic	no tòxic	no tòxic	fàcilment biodegradable	no tòxic	no tòxic	no tòxic	no tòxic	Tipus 2	potencial inhibitori baix	citotoxicitat moderada
2-HTEAPe	no tòxic	no tòxic	no tòxic	fàcilment biodegradable	no tòxic	no tòxic	Aguda 3	no tòxic	Tipus 3	potencial inhibitori baix	citotoxicitat moderada
[BMIM]Cl	no tòxic	Aguda 3	Aguda 3	no biodegradable	Aguda 3	no tòxic	no tòxic	no tòxic	Tipus 4	potencial inhibitori alt	citotoxicitat moderada
[OMIM]Cl	Aguda 1	Aguda 2	Aguda 2	no biodegradable	Aguda 3	Aguda 3	Aguda 3	no tòxic	Tipus 4	potencial inhibitori alt	citotoxicitat alta
[BPy]Cl	no tòxic	Aguda 3	Aguda 3	no biodegradable	Aguda 3	no tòxic	no tòxic	no tòxic	Tipus 4	potencial inhibitori alt	citotoxicitat moderada

D'acord amb el GHS (United Nations, 2013), la majoria dels PILs analitzats es poden classificar com a no tòxics per als **organismes aquàtics**, a causa de que els seus valors de CE_{50} són superiors a 100 mg L^{-1}, mentre que tots els AILs presenten un determinat grau de toxicitat aguda d'acord amb els valors de CE_{50} per a dos dels tres assaigs realitzats. En base als resultats de l'assaig de Microtox®, l'[OMIM]Cl pot ser classificat com a molt tòxic per als organismes aquàtics, i pertany a la categoria Aguda 1, mentre que els altres dos líquids iònics tenen valors de CE_{50} superiors a 100 mg L^{-1}. Tots els PILs analitzats han resultat ser no tòxics per *Vibrio fischeri* i *Pseudokirchneriella subcapitata*. En la prova d'inhibició del creixement de *P. subcapitata*, l'[OMIM]Cl pot ser classificat com a tòxic (categoria Aguda 2) i [BMIM]Cl i [BPy]Cl com a nociu per als organismes aquàtics (categoria Aguda 3). La planta aquàtica *Lemna minor* va resultar ser més sensible a la presència dels

PILs que l'alga verda *P. subcapitata* o els bacteri marí *V. fischeri*. El 2-HEAB, 2-HDEAB i 2-HDEAiB pertanyen a la categoria de nocius per al medi ambient aquàtic (categoria Aguda 3) en l'assaig amb *L. minor*. Aquest fet indica una major sensibilitat de *L. minor* envers els PILs amb àcids amb cadena alquílica més llarga com a part aniònica, especialment el butíric. Aquest fet va ser confirmat mitjançant l'anàlisi de correlació (vegeu capítol 3.2.). Tots els AILs mostren efectes tòxics cap a *L. minor*. L'[OMIM]Cl pot ser classificat com a tòxic (categoria Aguda 2), i els altres dos AILs analitzats com a nocius per al medi ambient aquàtic (categoria Aguda 3). Si el contaminant és més lipòfil, l'efecte tòxic per algues i bacteris és més fort, ja que la seva membrana cel·lular lipídica és el punt d'entrada per a les substàncies tòxiques. La paret cel·lular juga un paper crític en el transport de materials dins i fora de les cèl·lules d'algues, incloent substàncies tòxiques (Seine et al., 2010). Els estudis publicats sobre el mecanisme d'acció tòxica dels ILs generalment consideren que aquests s'acumulen a la membrana, hi produeixen alteracions, amb l'acumulació posterior dins de les cèl·lules (Ranke et al., 2006; Luczak et al., 2010). L'increment de la lipofília amb l'augment de la longitud dels substituents alquílics facilita l'augment de les interaccions de membrana (Megaw et al., 2013). Stolte et al. (2007b) atribueixen els efectes tòxics aguts dels líquids iònics a les cadenes laterals o a la lipofília del catió del líquid iònic, originant una interacció en la membrana semblant a la narcosi polar. Hi ha una similitud entre l'estructura química i el mecanisme d'acció d'alguns tensioactius i els líquids iònics. Com els surfactants, els líquids iònics podrien augmentar la permeabilitat de la membrana produint una narcosi cel·lular (Sena et al., 2010). Per tant, com els AILs tenen una major lipofilicitat que els PILs, el seu efecte més tòxic per a algues i bacteris no és d'estranyar. No obstant això, el cas de *L. minor* és diferent, ja que pren nutrients de l'aigua principalment a través de les frondes, el que significa que els compostos més hidròfils seran més disponibles per *L. minor*. A més, la durada de l'assaig en el cas de *L. minor* és superior que en el cas d'algues i bacteris, fet que també pot afavorir que els ILs manifestin una major toxicitat. Aquests resultats coincideixen amb els publicats per altres autors (Cedergreen i Madsen, 2003; Mohammad et al., 2005; Stolte et al., 2007a). L'efecte més fort de l'anió dels PILs sobre la toxicitat per a *L. minor* i un augment de la toxicitat amb l'augment del

nombre de carbonis en la part aniònica de la molècula dels PILs van ser demostrats tant per l'anàlisi de correlació com pel QSAR (vegeu els capítols 3.2 i 3.4.).

D'acord amb la directriu 301 de l'OCDE (1992) per a l'assaig de respirometria, els compostos que arriben a un nivell de **biodegradació** en aigua superior al 60% de la Demanda Teòrica d'Oxigen, dins dels 28 dies d'incubació, poden ser considerats com a "fàcilment biodegradables". Els PILs analitzats van mostrar taxes de biodegradació en aigua molt millors que els AILs. Després de 28 dies d'incubació, les taxes de biodegradació de la majoria dels PILs van ser superiors al 60%. Tres dels PILs assajats, 2-HDEAF, 2-HTEAB i 2-HTEAPe, no es poden considerar "fàcilment biodegradables" si s'aplica estrictament el criteri. Donat que els valors de 59 i 57% per al 2-HTEAB i 2-HTEAPe, respectivament, són molt a prop del límit de 60%, i per tant, poden ser considerats com a potencialment fàcilment biodegradables. El 2-HDEAF és l'únic PIL amb una taxa de biodegradació clarament per sota de 60%, de l'ordre del 13%. Els nivells de biodegradació normalment depenen de l'estabilitat i de la toxicitat del compost, com s'ha demostrat en el cas dels AILs analitzats, amb una taxa de biodegradació al voltant de l'1%, fet que indica la seva resistència a la biodegradació en l'aigua, la qual cosa serà també confirmada en l'assaig de mineralització del carboni en sòl.

La directriu 301 de l'OCDE dóna la possibilitat de realitzar proves addicionals de biodegradabilitat, per exemple, per mètodes cromatogràfics. Per aquesta raó es va realitzar periòdicament una quantificació cromatogràfica en les mostres de l'assaig de respirometria. L'objectiu va ser determinar si hi havia restes de la part catiònica després de 5 i 14 dies d'incubació i al final de la incubació, passats 28 dies. La degradació primària de 2-HEAF, 2-HEAB, 2-HDEAF i 2-HDEAPr va ser completa als 5 dies, mentre que les altres molècules de PILs (excepte la 2-HDEAPe) varen ser completament mineralitzades durant el període comprès entre el cinquè i el vint-i-vuitè dia. Després de la finalització de l'assaig de respirometria en aigua, als 28 dies, l'únic PIL que no va ser metabolitzat completament per la comunitat microbiana de l'inòcul de llots activats va ser 2-HDEAPe, quedant una concentració disponible del 38%. La resta de PILs varen ser completament degradats després de 28 dies, ja que no es va poder detectar cap resta catiònica en mostres d'aigua.

Aquesta anàlisi complementària confirma que tots els PILs són potencialment degradables en aigua, perquè fins i tot per al 2-HDEAF es va trobar un 100% de biodegradació del catió.

En un estudi realitzat per Stolte et al. (2008), ILs derivats de l'imidazoli i del piridini amb cadenes laterals curtes ([BMIM]Cl i [BPy]Cl) varen resultar ser altament resistents a la mineralització durant el període d'incubació. Es va poder detectar biodegradació primària per a l'[OMIM]Cl després de 28 dies, però només en relació amb la cadena lateral de vuit carbonis que es van sotmetre a diverses oxidacions i descarboxilacions per ser reduïda a acetat, mentre que l'anell aromàtic va quedar intacte, de manera que la biodegradació primària no va ser en realitat completa, com en el cas dels PILs.

La toxicitat terrestre i la biodegradabilitat en sòl són unes característiques molt poc estudiades quan es tracta de líquids iònics, i les dades disponibles sobre els efectes ecotoxicològics dels líquids iònics en **plantes terrestres** és escassa. Els estudis publicats fins a la data que tracten de la fitotoxicitat de ILs s'han centrat principalment en plantes aquàtiques (Zhu et al., 2009 i Pham et al., 2010). Pel que fa als PILs, només un representant va mostrar efecte tòxic per a les tres espècies analitzades, el 2-HDEAPe. El 2-HDEAPe ha resultat nociu (categoria Aguda 3) en l'assaig amb *Allium cepa*. El valor EC_{50} per a 2-HDEAA va ser de 996 mg kg^{-1}, molt a prop de 1000 mg kg^{-1}, el límit del GHS (United Nations, 2006), i que pot ser considerat com pràcticament innocu per a les plantes terrestres. *Lolium perenne* va ser l'espècie més sensible de les plantes terrestres analitzades, amb tres PILs que mostraren efectes tòxics (2-HEAB, 2-HDEAA i 2-HDEAPe) i es poden classificar com a nocius per a les plantes terrestres (categoria Aguda 3). El 2-HDEAPe i 2-HTEAPe poden ser classificats com a nocius per a les plantes terrestres (categoria Aguda 3) en l'assaig amb *Raphanus sativus*. Els PILs més tòxics eren els derivats del pentanoic, el que indica que els PILs amb cadenes alquíliques més llargues tenen major toxicitat. Aquesta fet també s'ha confirmat en el cas de l'[OMIM]Cl i el [BMIM]Cl, on l'[OMIM]Cl té una cadena lateral més llarga i ha resultat el més tòxic en tots els assaigs realitzats. Aquests resultats estan d'acord amb els observats per altres autors (Ranke et al., 2004; Matzke et al., 2008; Pham et al., 2010). Els PILs

analitzats són menys tòxics per a les plantes que els AILs estudiats. *A. cepa* va resultar ser l'espècie més sensible als efectes tòxics dels AILs, tots tres es classifiquen com a nocius per a les plantes terrestres (categoria Aguda 3). En el cas de *R. sativus* i *L. perenne*, només l'[OMIM]Cl va ser classificat com a nociu. La major toxicitat dels AILs per a plantes terrestres també es confirma per la clorosi de les fulles de *R. sativus* i *L. perenne* observada a les concentracions ≥ 100 mg kg^{-1} (vegeu el capítol 3.3.). L'efecte tòxic dels AILs observat podria ser causat per danys en les arrels o per bloqueig del transport de nutrients del sòl a les plantes.

La informació disponible a la bibliografia consultada referent a l'efecte dels ILs sobre la **microbiota del sòl i la biodegradació en el sòl** no és suficient, amb un nombre limitat d'ILs analitzats: tres derivats de l'imidazoli i un del piridini (Modelli et al., 2008 i Zhang et al., 2010). Els resultats del present estudi indiquen que no hi ha efecte tòxic dels PILs ni dels AILs analitzats sobre els microorganismes del sòl que participen en els cicles de mineralització de carboni i nitrogen. Per raó de que no es varen observar efectes tòxics (PILs) o aquests varen ser molt baixos (AILs), no es varen poder calcular els valors de CE_{50}. Aquest fet no és d'estranyar, si es té en compte que els assaigs es van dur a terme en la matriu sòl. El sòl té una gran capacitat de bloqueig i de neutralització de l'efecte tòxic de compostos químics així com de retenció de contaminants. La microbiota heteròtrofa del sòl presenta una gran diversitat de poblacions, de manera que si alguna d'elles es veu afectada per una substància química present en el sòl, les altres poden continuar amb el seu funcionament i, per tant, és molt difícil d'obtenir valors baixos de CE_{50} en mostres de sòl (Van Elsas et al., 2012).

En l'assaig de transformació del carboni es varen observar diferents tipus de corbes de respiració acumulada (cumulative respiration - CR), i les corbes van ser classificades en 4 tipus, corresponents als ILs analitzats. Tres d'ells descriuen els efectes dels PILs i el quart és representatiu del comportament dels AILs (vegeu capítol 3.3.). En el cas del primer tipus (2-HEAF, 2-HDEAF i 2-HDEAA), no hi ha efecte tòxic per a la microbiota del sòl i tots els valors d'oxigen consumit acumulat estan per sobre dels valors del control, després de 28 dies d'incubació. El segon tipus de corbes (2-HEAB, 2-HDEAiB i 2-HTEAB) també mostra una activitat

respiratòria ininterrompuda de la microbiota del sòl, però amb algunes diferències en la intensitat de la resposta de la mateixa. Les concentracions més altes (5000 i 10000 mg kg^{-1}) de PILs mostren un efecte inhibidor inicial sobre la microbiota del sòl, que posteriorment s'adapta i comença a respirar, amb un percentatge final d'acumulació d'oxigen per sobre del control. En el tercer tipus (2-HDEAPr, 2-HDEAB, 2-HDEAPe i 2-HTEAPe), les corbes presenten poca o cap diferència entre l'oxigen acumulat a les concentracions més baixes (1, 10, i 100 mg kg^{-1}) i el control, també s'observa una inhibició inicial a la concentració de 5000 mg kg^{-1} i una posterior recuperació de la respiració, amb valors finals d'oxigen consumit acumulat per sobre del control. La possible causa d'aquest comportament de la microbiota (una inhibició inicial i una posterior recuperació i estimulació de la respiració) podria ser la degradació de la matèria orgànica làbil procedent dels microorganismes afectats per la toxicitat inicial i/o per la biodegradació de la substància en si (Martí et al., 2011). La concentració més alta (10000 mg kg^{-1}) presenta una inhibició de l'activitat respiratòria durant tot l'assaig, que és un clar indicador de la toxicitat per a la microbiota del sòl a aquesta concentració. Aquests resultats varen ser confirmats pels resultats de la quantificació per cromatografia iònica. En el dia 28 de l'assaig de transformació de carboni, cap dels compostos, excepte 2-HDEAPe, va poder ser detectat en els extractes aquosos de sòl. Tots aquests resultats indiquen una biodegradació potencial dels PILs en sòl.

En el cas dels AILs, es va trobar un patró de comportament diferent. El quart tipus de corbes de respiració no exhibeix cap diferència entre els valors d'oxigen acumulat per les mostres control i les concentracions més baixes dels AILs (1, 10, i 100 mg kg^{-1}) en el cas del [BMIM]Cl i el [BPy]Cl, però hi ha un efecte inhibidor a partir de 1000 mg kg^{-1}, fet que indica que aquests compostos mostren certa toxicitat pels microorganismes implicats en el cicle de mineralització de carboni. L'[OMIM]Cl va ser el més tòxic dels tres AILs, amb inhibició de l'activitat respiratòria que ja comença a 100 mg kg^{-1}. En general, en el cas d'aquest assaig, els AILs analitzats també han demostrat ser més tòxics per a la microbiota del sòl que els PILs analitzats i no tenen potencial de biodegradació, tot i això, no van mostrar una inhibició prou forta per poder proporcionar valors de CE_{50}.

Els resultats de l'assaig de transformació del nitrogen mostren valors de CE_{50} molt elevats per a tots els PILs analitzats, el que indica que no hi ha toxicitat per a la microbiota que mineralitza el nitrogen en el sòl. Tots els valors de CE_{50} obtinguts pels AILs són també superiors a 100 mg kg^{-1}, el que significa, d'acord amb el sistema de classificació utilitzat, que no hi ha efecte tòxic dels líquids iònics analitzats sobre els processos de mineralització de nitrogen. Però, els valors de CE_{50} pels AILs varen ser significativament més baixos que els dels PILs.

Es van realitzar dos **assaigs de toxicitat addicionals**, la inhibició de l'enzim acetilcolinesterasa (AChE) i la toxicitat cel·lular per a IPC-81 (línia cel·lular promielocítica leucèmica de rata), amb la finalitat d'obtenir una avaluació més a fons de la toxicitat dels ILs. L'acetilcolina participa en un dels principals mecanismes de neurotransmissió en gairebé tots els organismes superiors, inclosos els humans. L'AChE és una diana molecular (eco)toxicològicament rellevant i l'assaig de la seva inhibició es pot realitzar en una placa de microtitració i per tant representa una eina ràpida i de baix cost per a anàlisi inicial de toxicitat de productes químics industrials (Arning et al., 2008). Els cultius de cèl·lules proporcionen també una visió ràpida i convenient de les activitats biològiques dels productes químics (Ranke et al., 2004).

En l'assaig d'inhibició de l'acetilcolinesterasa, els PILs no van mostrar toxicitat, amb valors de CE_{50} que van des de 1.27 (potencial inhibidor baix) a 59.02 mmol L^{-1} (sense potencial inhibidor). Els AILs presenten un alt potencial d'inhibició, amb valors d'CE_{50} entre 0.04 i 0.08 mmol L^{-1}. L'OMIM[Cl] és el més tòxic dels tres AILs. Hi ha una diferència de diversos ordres de magnitud en comparar els CE_{50} dels PILs i dels AILs, essent més elevats els dels PILs. Una altra manera de fer l'avaluació de la toxicitat dels ILs analitzats seria comparar els seus valors de CE_{50} amb el corresponent de l'aldicarb (2-metil-2(metiltio)propanal O-(N-metilcarbamoil) oxima), amb un valor de 0.0049 mmol L^{-1} (base de dades de UFT/Merck). L'aldicarb pertany a la família dels carbamats i és un potent inhibidor de l'enzim acetilcolinesterasa, que s'utilitza principalment com a insecticida. En comparació amb l'aldicarb, els PILs no van mostrar cap efecte tòxic, mentre que els AILs, encara que menys tòxics que l'aldicarb, van demostrar ser potents inhibidors de

l'acetilcolinesterasa. Quan es tracta de la citotoxicitat per IPC-81, els valors de CE_{50} per als PILs analitzats oscil.la entre 1.76 i 30.95 mmol L^{-1}, mentre que els ILs tenien CE_{50} entre 0.1 i 5 mmol L^{-1}. Un dels PIL, 2-HDEAF, no va mostrar cap efecte citotòxic. El 2-HEAB, el 2-HDEAB, el 2-HDEAiB, el 2-HTEAB i el 2-HTEAPe tenen un potencial inhibidor moderat. Els AILs es varen mostrar més tòxics que els PILs, sent l'OMIM[Cl] el més tòxic. La seva CE_{50} indica moderada ([BMIM]Cl i [BPy]Cl) i alta ([OMIM]Cl) citotoxicitat. En un estudi de Ranke et al. (2006) es va trobar una bona correlació entre la lipofília del catió i la citotoxicitat observada per a un conjunt d'aproximadament 70 líquids iònics, i això explica la major toxicitat dels AILs que són més lipòfils en comparació amb els PILs.

Els PILs en general mostren molt poca o gens de toxicitat per la majoria d'assaigs realitzats. El 2-HEAF, el 2-HDEAF, el 2-HDEAPr i el 2-HTEAB van resultar ser no tòxics en totes les proves d'ecotoxicitat. El 2-HEAB va resultar ser nociu per a *Lemna minor* i *Lolium perenne*, un fet que indica que és perillós per a les plantes. Dos dels tres PILs que van resultar ser tòxics per *L. minor* (2-HDEAB i 2-HDEAiB) no van mostrar efectes adversos en d'altres proves d'ecotoxicitat i exhibeixen citotoxicitat moderada. El tercer representant dels PILs tòxic per *L. minor*, el 2-HDEAB, va exhibir toxicitat per la planta terrestre *L. perenne* i també va mostrar citotoxicitat potencial moderada, a més es van obtenir valors propers al límit de la toxicitat en l'assaig de inhibició del creixement de les algues, la qual cosa indica que podria ser una amenaça si assolís el medi aquàtic. En canvi, el 2-HDEAPe va mostrar toxicitat per a totes les plantes superiors analitzades i té una corba de respiració del sòl del tipus 3, fet que indica que és un compost potencialment perillós per a la vida terrestre. L'altre PIL que va mostrar ser tòxic per a les plantes, el 2-HTEAPe, a més de ser nociu per a *Raphanus sativus*, també té una corba de respiració del tipus de 3, de manera que, en base dels seus valors de CE_{50}, també pot ser potencialment perillós per a la vida terrestre si es troba en concentracions més altes en el sòl, però menys tòxic que el 2-HDEAPe. El 2-HTEAB exhibeix citotoxicitat moderada, però, globalment es pot considerar com a no ecotòxic. Els PILs analitzats són potencialment biodegradables en aigua i sòl.

A diferència dels PILs, els AILs van demostrar ser tòxics en gairebé totes les proves realitzades. El [BMIM] Cl i [BPy] Cl són tòxics per a *P. subcapitata, L. minor i A. cepa*. El representant més tòxic, tant en medi aquàtic com terrestre, és l'[OMIM] Cl. A banda de l'ecotoxicitat mostrada en totes les proves realitzades, va mostrar un alt potencial d'inhibició de l'enzim AChE i elevada citotoxicitat. Tot i ser un compost soluble, és el més lipòfil de tots els ILs analitzats, per tant, és el més biodisponible enfront les membranes cel·lulars i, per tant, manifesta més toxicitat en aquells organismes on la membrana és la principal via d'entrada de contaminants. A més a més els AILs presenten una estructura química molt similar a l'acetilcolina, per tant no sorprèn que presentin elevada afinitat per l'enzim AChE. Els AILs analitzats no són biodegradables en aigua i són bastant menys biodegradables que els PILs en sòl.

Un dels requisits de REACH és demostrar que un producte químic no sigui persistent, bioacumulable i tòxic (Persistent, Bioaccumulative and Toxic – PBT). Els compostos PBT són substàncies químiques que són tòxiques, persistents en el medi ambient i es bioacumulen en la cadena alimentària i, en conseqüència, suposen un risc per a la salut humana i els ecosistemes (ECHA, 2012b). Segons el REACH (annex XIII), una substància compleix el criteri de persistència quan la seva vida mitjana en aigua és superior a 40 dies i la vida mitjana en sòl supera 120 dies. De tots els ILs analitzats, només els AILs podrien complir aquest criteri, com el [BMIM]Cl i [BPy]Cl, que no presenten cap biodegradació després de 31 dies (Stolte et al., 2008). En el sòl els AILs també mostren una lleugera inhibició de l'activitat microbiana, però donat que l'assaig només va durar 28 dies, es necessiten estudis de major durada per confirmar plenament aquestes hipòtesis. Els PIL no són persistents, donat que es biodegraden ràpidament, tant en aigua com en sòl. Tenint en compte que els PILs i els AILs tenen valors molt baixos o negatius de $\log K_{ow}$ (Ropel et al., 2005, Peric et al., resultats no publicats), seran poc bioacumulables. D'acord amb el REACH, si una substància compleix els criteris de persistència i bioacumulació, serà necessari com a mínim fer proves de toxicitat aguda amb la finalitat de determinar si la substància reuneix els criteris per a ser considerada com a tòxica. L'[OMIM]Cl seria un seriós candidat per a ser considerat com a

compost tòxic, ja que va mostrar un potencial tòxic en totes les proves de toxicitat aguda dutes a terme en aquest treball.

En aquesta tesi es va fer una **anàlisi de correlació** (Spearman) amb tots els resultats de les proves d'(eco)toxicitat amb la finalitat de relacionar l'estructura i la toxicitat/biodegradabilitat dels PILs i així obtenir més informació sobre els seus efectes tòxics i la possible biodegradació. El pes molecular dels PILs, el nombre d'àtoms de C del catió (amina) i de l'anió (àcid) de la molècula, com a possibles factors d'influència, es van correlacionar amb els valors de CE_{50} i el percentatge de biodegradació. Els resultats de les proves d'ecotoxicitat en els dos compartiments (aigua i sòl) mostren que la longitud de les cadenes alquíliques en la part aniònica de la molècula i l'augment del pes molecular estan en correlació directa amb la toxicitat. La part aniònica de la molècula dels PILs sembla tenir més influència sobre la toxicitat que la part catiònica, a diferència dels AILs. L'augment del pes molecular també té una forta influència sobre la toxicitat, la qual cosa indica que la complexitat de la molècula dels PILs té una influència positiva sobre la toxicitat potencial. El percentatge de biodegradació durant els primers 5 dies de l'assaig de biodegradabilitat es veu influenciat positivament per la mida de la part aniònica de la molècula. En les etapes posteriors de l'assaig, més a prop del dia 28, és la complexitat de l'amina la que redueix més la taxa de biodegradabilitat. La prova d'inhibició de l'acetilcolinesterasa mostra una forta influència dels tres factors estudiats, sent tots ells inversament correlacionats amb valors de CE_{50}. Tanmateix, els PILs tenen baix potencial inhibidor d'aquest enzim. La part aniònica també té força més influència en l'assaig de citotoxicitat. Les anàlisis de correlació realitzades per altres autors confirmen les dades prèviament esmentades. Stolte et al. (2007a) varen trobar correlació entre la lipofília i la toxicitat dels AILs en assaigs amb bacteris i algues. En el cas de les cèl·lules IPC-81, Stolte et al. (2007b) varen correlacionar positivament la lipofília amb la citotoxicitat. Ventura et al. (2012) va descriure correlacions significatives entre l'augment de longitud de les cadenes laterals del catió i la inhibició de l'enzim acetilcolinesterasa.

En el cas de l'assaig de transformació de carboni es varen obtenir correlacions inverses entre l'augment del nombre de carbonis en la part àcida de la molècula i

els valors de percentatge acumulat de la respiració, respiració basal i la respiració induïda per substrat. L'increment del pes molecular provoca la disminució d'aquests tres paràmetres, mentre que l'única funció que està influenciada per l'augment de la complexitat de la part catiònica dels PILs són els valors de respiració basal. És evident que a mida que augmenta la complexitat dels PILs menor és l'activitat de la microbiota del sòl pel que fa a la transformació del nitrogen. No obstant, els PILs no van mostrar efectes tòxics significatius en els assajos de nitrificació.

Existeix la possibilitat de sintetitzar un elevat nombre de possibles líquids iònics, però no és econòmicament factible estudiar-ne la toxicitat de tots ells. Una bona manera d'evitar realitzar tantes proves de toxicitat costoses és l'ús d'***in silico* QSAR mètodes** per a la predicció de la toxicitat, sent molt més ràpids i econòmics. El model QSAR basat en la contribució de grups (Luis et al., 2007) es va dur a terme per a cinc assaigs de toxicitat: Microtox®, inhibició de creixement de *P. subcapitata* i *Lemna minor*, inhibició de l'acetilcolinesterasa i citotoxicitat amb les cèl·lules IPC-81. Els resultats obtinguts indicaren la mateixa tendència que l'observada amb l'anàlisi de correlació. Tant la part catiònica com la part aniònica contribueixen a la toxicitat dels PILs, en el cas de les proves realitzades sobre toxicitat aquàtica i cel·lular. Els AILs seleccionats també es varen incloure en el model, i els resultats confirmen els publicats anteriorment per d'altres autors. És a dir, el catió influeix fonamentalment en la toxicitat, en canvi l'anió té efectes marginals o nuls sobre la toxicitat (Ranke et al., 2004, Matzke et al., 2007 i Stolte et al., 2007a). El nombre de carbonis en les cadenes laterals alquíliques varen mostrar una forta influència sobre la toxicitat dels ILs, de manera que com més llarga la cadena, la toxicitat és més elevada, un fet ben estudiat per altres autors (Ranke et al., 2004, Wells i Coombe, 2006, Pham et al., 2010 i Radošević et al., 2013). Els cations i anions dels PILs analitzats no van mostrar gran influència sobre la toxicitat general dels líquids iònics (on s'inclouen AILs també). Això coincideix amb el fet de que els PILs presenten molta menor toxicitat que els AILs en totes les proves de toxicitat assajades.

Amb l'aplicació del model de contribució de grup QSAR, es van predir els valors de CE_{50} per als vuit representants de la nova família dels PILs que no havien estat analitzats prèviament. Els resultats complets pels cinc assaigs de toxicitat esmentats es poden trobar en el capítol 3.4 d'aquesta tesi. La qualificació de les toxicitats dels PILs, basades en les CE_{50} predites, es presenta a la Taula 7.

Taula 7. Qualificació de la toxicitat dels vuit representants de la nova família de PILs, predita mitjançant QSAR per als assaigs de Microtox®, inhibició de creixement de algues i *L. minor*, inhibició de l'acetilcolinesterasa i citotoxicitat amb les cèl·lules IPC-81.

	Microtox®	**Algues**	***L. minor***	**AcHE**	**IPC-81**
2-HEAA	no tòxic	no tòxic	no tòxic	sense inhibició	citotoxicitat baixa
2-HEAPr	no tòxic	no tòxic	no tòxic	sense inhibició	citotoxicitat baixa
2-HEAiB	no tòxic	no tòxic	Aguda 3	sense inhibició	citotoxicitat moderada
2-HEAPe	no tòxic	no tòxic	Aguda 3	sense inhibició	citotoxicitat moderada
2-HTEAF	no tòxic	no tòxic	no tòxic	sense inhibició	citotoxicitat baixa
2-HTEAA	no tòxic	no tòxic	no tòxic	potencial inhibitori baix	citotoxicitat baixa
2-HTEAPr	no tòxic	no tòxic	no tòxic	potencial inhibitori baix	citotoxicitat baixa
2-HTEAiB	no tòxic	no tòxic	no tòxic	potencial inhibitori baix	citotoxicitat moderada

Les toxicitats estimades mitjançant el model QSAR mostren que, de manera anàloga als PILs analitzats experimentalment, la resta de membres de la família també tenen una baixa o nul·la toxicitat. En el cas de l'assaig de Microtox® i la inhibició del creixement d'algues, tots els valors de CE_{50} estan molt per sobre del llindar de toxicitat de 100 mg L^{-1}. En l'únic assaig de toxicitat aquàtica on s'observa l'efecte tòxic d'aquests PILs és en l'assaig d'inhibició del creixement de *L. minor* on dos dels PILs, 2-HEAiB i 2-HEAPe, tenen CE_{50} <100 mg L^{-1}, i d'acord amb el sistema GHS poden ser classificats com nocius per al medi ambient aquàtic (categoria

Aguda 3). Una altra vegada es tracta dels PILs amb les cadenes alquíliques més llargues (butíric i pentanoic) i la *L. minor* va resultar ser l'espècie més sensible en les proves de toxicitat aquàtica, com en el cas de la resta dels PILs analitzats experimentalment. Cap dels PILs va mostrar activitat inhibitòria enfront l'enzim acetilcolinesterasa. El 2-HEAiB, el 2-HEAPe i el 2-HTEAiB tenen moderat potencial citotòxic, mentre que els altres PILs tenen molt baix o cap efecte citotòxic, d'acord amb els criteris de classificació de la base de dades UFT/Merck Ionic Liquids Biological Database.

CONCLUSIONS

5. CONCLUSIONS

Com a resultat de la investigació realitzada en aquesta tesi, s'ha arribat a les següents conclusions:

- Els PILs analitzats en aquest estudi no han mostrat toxicitat en els assaigs efectuats amb organismes aquàtics, amb l'excepció de tres representants que es classifiquen com a nocius en el cas de l'assaig d'inhibició de creixement de la llentia d'aigua. L'[OMIM]Cl va resultar ser el més tòxic en totes les proves de toxicitat aquàtica i es classifica com a molt tòxic per a organismes aquàtics. Els altres dos representants d'AILs es classifiquen com a nocius per a organismes aquàtics.

- Entre els PILs només el 2-HDEAPe pot ser classificat com a nociu per a les tres plantes analitzades, mentre que altres tres PILs es classifiquen com a nocius per a una sola espècie de planta. Els AILs analitzats van resultar ser nocius per a la ceba i el [OMIM]Cl a més per al rave i per al raigràs anglès. Cap dels líquids iònics analitzats mostren efecte tòxic sobre la nitrificació ni l'activitat de la microbiota heteròtrofa del sòl, tanmateix hi ha una diferència considerable entre els valors de CE_{50} dels dos grups d'ILs en el test de transformació de nitrogen i en la forma de les corbes respiromètriques.

- En l'assaig de respirometria en aigua els PILs van mostrar taxes de biodegradació elevades. La forma de les corbes de respiració del sòl per als PILs indiquen que, en general, estimulen l'activitat de la microbiota. Aquests fets, juntament amb els resultats de la quantificació mitjançant cromatografia iònica, permeten concloure que els PILs analitzats són potencialment biodegradables en aigua i sòl. En canvi, els AILs semblen ser no biodegradables en aigua i mostren certa inhibició de l'activitat de la microbiota de sòl, fets que poden denotar resistència a la degradació.

- En el cas dels assaigs d'inhibició de l'acetilcolinesterasa, cap dels PILs mostren inhibició, a diferència dels AILs, que exhibeixen un alt potencial inhibidor de l'activitat enzimàtica. En la prova de citotoxicitat, cap dels líquids iònics analitzats

van mostrar una toxicitat molt alta, i el [OMIM]Cl, un cop més ha resultat ser el més tòxic.

- D'acord amb l'anàlisi de correlació, el pes molecular i la mida de l'àcid (part aniònica de la molècula) tenen més influència en la toxicitat dels PILs que l'amina (part catiònica de la molècula). En canvi, pels AILs la part catiònica és la més influent en termes de toxicitat. L'estudi comparatiu de l'(eco)toxicitat i de la biodegradabilitat d'ambdós grups indica que els ILs més complexos són els més tòxics. Aquest fet, contrastat amb dades d'altres autors, porta a la conclusió que els líquids iònics amb estructura més simple, sense cations orgànics voluminosos i amb cadenes laterals alquíliques curtes, mostren menor toxicitat i s'han de considerar com un camí a seguir en el futur per a la síntesi de nous líquids iònics.

- El model QSAR basat en la contribució de grups va mostrar que en el cas dels PILs tant la part aniònica com la catiònica de la molècula tenen influència sobre la toxicitat, tot i que la llargada de la cadena de carbonis de l'àcid sembla ser més influent. En canvi, pel que fa als AILs el model QSAR confirma que els cations tenen una influència predominant sobre la toxicitat, així com l'augment de la llargada de les cadenes laterals alquíliques.

- Els vuit representants de la nova família dels PILs, per als quals es va predir la CE_{50} mitjançant el model QSAR, s'han mostrar no tòxics, igual com altres representats de la nova família de PILs, i per tant, es podrien considerar com potencialment "verds".

- La baixa toxicitat i la bona biodegradabilitat dels PILs analitzats en aquest estudi, juntament amb el seu baix cost de producció, síntesi simple i nombroses aplicacions possibles, suggereix que podrien tenir bones perspectives per a un ús més ampli. L'estudi comparatiu indica que els PILs alifàtics de cadena curta es poden considerar com a alternatives ambientalment més segures per tal de substituir els AILs més tòxics que s'utilitzen freqüentment en l'actualitat. Tot i així, es necessari dur a terme més estudis per acabar de determinar la seva sostenibilitat i toxicitat. Seria convenient que els estudis de sostenibilitat es duguin a terme simultàniament amb els estudis de disseny i possibles aplicacions de líquids iònics.

REFERÈNCIES

6. REFERÈNCIES

Amberg, A., 2013. *In silico* methods, in: Vogel, H.G., Maas, J., Hock, F.J., Mayer, D. (Eds.), Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays. Springer, Berlin Heidelberg, pp. 1273–1296.

Arning, J., Stolte, S., Böschen, A., Stock, F., Pitner, W.R., Welz-Biermann, U., Jastorff, B., Ranke, J. 2008. Qualitative and quantitative structure activity relationships for the inhibitory effects of cationic head groups, functionalized side chains and anions of ionic liquids on acetylcholinesterase. Green Chem. 10, 47–58.

Azevedo, A.M., Ribeiro, D.M., Pinto, P.C., Lúcio, M., Reis, S., Saraiva, M.L. 2013. Imidazolium ionic liquids as solvents of pharmaceuticals: influence on HSA binding and partition coefficient of nimesulide. Int. J. Pharm. 443, 273–278.

Bałczewski, P., Bachowska, B., Bialas, T., Biczak, R., Wieczorek, W. M., Balińska, A. 2007. Synthesis and phytotoxicity of new ionic liquids incorporating chiral cations and/or chiral anions. J. Agric. Food Chem. 55, 1881–1892.

Barrosse-Antle, L.E., Bond, A.M., Compton, R.G., O'Mahony, A.M., Rogers, E.I., Silvester, D.S. 2010. Voltammetry in room temperature ionic liquids: comparisons and contrasts with conventional electrochemical solvents. Chem. Asian J. 5, 202–230.

Bernot, R. J., Brueseke, M.A., Evans-White, M.A., Lamberti, G.A. 2005a. Acute and chronic toxicity of imidazolium-based ionic liquids on *Daphnia magna*. Environ. Toxicol. Chem. 24, 87–92.

Bernot, R.J., Kennedy, E.E., Lamberti, G.A. 2005b. Effects of ionic liquids on the survival, movement, and feeding behavior of the freshwater snail, *Physa acuta*. Environ. Toxicol. Chem. 24, 1759–1765.

Biçak, N. 2005. A new ionic liquid: 2-hydroxy ethylammonium formate. J. Mol. Liq. 116, 15–18.

Biçak, N., Filiz Senkal, B., Sezer, E. 2005. Preparation of organo-soluble polyanilines in ionic liquid. Synthetic Met. 155, 105–109.

Blaise, C., Férard, J.F. (Eds.) 2005. Small-scale Freshwater Toxicity Investigations. Volume I. Toxicity test methods, Springer Science & Business, The Netherlands.

CAS registry http://www.cas.org/content/chemical-substances (data de consulta: 10 de setembre de 2014)

Cedergreen, N., Madsen, T.V. 2003. Light regulation of root and leaf NO_3^- uptake and reduction in the floating macrophyte *Lemna minor*. New Phytol. 161, 449–457.

Chiappe, C., Pieraccini, D. 2005. Ionic liquids: solvent properties and organic reactivity. J. Phys. Org. Chem. 18, 275–297.

Cho, C.W., Pham, T.P.T., Jeon, Y.C., Vijayaraghavan, K., Choe, W.S., Yun, Y.S. 2007. Toxicity of imidazolium salt with anion bromide to a phytoplankton *Selenastrum capricornutum*: effect of alkyl chain length. Chemosphere 69, 1003–1007.

Cho, C.W., Jeon, Y.C., Pham, T.P.T., Vijayaraghavan, K., Yun, Y.S. 2008. The ecotoxicity of ionic liquids and traditional organic solvents on microalga *Selenastrum capricornutum*. Ecotoxicol. Environ. Saf. 71, 166–171.

Cho, C.W., Ranke, J., Arning, J., Thöming, J., Preiss, U., Jungnickel, C., Diedenhofen, M., Krossing, I., Stolte, S. 2013. *In silico* modelling for predicting the cationic hydrophobicity and cytotoxicity of ionic liquids towards the *Leukemia* rat cell line, *Vibrio fischeri* and *Scenedesmus vacuolatus* based on molecular interaction potentials of ions. SAR QSAR Environ Res. 24, 863–882.

Costello, D.M., Brown, L.M., Lamberti, G.A. 2009. Acute toxic effects of ionic liquids on zebra mussel (*Dreissena polymorpha*) surviving and feeding. Green Chem. 11, 548–553.

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.

Cota, I., Gonzalez-Olmos, R., Iglesias, M., Medina, F. 2013. New Brønsted Ionic Liquids: Synthesis, Thermodinamics and Catalytic Activity in Aldol Condensation Reactions, in: Ionic Liquids - New Aspects for the Future, Kadokawa J. (Ed.), InTech. DOI: 10.5772/45605.

Cota, I., Medina, F., Gonzalez-Olmos, R., Iglesias, M. 2014. Alanine-supported protic ionic liquids as efficient catalysts for aldol condensation reactions. C. R. Chimie, 17, 18–22.

Couling, D.J., Bernot, R.J., Docherty, K.M., Dixon, J.K., Maginn, E.J. 2006. Assessing the factors responsible for ionic liquid toxicity to aquatic organisms via quantitative structure-property relationship modeling. Green Chem. 8, 82–90.

Cvjetko Bubalo, M., Hanousek, K., Radošević, K., Gaurina Srček, V., Jakovljević, T., Radojčić Redovniković, I. 2014. Imidiazolium based ionic liquids: effects of different anions and alkyl chains lengths on the barley seedlings. Ecotoxicol. Environ. Saf. 101, 116–23.

Das, R.N., Roy, K. 2013. Advances in QSPR/QSTR models of ionic liquids for the design of greener solvents of the future. Mol. Divers. 17, 151–96.

Deive, F.J., Rodríguez, A., Varela, A., Rodrígues, C., Leitão, M.C., Houbraken, J.A.M.P., Pereiro, A.B., Longo, M.A., Sanromán, M.Á., Samson, R.A., Rebelo, L.P.N., Silva Pereira, C. 2011. Impact of ionic liquids on extreme microbial biotypes from soil. Green Chem. 13, 687–696.

De Souza, R.L., Faria de, E.L., Figueiredo, R.T., Freitas, S.L., Iglesias. M., Mattedi, S., Zanin, G.M., dos Santos, O.A., Coutinho, J.A.P., Lima, Á.S., Soares, C.M. 2013. Protic ionic liquid as additive on lipase immobilization using silica sol-gel. Enzyme Microb. Technol. 52, 141–150.

Dobler, D., Schmidts, T., Klingenhöfer, I., Runkel, F. 2013. Ionic liquids as ingredients in topical drug delivery systems. Int. J. Pharm. 441, 620–627.

Docherty, K.M., Kulpa, C.F. 2005. Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. Green Chem. 7, 185–189.

Docherty, K.M., Dixon, J.K., Kulpa, C.F. 2007. Biodegradability of imidazolium and pyridinium ionic liquids by an activated sludge microbial community. Biodegradation 18, 481–493.

Domínguez de María, P. (Ed.) 2012. Ionic Liquids in Biotransformations and Organocatalysis: Solvents and Beyond, John Wiley & Sons Inc., USA.

Dos Santos, R.F., Falcade, T., Antonini, L.M., Ortega V., M.R. Mattedi, S., Iglesias, M., Malfatti, C.F. 2014. Ionic liquids (IL) corrosion on A285 carbon steel. Dyna 184, 122–128.

Du, F.Y., Xiao, X.H., Luo, X.J., Li, G.K. 2009. Application of ionic liquids in the microwave-assisted extraction of polyphenolic compounds from medicinal plants. Talanta 78, 1177–1184.

Earle, M.J., Seddon, K.R. 2000. Ionic liquids: green solvents for the future. Pure Appl. Chem., 72, 1391–1398.

ECHA http://echa.europa.eu/ (data de consulta: 14 d'agost de 2014)

ECHA. 2008. Guidance on Information Requirements and Chemical Safety Assessment Chapter R6: QSARs and grouping of chemicals, Helsinki, Finland.

ECHA. 2010. Practical Guide 10: How to avoid unnecessary testing on animals, Helsinki, Finland.

ECHA. 2012a. Practical Guide 5: How to report QSARs, Helsinki, Finland.

ECHA. 2012b. Guidance on information requirements and chemical safety assessment Chapter R.11: PBT Assessment, Helsinki, Finland.

Endres, F. 2012. Interfaces of ionic liquids. Phys. Chem. Chem. Phys. 14, 5008–5009.

European Union. 2006. Regulation 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.

Florindo, C., Araújo, J.M., Alves, F., Matos, C., Ferraz, R., Prudêncio, C., Noronha, J.P., Petrovski, Ž., Branco, L., Rebelo, L.P., Marrucho, I.M. 2013. Evaluation of solubility and partition properties of ampicillin-based ionic liquids. Int. J. Pharm. 456, 553–559.

Forsyth, S.A., Pringle, J.M., MacFarlane, D.R. 2004. Ionic liquids - An overview. Aust. J. Chem. 57, 113–119.

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

Frade, R.F.M., Rosatella, A.A., Marques, C.S., Branco, L.C., Kulkarni, P.S., Mateus, N.M.M., Afonso, C.A.M., Duarte, C.M.M. 2009. Toxicological evaluation on human colon carcinoma cell line (CaCo-2) of ionic liquids based on imidazolium, guanidinium, ammonium, phosphonium, pyridinium and pyrrolidinium cations. Green Chem. 11, 1660–1665.

Garcia, M.T., Gathergood, N., Scammells, P.J. 2005. Biodegradable ionic liquids. Part II: effect of the anion and toxicology. Green Chem. 7, 9–14.

Gathergood, N., Garcia, M.T., Scammells, P.J. 2004. Biodegradable ionic liquids: part I. Concept, preliminary targets and evaluation. Green Chem. 6, 166–175.

Gathergood, N., Scammells, P.J., Garcia, M.T. 2006. Biodegradable ionic liquids. Part III: the first readily biodegradable ionic liquids. Green Chem. 8, 156–160.

Greaves, T.L., Weerawardena, A., Fong, C., Krodkiewska, I., Drummond, C.J. 2006. Protic Ionic Liquids: Solvents with Tunable Phase Behavior and Physicochemical Properties. Phys. Chem. B 110, 22479-22487.

Greaves, T.L., Drummond, C.J. 2008. Protic Ionic Liquids: Properties and Applications. Chem. Rev. 108, 206–237.

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

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

Harjani, J.R., Singer, R.D., Garcia, M.T., Scammells, P.J. 2008. The design and synthesis of biodegradable pyridinium ionic liquids. Green Chem. 10, 436–438.

Hough, W.L., Rogers, R.D. 2007a. Ionic liquids then and now: from solvents to materials to active pharmaceutical ingredients. Bull. Chem. Soc. Jpn., 80 (12), 2262–2269.

Hough, W.L., Smiglak, M., Rodríguez, H., Swatloski, R.P., Spear, S.K., Daly, D.T., Pernak, J., Grisel, J.E., Carliss, R.D., Soutullo, M.D., Davis, J.H. Jr., Rogers, R.D. 2007b. The third evolution of ionic liquids: active pharmaceutical ingredients. New J. Chem., 31, 1429–1436.

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

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. 16, 2802–2808.

Iglesias, M., Andrade, R.S., Mazzer, H., Cardozo, L. 2014. Textile dyeing using protic ionic liquids. Poster presented at Expoquimia, 30 September - 3 October 2014, Barcelona.

IL Thermo NIST Standard Reference Database. http://ilthermo.boulder.nist.gov/ (data de consulta: 20 de juny de 2014)

Jaitely, V., Karatas, A., Florence, A.T. 2008. Water-immiscible room temperature ionic liquids (RTILs) as drug reservoirs for controlled release. Int. J. Pharm. 354, 168–173.

Jastorff, B., Störmann, R., Ranke, J., Mölter, K., Stock, F., Oberheitmann, B., Hoffmann, W., Hoffmann, J., Nüchter, M., Ondruschka, B., Filser, J. 2003. How hazardous are ionic liquids? Structure–activity relationships and biological testing as important elements for sustainability evaluation. Green Chem. 5, 136–142.

Jastorff, B., Mölter, K., Behrend, P., Bottin-Weber, U., Filser, J., Heimers, A., Ondruschka, B., Ranke, J., Schaefer, M., Schröder, H., Stark, A., Stepnowski, P., Stock, F., Störmann, R., Stolte, S., Welz-Biermann, U., Ziegert, S., Thöming, J. 2005. Progress in evaluation of risk potential of ionic liquids-basis for an eco-design of sustainable products. Green Chem. 7, 362–372.

Jastorff B., Störmann R., Ranke J. 2007. Thinking in structure-activity-relationships - a way forward towards sustainable chemistry. Clean: Soil, Air, Water. 35, 399–405.

Jin, R., Fan, L., An, X. 2011. Microwave assisted ionic liquid pretreatment of medicinal plants for fast solvent extraction of active ingredients. Sep. Purif. Technol. 83, 45–49.

Kaiser, K.L.E., Palabrica, V.S. 1991. *Photobacterium phosphoreum*, toxicity data index. Water Poll. Res. J. Can. 26, 361–431.

Keskin, S., Kayrak-Talay, D., Akman, U., Hortaçsu, O. 2007. A review of ionic liquids towards supercritical fluid applications. J. Supercrit. Fluids 43,150–180.

Khodadadi-Moghaddam, M., Habibi-Yangjeh, A., Gholami, M.R. 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.

Kruger, E. 2008. Ionic liquids as media for electro-organic synthesis. PhD thesis, Nelson Mandela Metropolitan University, Port Elisabeth, South Africa.

Kumar, S., Ruth, W. R., Sprenger, B., Kragl, U. 2006. On the biodegradation of ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate, Chim. Oggi 24, 70–72.

Kumar, R.A., Papaiconomou, N., Lee, J.M., Salminen, J., Clark, D.S., Prausnitz, J.M. 2009. *In vitro* cytotoxicities of ionic liquids: Effect of cation rings, functional groups, and anions. Environ. Toxicol. 24, 388–395.

Larson, J.H., Frost, P.C., Lamberti, G.A. 2008. Variable toxicity of ionic liquid-forming chemicals to *Lemna minor* and the influence of dissolved organic matter. Environ. Toxicol. Chem. 27, 676–681.

Latała, A., Stepnowski, P., Nędzi, 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.

Liu, T., Sui, X., Zhang, R., Yang, L., Zu, Y., Zhang, L., Zhang, Y., Zhang, Z. 2011. Application of ionic liquids based microwave-assisted simultaneous extraction of carnosic acid, rosmarinic acid and essential oil from *Rosmarinus officinalis*. J Chromatogr A. 1218(47), 8480–8489.

Luis, P., Ortiz, I., Aldaco, R., Irabien, A. 2007. A novel group contribution method in the development of a QSAR for predicting the toxicity (*Vibrio fischeri* EC_{50}) of ionic liquids. Ecotoxicol. Environ. Saf. 67, 423–429.

Luis, P., Garea, A., Irabien, A. 2010. Quantitative structure-activity relationships (QSARs) to estimate ionic liquids ecotoxicity EC_{50} (*Vibrio fischeri*). J. Mol. Liq. 152, 28–33.

Luczak, J., Jungnickel, C., Lacka, I., Stolle, S., Hupka, J. 2010. Antimicrobial and surface activity of 1-alkyl-3-methylimidazolium derivatives. Green Chem. 12, 593–601.

Luo, Y.R., San-Hu, W., Li, X.Y., Yun, M.X., Wang, J.J., Sun, Z.J. 2010. Toxicity of ionic liquids on the growth, reproductive ability, and ATPase activity of earthworm. Ecotoxicol. Environ. Saf. 73(5), 1046–50.

Martí, E., Sierra, J., Cáliz, J., Montserrat, G., Vila, X., Garau, M.A., Cruañas, R. 2011. Ecotoxicity of chlorophenolic compounds depending on soil characteristics. Sci. Total Environ. 409, 2707–2716.

Masten, S.A. 2004. Review of Toxicological Literature for Ionic Liquids, Integrated Laboratory Systems Inc., Research Triangle Park, NC, USA.

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 1-alkyl-3-methylimidazolium ionic liquids observed in an (eco)toxicological test battery. Green Chem. 9, 1198–1207.

Matzke, M., Stolte, S., Arning, J., Uebers, U., Filser, J. 2008. Imidazolium based ionic liquids in soils: effects of the side chain length on wheat (*Triticum aestivum*) and

cress (*Lepidium sativum*) as affected by different clays and organic matter. Green Chem. 10, 584–591.

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.

Megaw, J., Busetti, A., Gilmore, B.F. 2013. Isolation and characterization of 1-alkyl-3-methylimidazolium chloride ionic liquid-tolerant and biodegrading marine bacteria. PLoS ONE 8(4): e60806. DOI: 10.1371/journal.pone.0060806.

Modelli, A., Sali, A., Galletti, P., Samorì, C. 2008. Biodegradation of oxygenated and non-oxygenated imidazolium-based ionic liquids in soil. Chemosphere 73, 1322–1327.

Mohammad Ali, I. (Ed.), 2012. Green Solvents II: Properties and Applications of Ionic Liquids, Springer, New York, USA.

Mohammad, M., Kishimoto, T., Itoh, K., Suyama, K., Yamamoto, H., 2005. Comparative sensitivity of *Pseudokirchneriella subcapitata* vs. *Lemna* sp. to eight sulfonylurea herbicides. Bull. Environ. Contam. Toxicol. 75, 866–872.

Neumann, J., Steudte, S., Cho, C.-W., Thöming, J., Stolte, S. 2014. Biodegradability of 27 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium ionic liquid cations under aerobic conditions. Green Chem. 16, 2174–2184.

OECD. 1992. Guidelines for the Testing of Chemicals, Guideline 301: Ready biodegradability, Paris, France.

Olivier-Bourbigou, H., Magna, L., Morvan, D. 2010. Ionic liquids and catalysis: Recent progress from knowledge to applications, Applied Catalysis A: General 373, 1–56.

Pham, T.P.T., Cho, C.W., Yun, Y.S. 2010. Environmental fate and toxicity of ionic liquids: A review. Water Res. 44, 252–272.

Pretti, C., Chiappe, C., Pieraccini, D., Gregori, M., Abramo, F., Monni, G., Intorre, L. 2006. Acute toxicity of ionic liquids to the zebrafish (*Danio rerio*). Green Chem. 8, 238–240.

Pretti, C., Chiappe, C., Baldetti, I., Brunini, S., Monni, G., Intorre, L. 2009. Acute toxicity of ionic liquids for three freshwater organisms: *Pseudokirchneriella*

subcapitata, *Daphnia magna* and *Danio rerio*. Ecotoxicol. Environ. Saf. 72, 1170–1176.

Quijano, G., Couvert, A., Amrane, A., Darracq, G., Couriol, C., Le Cloirec, P., Paquin, L., Carrie, D. 2011. Toxicity and biodegradability of ionic liquids: new perspectives towards whole-cell biotechnological applications. Chem. Eng. J. 174, 27-32.

Radošević, K., Cvjetko, M., Kopjar, N., Novak, R., Dumić, J., Gaurina Srček, V. 2013. In vitro cytotoxicity assessment of imidazolium ionic liquids: biological effects in fish Channel Catfish Ovary (CCO) cell line. Ecotoxicol. Environ. Saf. 92, 112–118.

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 length in acute *Vibrio fischeri* and WST-1 cell viability assays. Ecotox. Environ. Saf. 58, 396–404.

Ranke, J., Cox, M., Müller, A., Schmidt, C. 2006. Sorption, cellular distribution, and cytotoxicity of imidazolium ionic liquids in mammalian cells - influence of lipophilicity. Toxicol. Environ. Chem. 88: 273–285

Ranke, J., Stolte, S., Störmann, R., Arning, J., Jastorff, B. 2007a. Design of sustainable chemical products – the example of ionic liquids. Chem. Rev. 107, 2183–2206.

Ranke, J., Stock, F., Müller, A., Stolte, S., Störmann, R., Bottin-Weber, U., Jastorff, B. 2007b. Lipophilicity parameters for ionic liquid cations and their correlation to *in vitro* cytotoxicity. Ecotoxicol. Environ. Saf. 67, 430–438.

Rogers, R.D., Seddon, K.R. 2002. Ionic liquids: industrial applications for green chemistry, American Chemical Society, Washington D.C.

Rogers, R.D., Zhang, S.J., Wang, J.J. 2012. Preface: An International Look at Ionic Liquids. Sci. China: Chem. 55 (8), 1475–1477.

Ropel, L., Belvèze, L.S., Aki, S.N.V.K., Stadtherr, M.A., Brennecke, J.F. 2005. Octanol-water partition coefficients of imidazolium-based ionic liquids, Green Chem. 7, 83–90.

Roth, M. 2009. Partitioning behaviour of organic compounds between ionic liquids and supercritical fluids. J. Chromatogr. A 1216, 1861–1880.

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.

Schuster, D., Laggner, C., Langer, T. 2005. Why drugs fail - a study on side effects in new chemical entities. Curr. Pharm. Des. 11, 3545–3559.

Sena, D.W., Kulacki, K.J., Chaloner, D.T., Lamberti, G.A. 2010. The role of the cell wall in the toxicity of ionic liquids to the alga *Chlamydomonas reinhardtii*. Green Chem. 12, 1066–1071.

Sheldon, R. 2001. Catalytic reactions in ionic liquids. Chem. Commun. 23, 2399–2407.

Składanowski, A.C., Stepnowski, P., Kleszczyński, K., Dmochowska, B. 2005. AMP deaminase *in vitro* inhibition by xenobiotics. A potential molecular method for risk assessment of synthetic nitro- and polycyclic musks, imidazolium ionic liquids and N-glucopyranosyl ammonium salts. Environ. Toxicol. Pharmacol. 19, 291–296.

Stasiewicz, M., Mulkiewicz, E., Tomczak-Wandzel, R., Kumirska, J., Siedlecka, E., Gołebiowski, M., Gajdus, J., Stepnowski, P. 2008. Assessing toxicity and biodegradation of novel, environmentally benign ionic liquids (1-alkoxymethyl-3-hydroxypyridinium chloride, saccharinate and acesulfamates) on cellular and molecular level. Ecotoxicol. Environ. Saf. 71, 157–165.

Stock, F., Hoffmann, J., Ranke, J., Störmann, R., Ondruschka, B., Jastorff, B. 2004. Effects of ionic liquids on the acetylcholinesterase - a structure-activity relationship consideration. Green Chem. 6, 286–290.

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

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

Stolte, S., Abdulkarim, S., Arning, J., Blomeyer-Nienstedt, A.-K., Bottin-Weber, U., Matzke, M., Ranke, J., Jastorff, B., Thöming, J. 2008. Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazoliumchloride and electrochemical wastewater treatment of poorly biodegradable compounds. Green Chem. 10, 214–224.

Studzińska, S., Buszewski, B. 2009. Study of toxicity of imidazolium ionic liquids to watercress (*Lepidium sativum* L.). Anal. Bioanal. Chem. 393, 983–990.

Swatloski, R.P., Holbrey, J.D., Memon, S.B., Caldwell, G.A., Caldwell, K.A., Rogers, R.D. 2004. Using *Caenorhabditis elegans* to probe toxicity of 1-alkyl-3-methylimidazolium chloride based ionic liquids. Chem. Commun, 668–669.

Tokuda, H., Hayamizu, K., Ishii, K., Susan, M.A.B.H., Watanabe, M.J. 2004. Physicochemical Properties and Structures of Room Temperature Ionic Liquids. 1. Variation of Anionic Species. J. Phys. Chem. B 108, 16593–16600.

Tokuda, H., Hayamizu, K., Ishii, K., Susan, M.A.B.H., Watanabe, M.J. 2005. Physicochemical Properties and Structures of Room Temperature Ionic Liquids. 2. Variation of Alkyl Chain Length in Imidazolium Cation. J. Phys. Chem. B 109, 6103–6110.

Tokuda, H., Ishii, K., Susan, M.A.B.H., Tsuzuki, S., Hayamizu, K., Watanabe, M.J. 2006. Physicochemical Properties and Structures of Room-Temperature Ionic Liquids. 3. Variation of Cationic Structures. J. Phys. Chem. B 110, 2833–2839.

UFT/Merck Ionic Liquids Biological Effects Database: http://www.il-eco.uft.uni-bremen.de (data de consulta: 15 de juliol de 2014)

United Nations. 2006. Globally Harmonized System of classification and labeling of chemicals. UN/SCEGHS/12/INF5. New York, USA.

United Nations. 2013. Globally Harmonized System of classification and labeling of chemicals (GHS), 5th revised edition, New York and Geneva.

Van Elsas, J.D., Jansson, J.K., Trevors, J.T. (Eds.) 2012. Modern Soil Microbiology, second edition. CRC Press, Taylor&Francis Group, Boca Raton, Florida, USA.

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

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

Visser, A.E., Swatloski, R.P., Reichert, W.M., Mayton, R., Sheff, S., Wierzbicki, A., Davis J.H., Rogers, R.D. 2002. Task-specific ionic liquids incorporating novel cations for the coordination and extraction of Hg^{2+} and Cd^{2+}: synthesis, characterization, and extraction studies. Environ. Sci. Technol. 36(11), 2523–2529.

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

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.

Wasserscheid, P., Welton, T. (Eds.) 2007. Ionic liquids in synthesis, 2nd edition. Wiley-VCH, Weinheim, Germany.

Web of science® https://webofknowledge.com (data de consulta: 22 de juliol de 2014)

Wells, A.S., Coombe, V.T. 2006. On the freshwater ecotoxicity and biodegradation properties of some common ionic liquids. Org. Pro. Res. Dev. 10, 794–798.

Welton, T. 1999. Room temperature ionic liquids. Solvents for synthesis and catalysis. Chem. Rev. 99, 2071–2083.

Werner, S., Haumann, M., Wasserscheid, P. 2010. Ionic liquids in chemical engineering. Annu. Rev. Chem. Biomol. Eng. 1, 203–230.

Wilkes, J.S. 2002. A short history of ionic liquids - from molten salts to neoteric solvents. Green Chem. 4, 73–80.

Yu, M., Li, S.M., Li, X.Y., Zhang, B.J., Wang, J.J. 2008. Acute effects of 1-octyl-3-methylimidazolium bromide ionic liquid on the antioxidant enzyme system of mouse liver. Ecotoxicol. Environ. Saf. 71, 903–908.

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.

Zhang, C., Wang, H., Malhotra, S.V., Dodge, C.J., Francis, A.J. 2010. Biodegradation of pyridinium-based ionic liquids *by an* axenic culture *of* soil *Corynebacteria.* Green Chem. 12, 851–858.

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.