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Ecotoxicological Assessment of Cetylpyridinium Chloride and 1-(2-Hydroxyethyl)-3-Methylimidazolium Chloride

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Dissertation submitted to the School of Health of the Polytechnic Institute of Porto (ESS|IPP) in partial fulfilment of the requirements for the Degree of Master in Health Biochemistry, conducted under the supervision of Prof. Dr. Piedade Barros, of the Department of Morphological Sciences of School of Health of Porto (ESS|IPP) and of the Research Centre in Health and Environment (CISA), and co-orientation of Prof. Dr. Ricardo Ferraz of the Department of Chemical Sciences and Biomolecules of School of Health of Porto (ESS|IPP), of the Research Centre in Health and Environment (CISA) and of the Departamento de Química e Bioquímica da Faculdade de Ciências da Universidade do Porto (REQUIMTE).

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ABSTRACT

Ionic liquids (ILs) are salts that are stable over their melting temperature and are made exclusively of ions (one organic cation and one organic or inorganic anion). This chemical class has received considerable interest due to their unique and tuneable properties. The growing interest in ILs predicts an increase of their manufacture and use at industrial scale, which may result in the increased release of these compounds into the environment. In the past years, ILs have been used as a greener alternative to hazardous conventional solvents, although few data regarding the toxicity and ecotoxicity of ILs is available.

In the present work, we assessed the ecotoxicological profile of one imidazolium based IL, 1-(2-hydroxyethyl)-3-methylimidazolium chloride ([C2OHMIM][Cl]), and one pyridinium based IL, cetylpyridinium chloride ([C16Pyr][Cl]). The ecotoxicological evaluation was performed using a battery of test organisms from different ecosystems and from different trophic levels: *Chlorella vulgaris, Daphnia magna, Artemia salina, Lactuca sativa* and *Allium cepa*.

[C16Pyr][Cl] demonstrated to be more toxic to all the test organisms than [C2OHMIM][Cl]. [C16Pyr][Cl] EC50 values varied between 0.0000205 and 35.404 mM. [C2OHMIM][Cl] EC50 values varied between 3.635 and 111.97 mM. [C16Pyr][Cl] hazard classification varied from relatively harmless for *A. cepa* to super toxic to *D. magna*. [C2OHMIM][Cl] hazard classification varied from relatively harmless for all test systems to practically nontoxic to *D. magna*. It was also shown that *D. magna* demonstrated the highest sensitivity for both ILs.

The present results showed to be useful to understand ILs toxicity profile and to provide additional information for the design of safer compounds.

Key words: ecotoxicity, ionic liquids, cetylpyridinium chloride, 1-(2-hydroxyethyl)-3-methylimidazolium chloride, *C. vulgaris*, *D. magna*, *A. salina*, *L. sativa*, *A. cepa*

RESUMO

Os líquidos iónicos (ILs) são sais que são estáveis acima da sua temperatura de fusão e são constituídos exclusivamente por iões (um catião orgânico e um anião orgânico ou inorgânico). Esta classe de substâncias químicas desperta considerável interesse devido às suas propriedades únicas e modificáveis. Devido ao crescente interesse nestes compostos, é previsível um aumento da sua produção e uso à escala industrial, o que pode resultar na libertação destes compostos no meio ambiente. Nos últimos anos, os ILs tem sido usados como uma alternativa aos solventes orgânicos convencionais, no entanto, poucos dados existem sobre a sua toxicidade e ecotoxicidade.

O presente trabalho tem como objetivo avaliar o perfil ecotoxicológico de um IL derivado do imidazólio, o cloreto de 1-(2-hidroxietil)-3-metilimidazólio ([C2OHMIM][Cl]), e um IL derivado do piridínio, o cloreto de cetilpiridínio ([C16Pyr][Cl]). A avaliação ecotoxicológica foi realizada utilizando uma bateria de organismos teste de diferentes ecossistemas e de diferentes níveis tróficos: *Chlorella vulgaris*, *Daphnia magna*, *Artemia salina*, *Lactuca sativa* e *Allium cepa*.

[C16Pyr][Cl] demonstrou maior toxicidade para todos os organismos teste do que o [C2OHMIM][Cl]. Os valores de EC50 para o [C16Pyr][Cl] variaram entre 0.0000205 e 35.404 mM. Os valores de EC50 para o [C2OHMIM][Cl] variaram entre 3.635 e 111.97 mM. Com base nos resultados, a classificação do [C16Pyr][Cl] variou de relativamente inofensivo para *A. cepa* a super tóxico para a *D. magna*. A classificação do [C2OHMIM][Cl] variou de relativamente inofensivo para todos os organismos teste a praticamente não tóxico para a *D. magna*. Foi também demonstrado que *D. magna* é o organismo mais sensível a ambos os ILs testados.

Os resultados do presente trabalho contribuem para melhor conhecer o perfil ecotoxicológico dos ILs e para fornecer informação adicional para a criação de compostos químicos mais seguros.

Palavras-chave: ecotoxicidade, líquidos iónicos, cloreto de cetilpiridínio, cloreto de 1-(2-hidroxietil)-3-metilimidazólio, *C. vulgaris*, *D. magna*, *A. salina*, *L. sativa*, *A. cepa*.

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LIST OF ABBREVIATIONS

API-ILs	Ionic liquids based on active pharmaceutical ingredients
APIs	Active pharmaceutical ingredients
[C2OHMIM][Cl]	1-(2-Hydroxyethyl)-3-methylimidazolium chloride
[C16Pyr][Cl]	Cetylpyridinium chloride
EC50	Median effective concentration; concentration that causes 50% effect on the population
ILs	Ionic liquids
LOEC	Lowest observed effect concentration; it is the lowest concentration that shows significant difference from the control
NOEC	No observed effect concentration; it is the highest concentration having response not significantly different from the control
OECD	Organization for Economic Co-operation and Development
QSAR	Quantitative structure–property relationship models
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
USEPA	United States Environmental Protection Agency
VOCs	Volatile organic compounds

I. INTRODUCTION

1.1. Ecotoxicology

Ecotoxicology is the study of the harmful effects of chemicals compounds in the environment (Walker *et al.*, 2012). Experimental bioassays are used in ecotoxicology to understand the impact of chemicals by dose-response relationships on wildlife populations and ecosystems. In addition, ecotoxicological models are used for the detection, control and monitoring of the presence of pollutants in the environment (Walker *et al.*, 2012). The insights provided by ecotoxicological studies are also essential for environmental regulatory frameworks, which rely on knowledge about the effects of contaminants on organisms to develop guidelines to manage chemicals release into the environment and for environmental or ecological hazard and risk assessment of chemicals. In addition of estimate the effect of chemicals on organisms, ecotoxicity tests also support the development of policies regulating the allowable level of contaminants in the environment (European Parliament and the Council of the European Union, 2008).

Many regulatory frameworks for environmental or ecological hazard and risk assessment of chemicals require standard test guidelines provided by international organizations, such as the Organization for Economic Co-operation and Development (OECD) and the U. S. Environmental Protection Agency (USEPA).

For example, the regulation that aims to protect human health and the environment against the risks from chemicals is REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) and its function is to regulate the registration, evaluation, authorization and restriction of chemicals. REACH regulation applies to all chemical substances, those used in industrial processes as well as those that are part of people's daily life. It establishes procedures for the collection and evaluation of information on the properties and hazards of substances, by evaluating individual records to verify chemicals conformity, in order to clarify any concerns with human health and the environment. REACH also promotes alternative methods for the assessment of the hazards of substances, aiming to reduce the number of vertebrate in toxicity tests, encouraging alternative strategies with invertebrates, plants as well as organ, tissue and cell cultures (European Chemicals Agency, n.d.b).

Toxicity bioassays are performed by exposing a representative population of organisms to a range of chemical concentrations and recording responses (endpoints) over a period of time (Wright & Welbourn, 2002). Ecotoxicity tests may be acute or chronic

toxicity tests conducted under standardized conditions. These assays consist of exposing the test organisms to a series of dilutions of a chemical to estimate the concentration causing 50% effect (i.e. mortality, growth inhibition, reproduction inhibition). Normally, tests are conducted for a period of 96 hours or less (Walker *et al.*, 2012).

The growing public demand for safer chemicals (Scheringer, 2017), regarding human health and environment protection, and the pressure to minimize the use of vertebrates in toxicological experiments, lead to alternative model organisms (Walker *et al.*, 1998). A number of bacteria, yeasts, invertebrates, algae, cyanobacteria, plants, but also fish and other organisms are used to assess the effects of chemicals on the environment (Walker *et al.*, 2012).

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) is a United Kingdom based independent scientific organization that aims to discover and implement new technologies and approaches that minimize the use of animals in research and improve animal welfare. This organization points acute and chronic toxicity assessment of chemicals as two of the four areas of potential current and future high vertebrate usage and identifies it as a potential area where reduction in both animal usage and testing severity can be achieved by the 3Rs principles (replacement, refinement, and reduction of animal testing) (Burden *et al.*, 2016).

1.2. Model Organisms

In ecotoxicology, a model organism, apart from physiology, should meet technical criteria such as easy and inexpensive maintenance in the laboratory, fast growth and high reproductive potential, genetic tractability or the availability of a broad spectrum of experimental methodologies (Segner & Baumann, 2016). Model organisms demonstrated to be valuable to identify toxic modes of action of chemicals, to predict the toxicological and ecological consequences of chemical exposure and to assess under which conditions the mode of action and toxic effect extrapolation between species can be done (Segner & Baumann, 2016).

The different individual characteristics and response to chemicals imply that a single bioassay cannot provide a full picture of the toxic effects of chemicals (Repetto *et al.*, 2001). Therefore, it is recommended, for a certain chemical, to conduct a representative battery of test organisms and indicators, so that different living organisms are tested in order to cover

a range of trophic levels and taxa (European Parliament and the Council of the European Union, 2008; Repetto *et al.*, 2001). It is also recommended that the selection of the test organisms take into consideration the representative biota that is aimed to be evaluated. In this manner, with the appropriate selection of the test organisms, it is possible to undertake terrestrial and/or aquatic ecotoxicological assessment.

Invertebrates have been extensively used in acute and chronic toxicity tests to assess the toxic effects of chemicals. Several characteristics of these living organisms contribute to their use in ecotoxicity tests, such as ease of cultivation and handling under laboratory conditions, high fertility rate, short shelf life, speed and simplicity of the tests (Guilhermino *et al.*, 2000; Walker *et al.*, 1998).

Microalgae are also usually used in toxicity tests, since they are sensitive indicators of environmental changes. They showed to be suitable for the prospective assessment of the potential cytotoxicity of aquatic pollutants (Prado *et al.*, 2015).

Aquatic and terrestrial vascular plants are often chosen to evaluate the phytotoxicity of chemicals. The responses to chemical exposure, such chlorophyll content, membrane permeability, root growth and genetic alterations, are valuable to understand the effect of chemicals on plants development. Phytotoxicity bioassays are simple and inexpensive methods that produce rapid results (Wright & Welbourn, 2002).

1.3. Ionic Liquids

Ionic liquids (ILs) are salts that are stable over their melting temperature and made exclusively of ions (one organic cation and one organic or inorganic anion) (Egorova *et al.*, 2017; Silva *et al.*, 2017). This chemical class has received considerable attention and interest due to their unique and tuneable properties (Welton, 2018).

One of the biggest advantages of ILs is the possibility to design a substantial number of ILs, as ions can be chosen to make the right pair (cation/anion). ILs easily tuneable physical and chemical properties make them attractive for a wide range of applications (Smiglak *et al.*, 2014). Also, some properties, such as negligible volatility near ambient temperature (Earle *et al.*, 2006), high thermal and chemical stability (Meine *et al.*, 2010), high ionic conductivity, lack of flammability and outstanding solvating potential (Welton, 1999), have attracted numerous applications across an extensive variety of research areas, in

particular related with chemistry and materials industry (Ghandi, 2014; Torimoto *et al.*, 2010).

ILs design flexibility and structural variety also allows the possibility to modify structural elements in order to optimise ILs physicochemical properties, which makes this chemical class very attractive in terms of technical applicability (Ahrens *et al.*, 2009). Task-specific design of ILs with optimal technological properties has been used in different fields of application, such as batteries (Balducci, 2017; Macfarlane *et al.*, 2014; Osada *et al.*, 2016), sensors (Wei & Ivaska, 2008), dye-sensitised solar cells (Kawano *et al.*, 2004), production and processing of fuels (Bösmann *et al.*, 2001; Gao *et al.*, 2012), lubricants (Mo *et al.*, 2008; Zhao *et al.*, 2008; Ye *et al.*, 2001), textile industry (Tavanaie, 2013), embalming fluids and tissue preservation (Majewski *et al.*, 2003), nanoparticle preparation (Antonietti *et al.*, 2004; Vollmer & Janiak, 2011) or in medicine and pharmaceutical industry (Choi *et al.*, 2011; Ferraz *et al.*, 2012; Hauss, 2007; Hough *et al.*, 2007; Kumar & Malhotra, 2010; Marrucho, Branco, & Rebelo, 2014).

Based on their organic cation segment, ILs are usually categorized into four types: ammonium-, imidazolium-, phosphonium- and pyridinium based ILs (Figure 1) (Davis, 2004; Welton, 1999). The cation core can be combined with a side chain (typically, saturated hydrocarbon fragments) and a large number of different anions (Figure 2), which justifies the wide range of possible combinations (Davis, 2004).

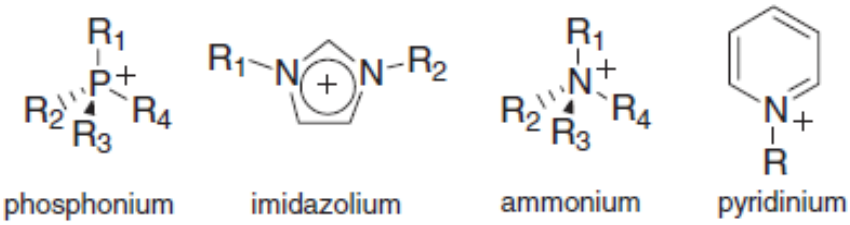

Figure 1 - Common organic cations used in the design of ILs. Ammonium, phosphonium, imidazolium and pyridinium cations (left to right). The"R" group represents the alkyl side chain. Adapted from "Task-Specific Ionic Liquids", by Davis J, 2004, *Chemistry Letters, 33(9)*.

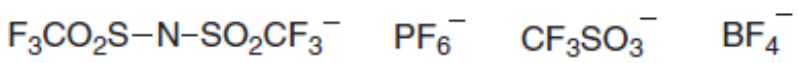

Figure 2 - Common anions often combined with the cation core in the design of ILs. Adapted from "Task-Specific Ionic Liquids", by Davis J, 2004, *Chemistry Letters, 33(9)*.

Due to their remarkable properties, ILs enable new applications that are not possible with conventional solvents. One of the most attractive advantages of ILs, and an important primary driving force behind research into ILs design, is the possibility to replace conventional solvents, most of which are toxic, hazardous and flammable, by nonvolatile ILs. Due to their negligible volatility, which prevents the emission of volatile organic compounds (VOCs) (a major source of environmental pollution), ILs gained the reputation of being green solvents (Rogers & Seddon, 2003).

According to their characteristics ILs are classified into three generations (Hough *et al.*, 2007). First generation ILs solvents were particularly fascinating due to their tuneable physical properties, including lower melting point, high thermal and chemical stability, negligible vapour pressure and non-flammable properties when compared to traditional solvents (Rogers & Seddon, 2003). The second generation focused on the deliberate modification of physicochemical properties for designing ILs competent in a specific task, leading to the creations of task-specific ILs (Ahrens *et al.*, 2009; Davis, 2004). The third generation appeared with the pharmaceutical application of ILs, where ILs can act just as solvents for active pharmaceutical ingredients (APIs) or APIs can be transformed into ILs themselves (API-ILs) (Hough *et al.*, 2007).

1.4. Ionic Liquids Ecotoxicology

Although the information about physical, thermodynamic, kinetic or engineering data have been deeply investigated, few data regarding the toxicity and ecotoxicity of ILs is available (Costa *et al.*, 2017). In the past years, ILs have been seen and used as a greener alternative to hazardous conventional solvents. The growing interest in ILs predicts an increase of their manufacture and use at industrial scale, which will result in the increased release of these compounds into the environment. Although ILs can reduce air pollution risk, due to their negligible volatility, even the hydrophobic ones present some water solubility (Freire *et al.*, 2007) which can lead to aquatic and soil contamination when released into the environment.

Despite the attractive green character of these compounds, some studies revealed that some ILs are very toxic (Pretti *et al.*, 2009; Stolte *et al.*, 2007; Wells & Coombe, 2006), with cation side chain length, cation core and nature of anion as main contributors for the toxic character (Costa *et al.*, 2017). It is, therefore, essential the assessment of these compounds' toxicity prior to their indiscriminate use. Various recent studies have been performed aiming

to disclose their toxic effect on several biological organisms (Costa *et al.*, 2017; Heckenbach *et al.*, 2016; Ostadjoo *et al.*, 2017; Stolte *et al.*, 2012). Bacteria (Azevedo *et al.*, 2017; Costa *et al.*, 2014; Costa *et al.*, 2015; Hernández-Fernández *et al.*, 2015; Stolte *et al.*, 2012; Ventura *et al.*, 2012), microalgae (Stolte *et al.*, 2012; Zhang *et al.*, 2017), crustacean (Stolte *et al.*, 2012; Zhang *et al.*, 2017), fish (El-Harbawi, 2014; Pretti *et al.*, 2006; Ruokonen *et al.*, 2016) and aquatic plants (Larson *et al.*, 2008; Stolte *et al.*, 2007) are some of the living organisms most used to evaluate ILs' aquatic toxicity. Likewise, terrestrial toxicity has been studied by the assessment of ILs ability to sorption onto soils (Matzke *et al.*, 2009b) and the influence of different soil types (Matzke *et al.*, 2009a) as well as their effect on plants (Biczak *et al.*, 2014; Liu *et al.*, 2015; Tot *et al.*, 2018) and earthworms (Liu *et al.*, 2016; Shao *et al.*, 2018).

In addition to traditional ecotoxicity testing, several recent quantitative structure–property relationship (QSAR) models have been developed to assess the factors that contribute to the toxic behaviour of a range of different ILs to living organisms (Cho & Yun, 2016; Couling *et al.*, 2006; Grzonkowska *et al.*, 2016).

Ecotoxicity studies showed to be useful to understand ILs toxicological interactions and to provide additional information for the design of safer compounds.

1.5. Imidazolium based Ionic Liquids

Imidazolium based ILs are among the most studied classes of ILs. These ILs have an imidazolium ring as a cation core, which is a five-member aromatic heterocycle with two nitrogen heteroatoms (Figure 3) (Ghandi, 2014). There are several reports regarding the application of imidazolium based ILs to energy engineering, medicine or biotechnology (Bösmann *et al.*, 2001; Mo *et al.*, 2008; Smiglak *et al.*, 2014; Torimoto *et al.*, 2010; Vollmer & Janiak, 2011).

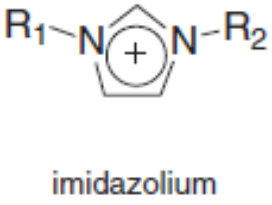

Figure 3 - Imidazolium cation core. Adapted from "Task-Specific Ionic Liquids", by Davis J, 2004, *Chemistry Letters, 33(9)*.

1-(2-Hydroxyethyl)-3-methylimidazolium chloride

1-(2-Hydroxyethyl)-3-methylimidazolium chloride ([C2OHMIM][Cl]) is an IL formed by the 1-alkyl-3-methylimidazolium cation core which is paired with a chloride anion. The ethyl side chain, with a hydroxyl group incorporated, is appended to the cation core (Figure 4). [C2OHMIM][Cl] has been successfully applied in different areas, like heat pumps manufacture (Nie *et al.*, 2012), nanotechnology industry (Cao *et al.*, 2004; Choi *et al.*, 2007) and in a variety of chemical reactions (Parveen *et al.*, 2016; Pereira *et al.*, 2013). It has also shown good results for its potential use in dechlorination processes (Wang *et al.*, 2009), in electrodes (Dedzo *et al.*, 2012; Tonle *et al.*, 2009) and wood floor fabrication (Ou *et al.*, 2014) and in the pharmaceutical industry, with good results in terms of antitumoral activity when used to prepare ILs based on ampicillin (Ferraz *et al.*, 2012, 2015).

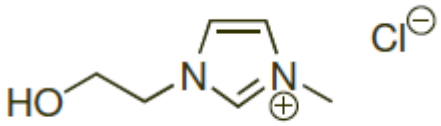

Figure 4 - 1-(2-Hydroxyethyl)-3-methylimidazolium chloride molecule structure. Retrieved from IoLiTec (2018).Retrieved May 6, 2018, from https://iolitec.de/en/products/ionic_liquids/catalogue/imidazolium-based/il-0039-hp

1.6. Pyridinium based Ionic Liquids

Pyridinium based ILs are salts containing a pyridinium ring, which is a six-member aromatic heterocycle with one nitrogen heteroatom (Figure 5). Many applications of this group of ILs has emerged, such as a green catalyst and solvent (Anvar *et al.*, 2014), synthesis of some pharmaceutical agents (Ferraz *et al.*, 2012), removal of aromatic heterocyclic sulphur compounds from diesel (Gao *et al.*, 2012) and textile processing (Tavanaie, 2013). Despite the importance of these applications, the most important one lies in their effective antimicrobial activity. In fact, pyridinium based ILs have demonstrated a great potential as antimicrobial agent (Choi *et al.*, 2011; Cornellas *et al.*, 2011; Messali, 2015). They have already been effectively applied in daily´s life products, such as dental hygiene products (Albert-Kiszely *et al.*, 2007; Latimer *et al.*, 2015; Lee *et al.*, 2017; Teng *et al.*, 2016) and throat sprays (Shima *et al.*, 2015).

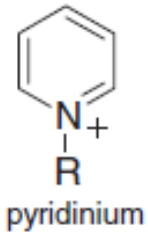

Figure 5 - Pyridinium cation core. Adapted from "Task-Specific Ionic Liquids", by Davis J, 2004, *Chemistry Letters, 33(9)*.

Cetylpyridinium chloride

Cetylpyridinium chloride ([C16Pyr][Cl]) is an IL formed by the pyridinium ring linked to a sixteen carbon saturated hydrocarbon chain as cation core and the halide chloride as the anion constituent (Figure 6) (Drug Bank, 2018). It is a cationic surfactant, with antimicrobial properties, widely used as an active ingredient in mouthwashes (Albert-Kiszely *et al.*, 2007; Latimer *et al.*, 2015; Lee *et al.*, 2017), toothpastes (Teng *et al.*, 2016), lozenges, throat sprays (Shima *et al.*, 2015), breath sprays, and nasal sprays. In dental hygiene products, it mediates a protective action against dental plaque and reducing gingivitis (Latimer *et al.*, 2015; Teng *et al.*, 2016) and shows significant benefit in reduction of bleeding (Albert-Kiszely *et al.*, 2007; Lee *et al.*, 2017).

[C16Pyr][Cl] also demonstrates valuable properties in other fields of applications. It exhibits potent and rapid activity against susceptible and resistant strains of influenza virus by targeting and disrupting the viral envelope (Popkin *et al.*, 2017). It also demonstrated to be an effective preservative of sputum samples for tuberculosis cultures (Hiza *et al.*, 2017) and good antibacterial properties when used to design ILs based on ampicillin (Ferraz *et al.*, 2014).

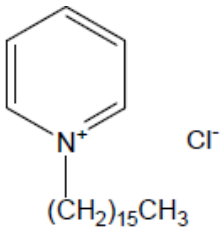

Figure 6 - Cetylpyridinium chloride molecule structure. Retrieved from Drug Bank. (2018). Cetylpyridinium. Retrieved May 6, 2018, from https://www.drugbank.ca/drugs/DB11073.

1.7. Present Work Overview

1.7.1 Study Approach

In the present work, we proposed to assess the ecotoxicological profile of one imidazolium based IL, [C2OHMIM][Cl], and one pyridinium based IL, [C16Pyr][Cl]. The ecotoxicological evaluation was performed using a battery of test organisms from different ecosystems and from different trophic levels, as described in Table 1. The organisms were exposed to the tested ILs in a range of different concentrations and the toxic effect evaluated.

For each organism, one or more acute endpoints were determined, such as culture growth for the microalgae, survival in the case of marine and freshwater crustacean, germination, shoot or root growth for terrestrial plants. The median effective concentration (EC50) results for each of the previous endpoints were compared with the acute toxicity ranking scale by Passino and Smith (1987) for aquatic organisms, as described in Table 2. For the terrestrial ecotoxicity assessment, the hazard classification also followed the same hazard ranking system to allow comparison (Passino & Smith, 1987). When possible, the highest concentration having response not significantly different from the control (no observed effect concentration - NOEC) and the lowest concentration that shows significant difference from the control (lowest observed effect concentration - LOEC) values were also determined.

Table 1 – Description of the present work design. Test organism's characterization, endpoints and test period evaluated.

Ecossystem	**Organism**	**Trophic level**	**Endpoint**	**Test period**
Aquatic/Freshwater	*Chlorella vulgaris*	Producer	Growth inhibition	96h
	Daphnia magna	Primary consumer	Immobilisation	24/48h
Aquatic/Saltwater	*Artemia salina*	Primary consumer	Mortality	24/48h
Terrestrial	*Lactuca sativa*	Producer	Germination inhibition Root and shoot growth inhibition	96h
	Allium cepa	Producer	Number of emerging roots inhibition Root growth inhibition	96h

Table 2 – Acute toxicity ranking scale by Passino and Smith (1987) and hazard representation that is used in the present work for hazard assessment.

EC50 (mg L^{-1})	**Toxicity level**	
	Classification	**Representation**
<0.01	Super toxic	++++++
0.01 – 0.1	Extremely toxic	+++++
0.1 – 1	Highly toxic	++++
1 – 10	Moderately toxic	+++
10 – 100	Slightly toxic	++
100 - 1000	Practically nontoxic	+
>1000	Relatively harmless	-

1.7.2. General Plan

The present work consists of eleven chapters, including the current chapter describing the state of the art, the work overview and the objectives (**Chapter I**). **Chapter II** has a general description of the experimental section used during this work, including the stock solutions preparation, equipment and organisms used, preliminary assays and statistical analysis description. **Chapter III**, **IV**, **V**, **VI** and **VII** describe the work developed with: *C. vulgaris*, *D. magna*, *A. salina*, *L. sativa* and *A. cepa*, respectively. Each one of these five chapters include an overview of the organism and the principles of the assay, the objectives, materials and methods, results, discussion and conclusion of the assay. These chapters are organized as follows:

Chapter III– *Chlorella vulgaris* growth inhibition assay;

Chapter IV– *Daphnia magna* acute immobilisation assay;

Chapter V– *Artemia salina* acute mortality assay;

Chapter VI– *Lactuca sativa* germination and root and shoot growth inhibition assay;

Chapter VII– *Allium cepa* root growth inhibition assay.

The remaining chapters are organized as follows:

Chapter VIII– General Discussion, gives a general discussion of all the work;

Chapter IX– Conclusion, summarizes the main conclusions and presents the future perspectives;

Chapter X – References, this presents the bibliography used;

Chapter XI – Appendix, this presents the preparation protocol of the Z8 Kotai medium (Appendix I) and the M4 Elendt medium (Appendix II).

1.7.3 Objectives

The main objective of the present work was to assess the ecotoxicological effect of 1-(2-hydroxyethyl)-3-methylimidazolium chloride ([C2OHMIM][Cl]) and cetylpyridinium chloride ([C16Pyr][Cl]) towards a battery of test organisms from:

i. different ecosystems: aquatic and terrestrial ecosystems;
ii. different trophic levels: producers and primary consumers.

It was also our goal to find the most sensitive organism, among the organisms tested, to the studied ILs.

The particular objectives of each chapter are described as follows:

Chapter III – The goals of the *C. vulgaris* growth inhibition assay were to determine the:

i. EC50 (concentration that inhibits 50% of the culture growth) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 96 hours of exposure;
ii. NOEC and LOEC values.

Chapter IV – The main goals of the *D. magna* acute immobilisation assay were to determine the:

i. EC50 (concentration that causes immobilisation of 50% of the daphnids) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 24 and 48 hours of exposure;
ii. NOEC and LOEC values.

Chapter V – The main goals of the *A. salina* acute mortality assay were to determine the:

i. EC50 (concentration that causes mortality of 50% of the nauplii) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 24 and 48 hours of exposure;
ii. NOEC and LOEC values.

Chapter VI – The main goals of the *L. sativa* germination and root and shoot growth inhibition assay were to determine the:

i. EC50 (concentration that causes 50% inhibition) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 96 hours of exposure for:
    a. germination;
    b. root growth;
    c. shoot growth;
ii. NOEC and LOEC values for all the three endpoints evaluated.

Chapter VII – The main goals of the *A. cepa* root growth inhibition assay were to determine the:

i. EC50 (concentration that causes 50% inhibition) of [C16Pyr][Cl] and [C_2OHMIM][Cl] after 96 hours of exposure for:
    a. number of emerging roots;
    b. root growth;
ii. to determine the NOEC and LOEC values for the two endpoints tested.

II. EXPERIMENTAL SECTION

2.1. Stock Solutions

[C16Pyr][Cl] was purchased from Sigma-Aldrich. It has a molecular weight of 358.00 g mol^{-1} and a purity >99% (Table 3). A 50 mM stock solution was prepared by dissolution in distilled water to use as stock solution, except for the *Chlorella vulgaris* test, where it was prepared a 25 mM stock solution.

[C2OHMIM][Cl] was purchased from Ionic Liquids Technologies. It has a molecular weight of 162.62 g mol^{-1} and a purity >99% (Table 3). A 50 mM stock solution was prepared by dissolution in distilled water, except for the *C. vulgaris* test, where it was prepared a 100 mM stock solution.

Table 3 - Chemical properties of [C16Pyr][Cl] and [C2OHMIM][Cl].

Compound	Molecular weight	Purity	log Kow	Empirical Formula
[C16Pyr][Cl]	358.00 g mol^{-1}	>99%	1.71	$C_{21}H_{38}ClN \cdot H_2O$
[C2OHMIM][Cl]	162.62 g mol^{-1}	>99%	-2.32	$C_6H_{11}ClN_2O$

Data collected from Pubchem and EPA databases.

Test solutions were prepared by serial dilutions using the corresponding organism culture medium as dilution solution (Table 4).

Table 4 - Dilution medium used to prepare test solutions for each assay.

Assay	Dilution solution
C. vulgaris	Kotai Z8 medium
D. magna	Elendt M4 medium
A. salina	Saltwater (35g L^{-1})
L. sativa	Distilled water
A. cepa	Distilled water

2.2. Equipment

The present work required the use of the following equipment:

- Laminar air flow chamber, Telstar model PV-30/70;
- Automate orbital shaker, Heidolph Roramax 120;
- Microplate reader, Biotek Synergy HT;
- Autoclave chamber, JSM.

2.3. Test Organisms

Organisms from distinct trophic levels, producers and primary consumers, and from different ecosystems, aquatic and terrestrial, were tested. Test solutions of [C16Pyr][Cl] and [C2OHMIM][Cl] were tested on an aquatic producer, the microalgae *Chlorella vulgaris*, on a freshwater primary consumer, *Daphnia magna*, on a saltwater primary consumer, *Artemia salina* and on two terrestrial producers, *Lactuca sativa* and *Allium cepa.*

All organisms were handled and maintained under controlled laboratory conditions previously and during the test period, according to test standards.

2.4. Preliminary Assays

Preliminary assays were carried out under the same conditions as the definitive test. Preliminary assays aimed to determine the range of concentrations between the lowest concentration causing effect at 100% of the organisms and the highest concentration at which no effect occurs. This range-finding study was performed to determine the range of concentrations to be used in the definitive tests. Then, definitive toxicity experiments were carried out to determine the concentration that causes effect on 50% of the individuals (EC50) (Walker *et al.*, 2012). The range of tested concentrations was 10; 1; 0,1; 0,01 and 0,001 mM in every preliminary test performed for each organism.

2.5. Statistical Analysis

For each organism tested, a statistical analysis was performed using *IBM SPSS Statistics 25* software.

The EC50 values were determined by statistical probit analysis with 95% confidence limits ($\alpha = 0.05$) (Finney, 1952). The Kruskal-Wallis test was performed to determine if there were statistically significant differences between groups ($\alpha = 0.05$) (Kruskal & Wallis, 1952) and the Mann-Whitney test was performed to make pairwise groups comparisons ($\alpha = 0.05$) (Mann & Whitney, 1947). All the conclusions were obtained with this significance level. The NOEC and LOEC results were also determined according to the statistical analysis described.

A detailed description will be given in the next chapters.

III. *Chlorella vulgaris* growth inhibition assay

3.1. *Chlorella vulgaris* assay overview

Microalgae are an important class of test organisms for ecotoxicity studies since they belong to the first level of the trophic chain (they incorporate solar energy, to produce organic matter and oxygen, and serve as food for animals), which means that any disturbance in their dynamics might affect the higher levels of the ecosystem (Elser *et al.*, 2001).

Microalgae are involved in many hazard assessment schemes as representatives of the aquatic plant community. Apart from being very sensitive to changes in their environment (Ma, 2005), microalgae allow the evaluation of pollutants effects over several generations, due to their short life cycle. Microalgae species demonstrated to be suitable for the prospective assessment of the potential cytotoxicity of major aquatic pollutants classes which indicates that they are good biosensors in aquatic toxicity evaluation (Buckova *et al.*, 2017; Prado *et al.*, 2015). In addition, the ease of cultivation and handling in laboratory conditions, as well as well standardized testing protocols make microalgae a preferential candidate to be used as model organism in ecotoxicity testing (OECD, 2006b). Several parameters can be measured to assess the effects of toxicants on microalgae, being culture growth and photosynthetic activity commonly evaluated (Buckova *et al.*, 2017; Campanella *et al.*, 2001).

The main objective of the algae growth inhibition test is to determine the effects of a chemical on a green algae population growing exponentially in a nutrient-enriched medium for 72 or 96 hours. Cell density is determined using either a direct measurement (microscope counting) or one of several indirect techniques (spectrophotometric method or electronic particle counter) and it is used to understand the changes on the growth rate of the test population when exposed to test solutions (Walker *et al.*, 2012).

Chlorella vulgaris is a green eukaryotic unicellular microalgae that grows in freshwater ecosystems (Safi *et al.*, 2014). *C. vulgaris* demonstrated high sensitivity to different classes of pollutants (Ma *et al.*, 2004). The use of *C. vulgaris* as test organism is considered an economic and easy strategy since they are easily cultured and handled in laboratory (Silva *et al.*, 2009). It is a commonly used organism as representative of the aquatic producers' trophic level. It has been extensively used in the assessment of the toxic effects of chemicals through the evaluation of cell culture growth, photosynthetic activity, response to oxidative stress or gene transcription analysis (Qian *et al.*, 2009; Smutná *et al.*, 2014). It is a frequently used specie in the ecological evaluation of different compounds,

such as pesticides (Qian *et al.*, 2009), pharmaceuticals (Geiger *et al.*, 2016), nanoparticles (Smutná *et al.*, 2014), water effluents (Silva *et al.*, 2009) and in the toxic evaluation of emerging chemicals class such as ILs (Ventura *et al.*, 2010). It has also been applied in biotechnology, in the development of novel biosensors based on immobilised whole cell (Chouteau *et al.*, 2004).

3.2. Objectives

The main goals of the *C. vulgaris* growth inhibition assay were:

i. to determine the EC50 (concentration that inhibits 50% of the culture growth) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 96 hours of exposure (96h EC50);
ii. to determine the NOEC and LOEC values of [C16Pyr][Cl] and [C2OHMIM][Cl] after 96 hours of exposure (96h NOEC and 96h LOEC).

3.3. Materials and Methods

3.3.1. Assay

The test followed the OECD guidelines for testing of chemicals number 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (OECD, 2006b). The purpose of this test was to determine the effects of the compounds on the growth of freshwater microalgae *C. vulgaris*. Growth inhibition was quantified from measurements of the algal biomass as a function of time by optical density. The endpoints assessed were the EC50 (effective concentration that causes a 50% inhibition in the algae growth) and the NOEC and LOEC values at the end of the test period (96 hours).

3.3.2. *Chlorella vulgaris* handling

The ecotoxicity tests were carried out with the freshwater unicellular green algae *C. vulgaris*. The cultures of the algae *C. vulgaris* were prepared and maintained in a sterile environment, with a 14h light: 10h dark photoperiod and constant temperature (21 ± 1º C) in Z8 culture medium (Appendix I) (Kotai, 1972). The algae cell culture used for testing was in the exponential growth phase at the start of the test.

3.3.3. Test solutions

Test solutions were obtained by serial dilutions of the stock solution in Z8 culture medium, arranged in a geometric series with a separation factor of 10.0 in the range-finding.

Seven concentrations of the ILs were tested in the definitive test. The concentrations tested are described in Table 5 and were based on the results from the preliminary test.

Table 5 – Concentrations range tested in the *C. vulgaris* growth inhibition assay.

Test organism	**IL**	**Concentrations range tested (mM)**
C. vulgaris	[C16Pyr][Cl]	0.00047; 0.00149; 0.005; 0.015; 0.049; 0.156; 0.5
	[C2OHMIM][Cl]	1.25; 2.5; 5; 10; 20; 40; 80

3.3.4. Test procedure

Aseptic techniques were used in the algal culture preparation, handling and test procedure to avoid contamination. All the glass material, micropipette tips and water used were sterilized at 121 °C for 20 minutes in an autoclave chamber. A laminar air flow chamber, Telstar model PV-30/70, was used to handle the culture and prepare the assay.

The *C. vulgaris* growth inhibition test was performed in 96-well microplates. Each well was filled with 180 µL of test solution, or Z8 culture medium for the control group, and 20 µL of *C. vulgaris* cell culture in the exponential growth phase (initial cell density between 10^5 to 10^6 cell mL^{-1}). Six replicates were performed for each test solution and control. The microplates were incubated for a period of 96 hours with a 14h light: 10h dark photoperiod, temperature of 21 ± 1 °C and with constant shaking (30 rpm). Biomass evaluation was done by daily optical density measurement at 450 nm since the beginning of the test (0 hours) until the end of the test period (96 hours), using a Biotek Synergy HT microplate reader. The test was performed three times. In each experiment, growth rate inhibition, relative to growth in the control, was calculated using optical density data (Ma *et al.*, 2004).

3.3.5 Statistical analysis

Growth assessment was performed by reading the optical density in microplate reader at 0, 24, 48, 72 and 96 hours and by further conversion into number of cells per milliliter using a predetermined linear regression equation (1):

$$y = (40000000 \times DO) - 1000000 \quad (r^2=0.998) \qquad (1)$$

The growth rate at 96 hours was calculated by the equation (2):

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \quad (2)$$

where μ_{i-j} represents the average growth between time i and j, $\ln X_i$ is the biomass at time i and $\ln X_j$ represents the biomass at time j.

The growth inhibition rate was determined by the equation (3):

$$\%I_r = \frac{\mu c - \mu T}{\mu c} \times 100 \quad (3)$$

where %I represents the inhibition of the growth rate, μc is the growth rate of the control and μT represents the growth rate of the applied treatment (OECD, 2006b).

The growth rate inhibition after 96 hours of exposure were plotted against test concentrations. The EC50 values were determined by statistical probit analysis with 95% confidence limits (α = 0.05) (Finney, 1952) using *IBM SPSS Statistics 25* software.

3.4. Results

The growth curves of both compounds reflect the variation of cell density over time, which makes possible to analyse the evolution of the cell culture growth in the presence of the tested ILs in a range of concentrations. The microalgae growth inhibition rate was determined on the basis of the algae growth rate related to the growth rate on the control group.

The graphics data are expressed as the mean of three independent experiments, with six replicates in each run. Error bars represent standard deviation (SD).

Statistically significant differences between groups are pointed out in each graphic. Each letter (a, b, c, …) corresponds to a set of groups with no significant differences found among them. Only the relevant significant differences for the results interpretation are pointed out.

3.4.1. Acute toxic effects of [C16Pyr][Cl] to *Chlorella vulgaris*

When exposed to [C16Pyr][Cl], *C. vulgaris* biomass varied over time with the increase of the [C16Pyr][Cl] concentration, as shown by the growth curves in Figure 7. This dose-

response relation is also shown by the growth rate and growth rate inhibition graphics (Figure 8, Figure 9 and Figure 10).

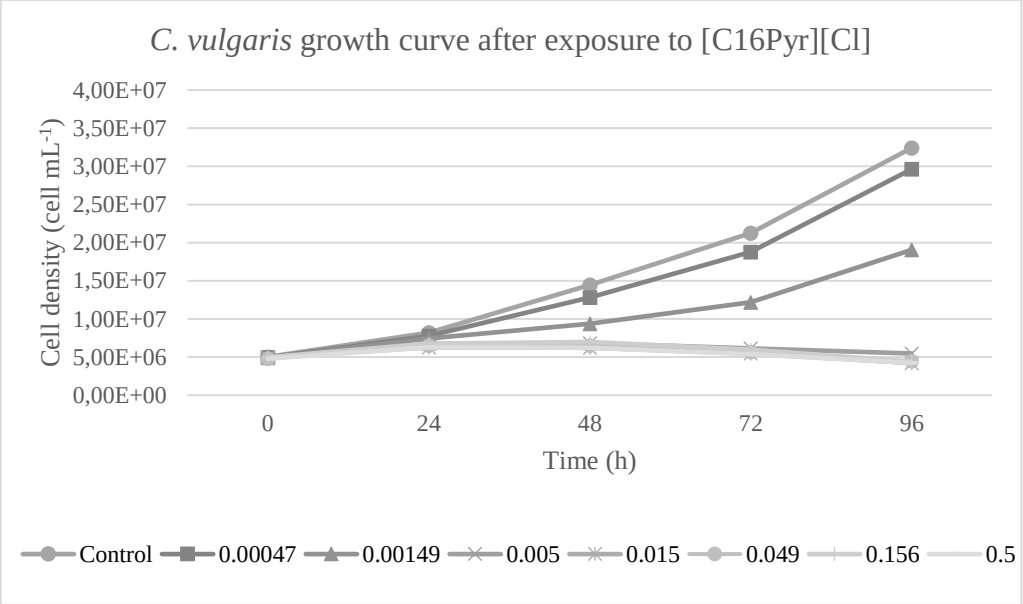

Figure 7- *C. vulgaris* growth curves, expressed as cell density over time, for different concentrations (mM) of [C16Pyr][Cl] and control group.

The results regarding *C. vulgaris* growth when exposed to [C16Pyr][Cl] showed that there is an increase in cell density at the control group and at lower concentrations (particularly, at the concentrations 0.00047 and 0.00149 mM) over time, as demonstrated in Figure 7. For all the tested concentrations, there is an increase of cell density in the first 48h of exposure, but there is a decline of cell density after 48h of exposure to [C16Pyr][Cl] to the 0.005 mM and higher concentrations.

The growth rate over the test period is shown in Figure 8. Here, it is possible to confirm the decrease of culture growth after 48 hours of exposure to the highest concentrations. The control group and lower concentrations (0.00047 and 0.00149 mM) demonstrated a continuous growth during the test period.

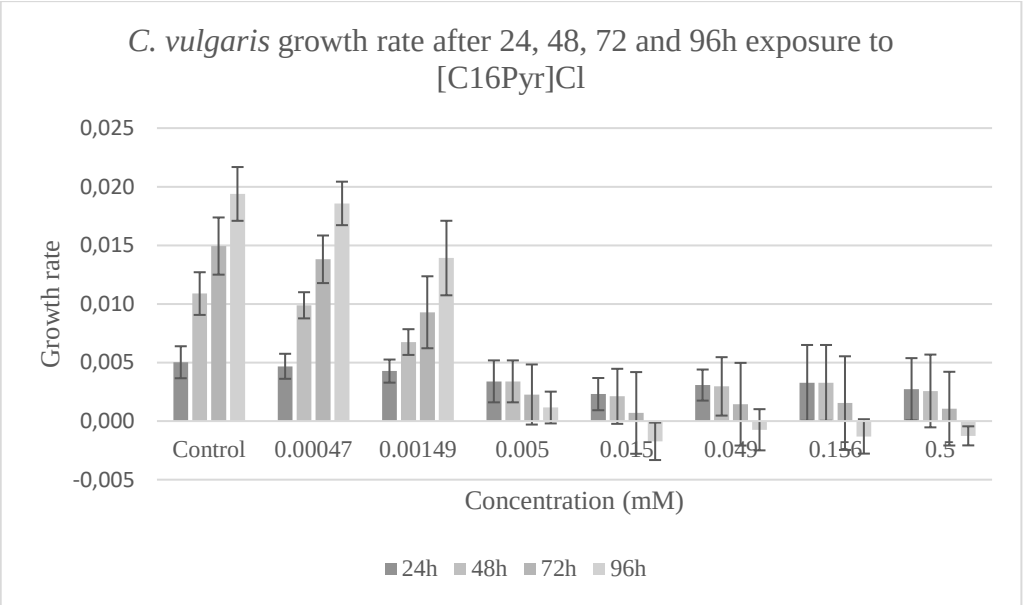

Figure 8 - *C. vulgaris* growth rate after 24, 48, 72 and 96 hours of exposure to [C16Pyr][Cl] and control group. Error bars represent SD.

The growth rate after 96h exposure to [C16Pyr][Cl] decreased with the increasing of the test concentration (Figure 9). It was noted that the growth rate for the culture exposed to the concentrations above 0.005 mM is negative. The Kruskal-Wallis test showed that there was a statistically significant difference (H=116.18, p<0.001) in the growth rate between the different concentrations. The Mann-Whitney test indicated that no significant differences were found between the control group and the growth rate of the culture exposed to the lowest concentration (0.00047 mM) (U=129.0, p=0.308) (groups marked as “a”). Also, no significant differences were found among the four highest concentrations (0.015, 0.049, 0.156 and 0.5 mM) (groups marked as “d”) (Figure 9).

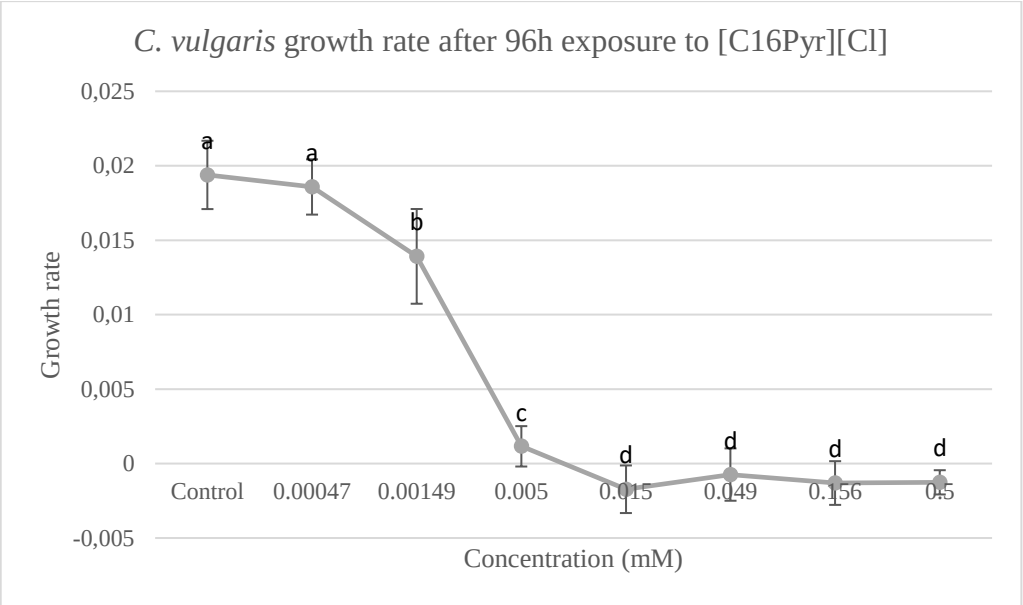

Figure 9 - *C. vulgaris* culture growth rate after 96 hours exposure to the tested concentrations of [C16Pyr][Cl] and control group. Error bars represent SD. a, b, c, d: no statistically significant differences found between groups.

The culture growth rate inhibition increases with the increasing of the [C16Pyr][Cl] concentration, as shown in Figure 10. The 100% growth rate inhibition was already reached at the 0.015 mM concentration.

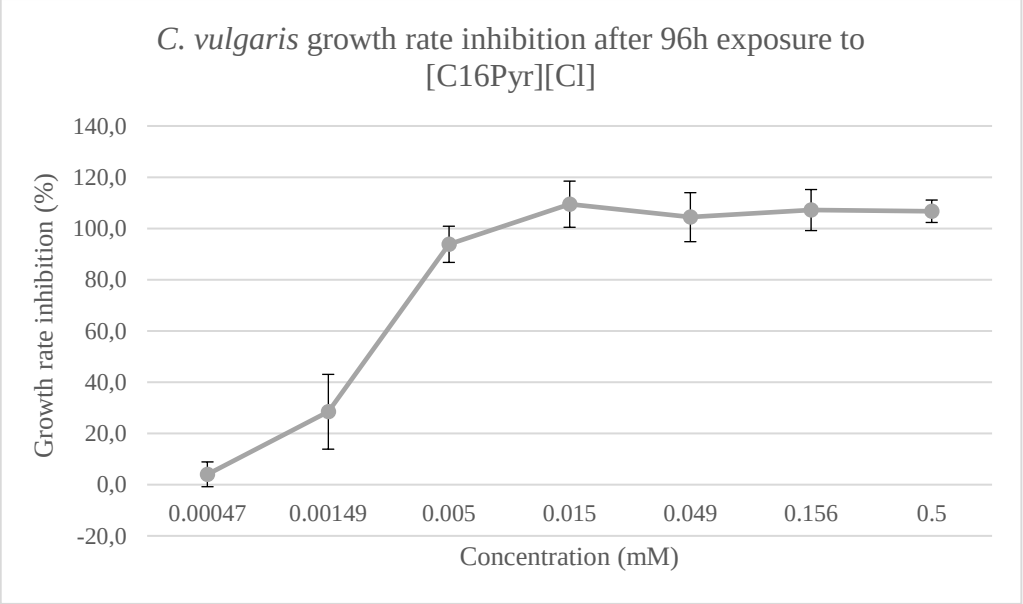

Figure 10 - *C. vulgaris* growth rate inhibition after 96h exposure to the [C16Pyr][Cl] test concentrations. Error bars represent SD.

The EC50 values obtained by the probit test in the three assays performed are shown in Table 6. The 96h EC50 values of [C16Pyr][Cl] varied between 0.003 and 0.015 mM with a mean 96h EC50 value of 0.011 mM, being classified as moderately toxic to *C. vulgaris*.

As seen in Table 7, the NOEC and LOEC values obtained were 0.00047 and 0.00149 mM, respectively.

3.4.2 Acute toxic effects of [C2OHMIM][Cl] to *Chlorella vulgaris*

In what concerns the exposure of *C. vulgaris* to [C2OHMIM][Cl], it was demonstrated that there is an increase of cell culture density over time (Figure 11), and growth rate (Figure 12) for all the treatments, from the beginning until the end of the test period, with the exception of the culture exposed to the 80 mM treatment, which showed a slightly decrease of cell density after 72 hours of exposure. This decrease of growth rate is seen in Figure 12.

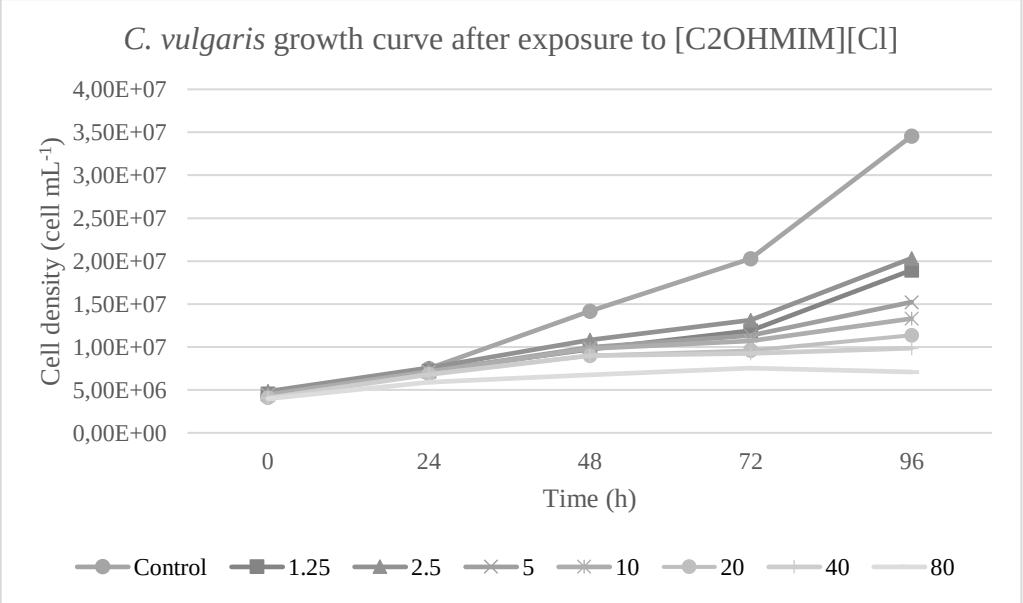

Figure 11 - *C. vulgaris* growth curves, expressed as cell density over time, for different concentrations (mM) of [C2OHMIM][Cl] and control group.

It was also evident that the growth curve is more accentuated at the control group and at lower concentrations. The curve slope decreases as the concentration increases (Figure 11). By the analysis of Figure 11, it is also possible to realize that the growth curve for the concentration 2.5 mM is in a higher position than for the 1.25 mM treatment. Although the cell culture exposed to concentration 2.5 mM had a higher cell density over time than the immediately lower concentration (1.25 mM), this is due to the fact that at the beginning of the test there was also a higher cell density, and not due to a stimulation of growth rate, which is confirmed by the growth rate graphic (Figure 13).

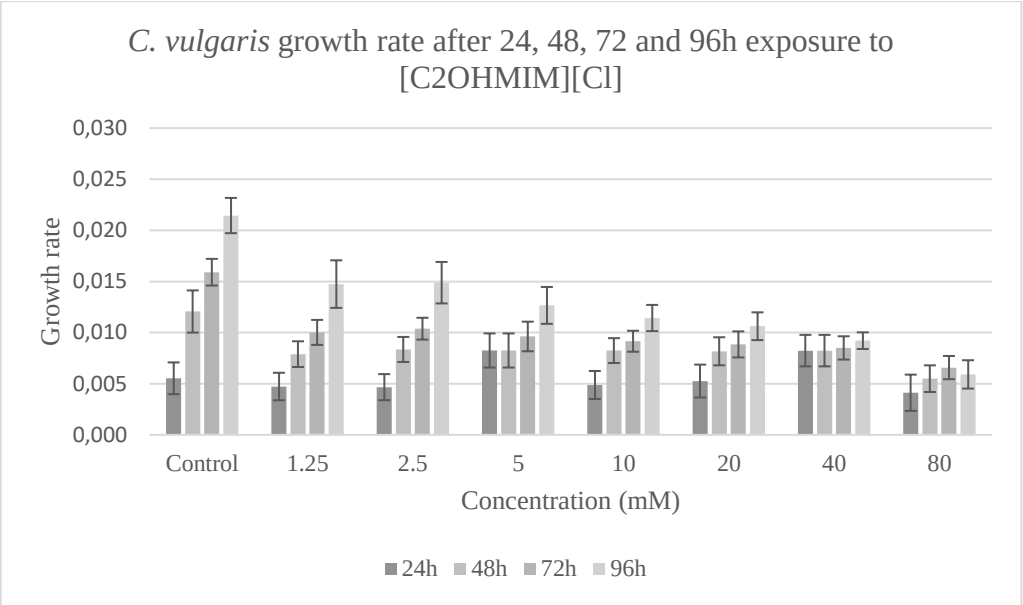

Figure 12 - *C. vulgaris* growth rate after 24, 48, 72 and 96 hours of exposure to [C2OHMIM][Cl] and control group. Error bars represent SD.

The growth rate after 96h exposure to [C2OHMIM][Cl] decreases with the increase of the test concentration (Figure 13). The control group had the highest growth rate.

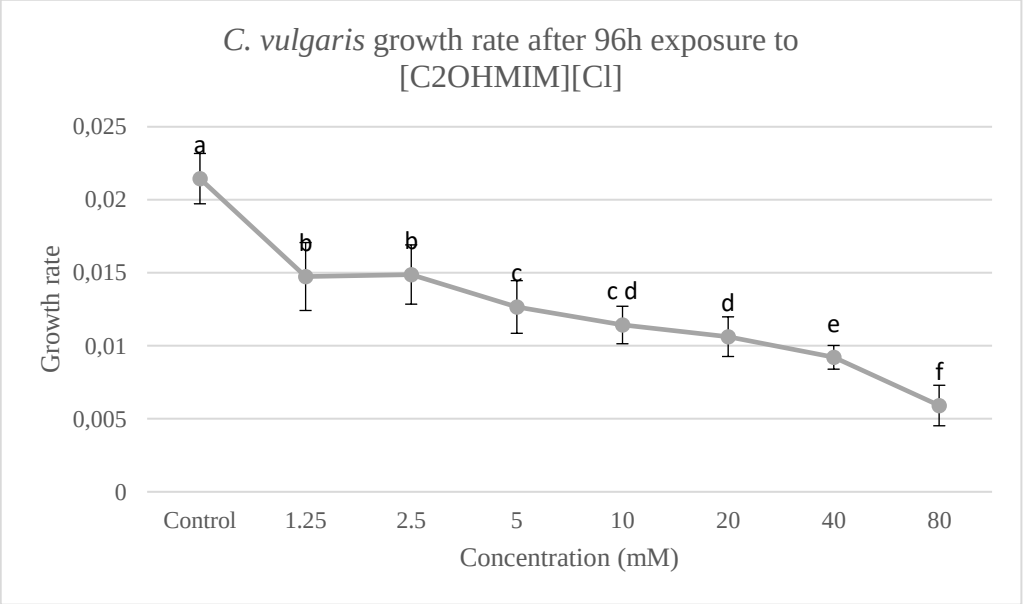

Figure 13 - *C. vulgaris* culture growth rate after 96 hours exposure to the tested concentrations of [C2OHMIM][Cl] and control group. a, b, c, d, e, f: no statistically significant differences found between groups. Error bars represent SD.

The culture growth rate inhibition increases with the increase of the [C2OHMIM][Cl] concentration, as shown in Figure 14. The 100% inhibitory effect was not reached with the tested concentrations.

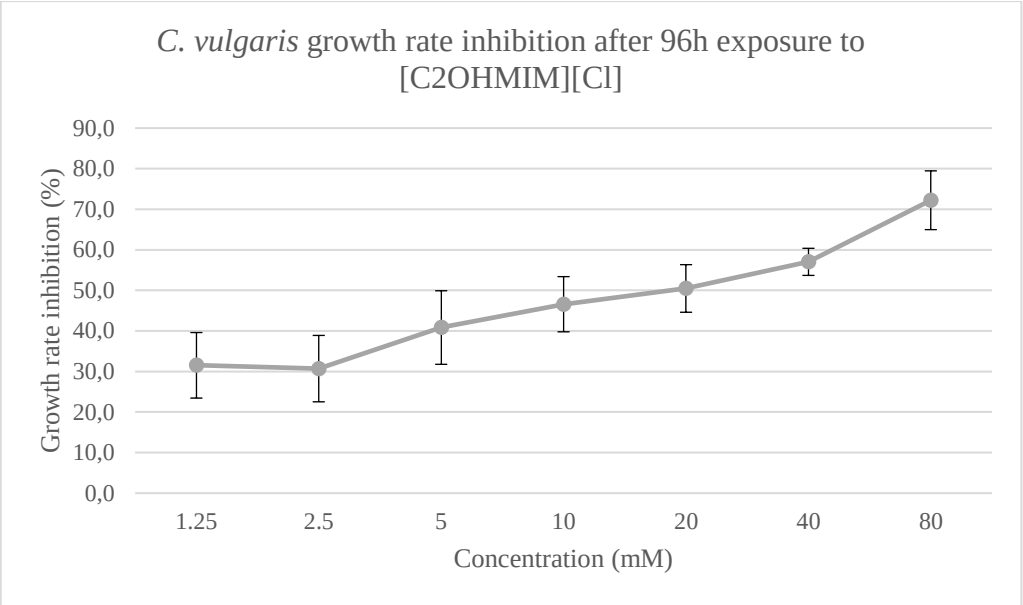

Figure 14 - *C. vulgaris* growth rate inhibition after 96h exposure to the [C2OHMIM][Cl] tested concentrations. Error bars represent SD.

The Kruskal-Wallis test showed that there was a statistically significant difference (H=122.25, p<0.001) in the growth rate between the different concentrations. The Mann-Whitney test indicated that there were significant differences between the control group and the growth rate of the culture exposed to all the tested concentration (p<0.05).

The EC50 values obtained in the three tests performed are shown in Table 6. The 96h EC50 values of [C2OHMIM][Cl] varied between 29.95 and 38.23 mM with a mean 96h EC50 value of 34.03 mM, which classifies it as relatively harmless to *C. vulgaris*. With the tested concentrations, it was not possible to determine the NOEC value, although the LOEC was assessed and corresponds to the 1.25 mM concentration (Table 7).

Table 6 - Acute toxic effects of [C16Pyr][Cl] and [C2OHMIM][Cl] to *C. vulgaris*. 96h EC50 values for the three assays performed are shown in mM.

ILs	**96h EC50 (mM)**	**95% Confidence interval**	**Mean 96h EC50 (mM)**	**Hazard Ranking***
[C16Pyr][Cl]	0.015 0.003 0.014	nd 0.002 - 0.004 nd	0.011 (4.654 mg L^{-1})	+++
[C2OHMIM][Cl]	29.95 33.91 38.23	-6.75 – 51.93 -32.02 – 64.87 -48.31 – 77.10	34.03 (5533.8 mg L^{-1})	-

*: The following standard was used for the 96h EC50 (mg L^{-1}) to evaluate the toxicity of the ILs to *C. vulgaris* according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987). nd: not possible to determine.

Table 7 - *C. vulgaris* NOEC and LOEC results after 96 hours exposure to [C16Pyr][Cl] and [C2OHMIM][Cl] .

ILs	**96h NOEC (mM)**	**96h LOEC (mM)**
[C16Pyr][Cl]	0.00047	0.00149
[C2OHMIM][Cl]	nd	1.25

nd: not possible to determine.

3.5. Discussion and Conclusion

Microalgae play an important role in the equilibrium of aquatic ecosystems. Since they belong to the first level of the trophic chain, the rest of the aquatic organisms are dependent upon the photosynthetic activity of these organisms. Thus, perturbations to its welfare have repercussions on higher levels of the ecosystem (Elser *et al.*, 2001). Because of their ecological importance and sensitivity to environmental changes (Ma, 2005), microalgae are used in toxicity testing.

The unicellular green microalga *C. vulgaris* was selected as test organism in the present study because it has a good sensitivity to toxicants (Ma *et al.*, 2004), it is easily cultured in laboratory and the tests are easy and economic. *C. vulgaris* growth rate was

assessed based on the assumption that growth rate is a better response variable than biomass (Nyholm, 1985).

According to the present results, [C16Pyr][Cl] have a negative effect towards the *C. vulgaris* growth. When exposed to [C16Pyr][Cl], *C. vulgaris* cell density decreased with the increase of the [C16Pyr][Cl] concentration. This dose-response relation was also shown by increasing growth rate inhibition with the increase of the concentration.

Apart from the initial cell density increase, it was noted a decline of cell density after 48h of exposure to [C16Pyr][Cl] to the 0.005 mM and higher concentrations, which may be due to the cumulative effect over time. It was also demonstrated that the growth rate for the culture exposed to the concentrations above 0.005 mM is negative, which means that the cell density at the end of the exposure period is lower than the cell density at the beginning of the test. This variation means that [C16Pyr][Cl] triggered cellular loss. It would be necessary an extension of the exposure period to reveal if the cell loss was permanent or if the culture would be able to recover.

Statistical analysis demonstrated that no significant differences were found on the toxic effect of the highest concentrations (0.015, 0.049, 0.156 and 0.5 mM). Therefore, it is possible to conclude that, for the test conditions, the maximum toxic effect to *C. vulgaris* after 96 hours exposure to [C16Pyr][Cl] was reached at the 0.015 mM concentration.

There are no previous published results for microalgae response to [C16Pyr][Cl] apart from the REACH evaluation results which pointed a 72h EC50 for *Pseudokirchneriella subcapitata* of 0.075 mM (Environmental Chemicals Agency, n.d.a). In comparison, our results demonstrated a lower EC50 value, which may be due to different organism sensitivity. If so, *C. vulgaris* is more sensitive to [C16Pyr][Cl] than *P. subcapitata*.

Among the published data about pyridinium based ecotoxicity to microalgae, *P. subcapitata* and *Scenedesmus vacuolatus* are the most studied species. The EC50 values are diverse and dependent on the species, IL tested and time of exposure (Cho *et al.*, 2008; Stolte *et al.*, 2007; Wells & Coombe, 2006). Therefore, the comparison between the present results and previous works is difficult. The published EC50 varied between 0.0004 and 5.012 mM (Cho *et al.*, 2008; Pretti *et al.*, 2009; Stolte *et al.*, 2007; Wells & Coombe, 2006).

Pretti *et al.* (2009) evaluated the acute toxicities of eighteen ILs, including a pryridinium based IL (butylpyridinium bis(triflimide)), for the microalgae *P. subcapitata*.

The results showed an EC50 of 0.0169 mM which may be considered as slightly toxic to the organism (Pretti *et al.*, 2009). In a different study, Stolte *et al.* (2007) investigated the effects of different head groups and functionalised side chains on the aquatic toxicity of ILs. The study was performed in three different aquatic organisms, including the microalgae *S. vacuolatus*. Forty ILs with different head groups, side chains and anions combinations were analysed, including the compounds with the aromatic head group 4-(dimethylamino)pyridinium and pyridinium. For the microalgae *S. vacuolatus* it was found a drastic toxicity for 1-butyl-4-(dimethylamino)pyridinium (Stolte *et al.*, 2007).

In the [C2OHMIM][Cl] toxic assessment towards *C. vulgaris*, it was demonstrated its negative effect towards the culture growth. When exposed to [C2OHMIM][Cl], *C. vulgaris* cell density decreased with the increase of the concentration. This dose-response relation was also shown by the increasing growth rate inhibition values.

It was demonstrated that there was an increase of culture cell density over time for all the treatments, except for the culture exposed to the 80 mM treatment, which showed a slightly decrease of cell density after 72 hours of exposure. Further extension of the exposure period would reveal if this decline in cell density was significant and could lead to higher cellular loss. The growth rate after 96h exposure to [C2OHMIM][Cl] was also affected by the increasing test concentration. The growth rate decreased with the increase of the test concentration, which proves that higher doses slow down the culture growth.

Some studies have revealed the effects of imidazolium based ILs towards microalgae (Cho *et al.*, 2008; Kulacki & Lamberti, 2008; Matzke *et al.*, 2007; Pretti *et al.*, 2009; Wells & Coombe, 2006; Zhang *et al.*, 2017) . The EC50 values range is very wide and the imidazolium based ILs are classified from practically harmless to highly toxic to algae species. The species most frequently used are *S. vacuolatus* and *P. subcapitata* (Cho *et al.*, 2008; Matzke *et al.*, 2007; Pretti *et al.*, 2009; Wells & Coombe, 2006). *C. vulgaris* has also been used to assess the effects of imidazolium based ILs by some authors (Latała *et al.*, 2009; Zhang *et al.*, 2017).

Latala et al (2009) assessed the toxicity of 1-alkyl-3-methylimidazolium ILs towards *C. vulgaris*. The EC50 values varied from 0.00368 to 6.33051 mM and were correlated with the length of the alkyl chain, since the lowest EC50 values were correlated with longer alkyl chains. The results also demonstrated that the anion tetrafluoroborate was the most toxic and dicyanamide the least toxic anion to *C. vulgaris* (Latała *et al.*, 2009).

Zhang *et al.* (2017) evaluated the acute toxic effects of 1-alkyl-3-methylimidazolium nitrate ILs with different alkyl chain length on *C. vulgaris*. In their study, they found out that as the alkyl chain length increased, also increased the toxic potential of the compounds. Therefore, the IL with the shorter alkyl chain, $[C_2mim]NO_3$, showed the highest EC50 value (96 h EC50, 637.4 mg L^{-1}) and was considered slightly toxic to the organism (Zhang *et al.*, 2017). This result is in accordance with our results, since [C2OHMIM][Cl] also has a two carbon alkyl chain (n = 2) and showed low toxic effect towards *C. vulgaris* growth, being classified as practically harmless to *C. vulgaris*. The lower toxic effect observed in our work, comparing with Zhang results, may be due to the incorporation of a hydroxyl group to the alkyl chain, which is known as a factor able to reduce ILs toxicity (Pretti *et al.*, 2009). Likewise, the halide chloride present in our tested compound is also related to lower toxicity effects, in comparison with NO_3 present in Zhang´s tested ILs (Cho *et al.*, 2008).

Pretti *et al.* (2009) studied the effect of [C2OHMIM][Tf2N] (3-(2-hydroxyethyl)-1-methylimidazolium bis-triflimide) on the growth of the microalgae *P.subcapitata*. The 72h EC50 obtained was 0.1392 mM and was classified as moderately toxic. This value is much lower than the EC50 obtained in the present study. It may be due to the different configuration of the molecule or to the different anion present in the structure of the molecule, since it was shown that Tf2N anion increases the toxicity of compounds, compared to the halides (Pretti *et al.*, 2009). Also, it has been demonstrated that the halide chloride is one of the anion with less toxic influence on the organisms (Matzke *et al.*, 2007). In the same work, Pretti evaluated the effect of $[C_3OHmim][Cl]$ on the growth of the microalgae *P.subcapitata* (Pretti *et al.*, 2009). The 72h EC50 was 0.1694 mM and was also classified as moderately toxic. Apart from the anion composition, the two ILs differ in the side chain length. It is in accordance with previous results that correlate the longer alkyl chains with higher toxic effect (Zhang *et al.*, 2017).

The relation between the alkyl chain length of imidazolium based ILs and its toxicity towards algae is well-known (Cho *et al.*, 2007; Zhang *et al.*, 2017). Some studies have demonstrated the effects of alkyl chain length on the toxicity of imidazolium ILs towards various algae species, reporting that longer chain length exhibited stronger inhibition on the algal growth (Cho *et al.*, 2007; Latała *et al.*, 2009; Zhang *et al.*, 2017).

Comparing the toxicity of the two ILs tested in the present study towards *C. vulgaris*, it is possible to conclude that [C16Pyr][Cl] presents a higher toxic effect than [C2OHMIM][Cl].

IV. *Daphnia magna* acute immobilisation assay

4.1. *Daphnia magna* assay overview

Daphnia magna is a world widespread parthenogenetic planktonic invertebrate organism inhabiting freshwater ecosystems such as lakes and ponds (Bekker *et al.*, 2018). *D. magna* is used as a reference organism for regulatory toxicity testing of chemicals and the acute toxicity test with this species is standardized by international organizations (OECD, 2004; USEPA, 2002). In natural freshwater ecosystems, *D. magna* is an important link between microbiome and higher trophic levels (Dodson & Hanazato, 1995) and have been the focus of many physiological, evolutionary and ecological studies (Ashauer & Jager, 2018; Bekker *et al.*, 2018; Tatarazako & Oda, 2007).

Several characteristics make *D. magna* the ideal organism for ecotoxicological studies, such as its ease of cultivation and handling under laboratory conditions, high fertility rate, speed of the tests and reduced genetic variability due to parthenogenic reproduction (Guilhermino *et al.*, 2000; Lagadic & Caquet, 1998). Tests can be performed with organisms from laboratory cultures or hatched from dormant eggs (Persoone *et al.*, 2009). Several short-term or long-term biomarkers can be assessed, such as the swimming behaviour (Bownik, 2017; Dodson & Hanazato, 1995), growth (Flaherty & Dodson, 2005), reproduction (Cui *et al.*, 2017), embryonic development (Abe *et al.*, 2001), heart rate (Lovern *et al.*, 2007) and mortality/immobilisation (OECD, 2004). International organisations such as OECD have adopted the "immobility" criteria, which relates to the inability of the test organisms to swim within 15 seconds after gentle agitation (OECD, 2004). In the present work, the immobilisation criteria was taken as the mortality endpoint.

4.2. Objectives

The main goals of the *D. magna* acute immobilisation assay were:

i. to determine the EC50 (concentration that causes immobilisation of 50% of the daphnids) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 24 and 48 hours of exposure (24h EC50 and 48h EC50);
ii. to determine the NOEC and LOEC values of [C16Pyr][Cl] and [C2OHMIM][Cl] after 24 and 48 hours of exposure (24/48h NOEC and 24/48h LOEC).

4.3. Materials and Methods

4.3.1. Assay

The test was performed according to the OECD guidelines for testing of chemicals number 202: *Daphnia sp*. immobilisation test (OECD, 2004). The purpose of this test was to determine the effects of the compounds on the survival of the freshwater crustacean *D. magna*. Neonates less than 24 hours of age were exposed to the test solutions at a range of concentrations for a period of 48 hours. Immobilisation was recorded and compared with control group immobilisation values and the EC50 (concentration estimated to immobilise 50% of the juveniles) were determined after 24 and 48 hours of exposure to the test solutions.

4.3.2. *Daphnia magna* handling

All organisms used were originated from cultures established from the same stock of daphnids. *D. magna* cultures consist of glass containers holding Elendt M4 culture medium (Elendt & Bias, 1990) (Appendix II) with 10 daphnids per litre. Culture medium was renewed three times a week. Cultures were maintained at 21± 1°C under 14h light:10h dark photoperiod. Daphnids were fed daily with a suspension of the unicellular green alga *C. vulgaris*. The food concentration was about 5 x 10^5cell mL^{-1}. The food suspension was prepared by centrifugation of *C. vulgaris* culture at 2900 rpm for 10 minutes, to remove Z8 medium, and resuspension of the cellular pellet in Elendt M4 medium. Cell density was determined by Neubauer chamber cell counting.

4.3.3. Test solutions

Test solutions were obtained by serial dilutions of the stock solution in Elendt M4 medium, arranged in a geometric series with a separation factor of 10.0 in the range-finding preliminary test and 2.0 in the definitive test. Six test solutions were tested in the final definitive test. The concentrations tested in the definitive test are described in Table 8.

Table 8 - Concentrations range tested in the *D. magna* acute immobilisation assay.

Test organism	IL	Concentrations range tested (mM)
D. magna	[C16Pyr][Cl]	0.0000125; 0.000025; 0.00005; 0.0001; 0.0002; 0.0004
	[C2OHMIM][Cl]	0.156; 0.313; 0.625; 1.25; 2.5; 5

4.3.4. Test procedure

Tests were conducted as 48 hours static acute tests and *D. magna* neonates (aged less than 24 hours) born from parthenogenic females were used. In order to reduce variability, first and second brood progenies were discarded and only succeeding brood progenies were used for testing. Absence of signs of stress was checked and all organisms used for a test were originated from cultures established from the same stock culture. Stock was maintained in the same culture conditions (light, temperature and medium) used in the test.

The acute toxicity test was performed in 100 mL beakers, each containing 25 mL of test solution, or Elendt M4 medium for the control group. Five neonates were placed in each of the glass beakers. Four replicates for each of the six treatment solutions (control plus five test concentrations) were performed. The test was conducted at 21 ± 1 °C with a 14h light:10h dark photoperiod. The organisms were not fed during the entire test period. Neonates were inspected for mortality after 24 and 48 hours of exposure. The juveniles without detectable movement after 15 seconds light stimulus were considered to be dead (OECD, 2004).

4.3.5. Positive control test with the reference toxicant ($K_2Cr_2O_7$)

To evaluate the sensitivity of the test organisms, a standard test with $K_2Cr_2O_7$ was performed previously to the ILs testing. The test was performed in the same conditions as the ILs test (as described above) and the $K_2Cr_2O_7$ concentrations tested were 0.31; 0.625; 1.25; 2.5; 5. As acceptability criteria, the EC50 obtained to the reference toxicant had to be within the range 0.6 to 2.1 mg L^{-1} (ISO, 2012). If so, the organisms were considered to have an acceptable sensitivity to proceed with the tests.

4.3.6. Statistical analysis

The percentage of daphnids immobilised at 24 and 48 hours observations were plotted against test concentrations. Data was analysed by probit analysis to calculate the EC50 with 95% confidence limits (α = 0.05) (Finney, 1952) using *IBM SPSS Statistics 25* software.

4.4. Results

D. magna was used to test [C16Pyr][Cl] and [C2OHMIM][Cl] acute toxic effect on freshwater crustacean. The *D. magna* sensitivity to $K_2Cr_2O_7$ assessment results are also presented.

Statistically significant differences between groups are pointed out in each graphic. Each letter (a, b, c, …) corresponds to a set of groups with no significant differences found among them. Only the relevant significant differences for the results interpretation are pointed out.

4.4.1. Sensitivity of *Daphnia magna* to the reference toxicant ($K_2Cr_2O_7$)

In the sensitivity assessment of *D. magna* to the reference toxicant ($K_2Cr_2O_7$), it was not observed immobilised individuals in the control group (Figure 15). The 24 h EC50 for the $K_2Cr_2O_7$ to *D. magna* was 0.863 mg L^{-1}, with a 95% confidence interval of 0.717 mg L^{-1} to 1.034 mg L^{-1} (Table 9). This result was within the range of acceptability criteria, 0.6 mg L^{-1} to 2.1 mg L-1 (ISO, 2012). Thus, the sensitivity of *D. magna* demonstrated to be acceptable to proceed with the tests.

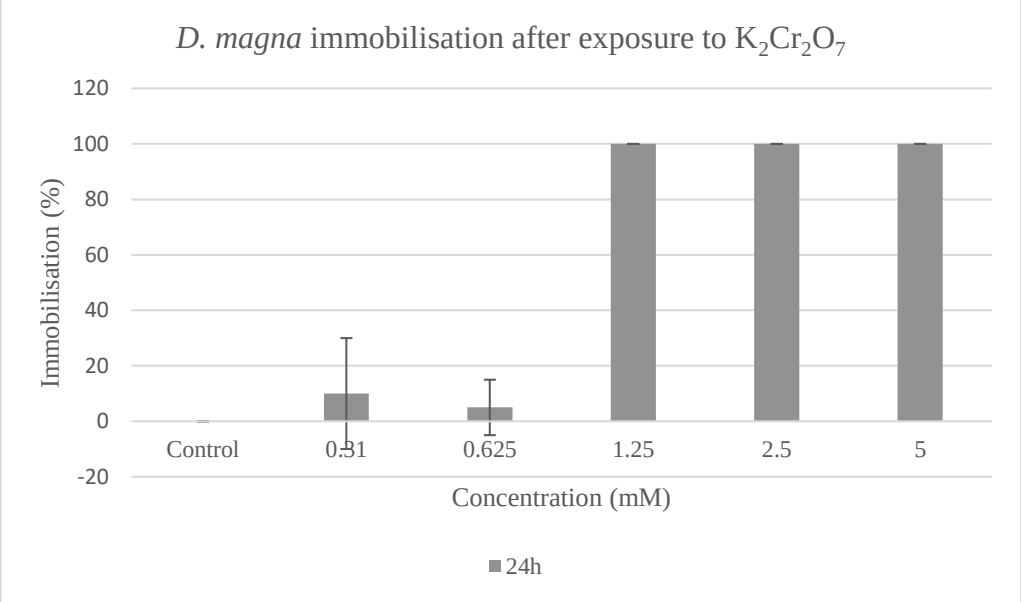

Figure 15 - *D. magna* immobilisation data of the exposure to the reference toxicant, $K_2Cr_2O_7$, for 24 hours. Error bars represent SD.

Table 9 - *D. magna* EC50 after 24h exposure to the reference toxicant, $K_2Cr_2O_7$, and acceptability criteria.

Reference toxicant	**24h EC50 (mg L^{-1})**	**95% Confidence intervals (mg L^{-1})**	**Acceptability criteria (mg L^{-1})***
$K_2Cr_2O_7$	0.863	0.717 – 1.034	0.6 – 2.1

* Acceptability criteria according to (ISO, 2012).

4.4.2. Acute toxic effects of [C16Pyr][Cl] to *Daphnia magna*

The effect of [C16Pyr][Cl] towards *D. magna* immobilisation was assessed after 24 and 48 hours of exposure to a range of concentrations. The 24 and 48 hours immobilisation data is illustrated in Figure 16.

In the daphnids exposed to the [C16Pyr][Cl] solutions, it was observed that the immobilisation percentage increases with the increase of the [C16Pyr][Cl] concentration. It was also demonstrated that the immobilisation percentage is higher at the 48 hours observations than it was at 24 hours. After 24 hours of exposure, the concentrations 0.0002 and 0.0004 mM reached 100% immobilisation. After 48 hours of exposure, all the four highest concentrations (0.00005, 0.0001, 0.0002, 0.0004 mM) also presented 100% immobilisation.

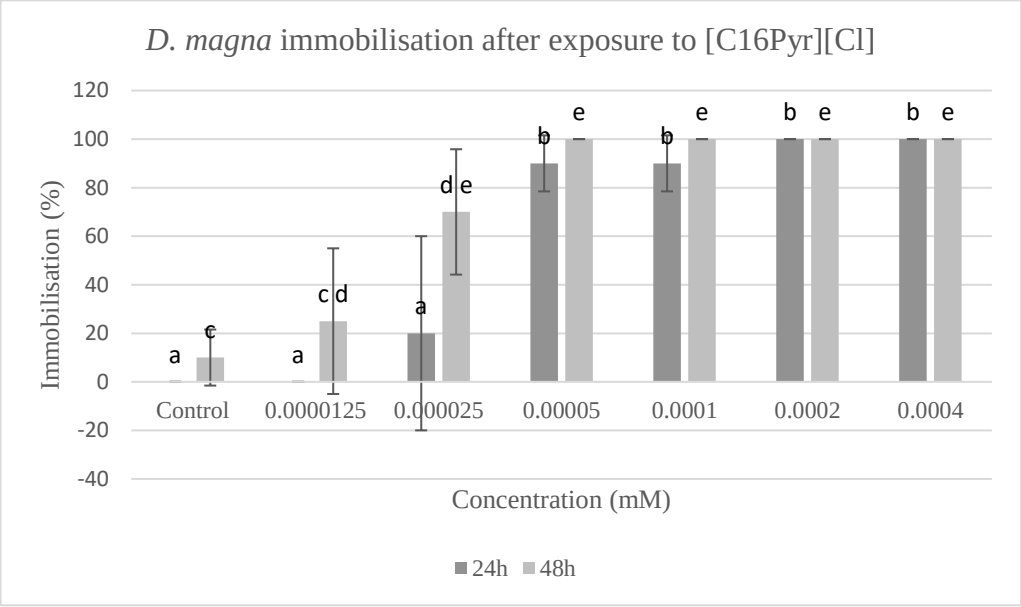

Figure 16 - *D. magna* immobilisation data after 24h and 48h exposure to [C16Pyr][Cl]. Error bars represent SD. a, b, c, d, e: no statistically significant differences found between groups.

The Kruskal-Wallis test showed that there was a statistically significant difference in the immobilisation between the different concentrations after 24 hours (H=23.677, p<0.001) and after 48 hours (H=24.353, p<0.001) of exposure to the [C16Pyr][Cl] concentration. After

24 hours, the Mann-Whitney test indicated that there were no significant differences found between the control group and the immobilisation percentage of daphnids exposed to the two lowest concentration (0.0000125 and 0.000025 mM) (U=8.0, p=1.0 and U=6.0, p=1.0, respectively) (groups marked as "a" in Figure 16). After 48 hours of exposure, no significant differences were found in the immobilisation percentage of daphnids exposed to the control group and to the lowest concentration (0.0000125 mM) (U=6.0, p=0.657) (groups marked as "c" in Figure 16).

The 24h EC50 and 48h EC50 values obtained by the probit test are shown in Table 10. The 24h EC50 and 48h EC50 of [C16Pyr][Cl] was 0.0000449 and 0.0000205 mM, respectively, which classifies it as extremely toxic after 24 hours and super toxic after 48 hours to *D. magna*. As seen in Table 11, the 24h NOEC and 24h LOEC values obtained were 0.000025 and 0.00005 mM, respectively. The 48h NOEC and 48h LOEC values were 0.0000125 and 0,000025 mM, respectively.

4.4.3. Acute toxic effects of [C2OHMIM][Cl] to *Daphnia magna*

The effect of [C2OHMIM][Cl] towards *D. magna* immobilisation was assessed after 24 and 48 hours of exposure to a range of concentrations. The 24 and 48 hours immobilisation data is demonstrated in Figure 17. It was observed that the immobilisation percentage tends to increase over time. The 100% immobilisation was reached after 48 hours of exposure to the 5 mM concentration. The dose-response relation (Figure 17) and the statistical analysis performed showed that no significant differences in the immobilisation were found among the concentrations tested, with the exception of the highest concentration tested (5 mM).

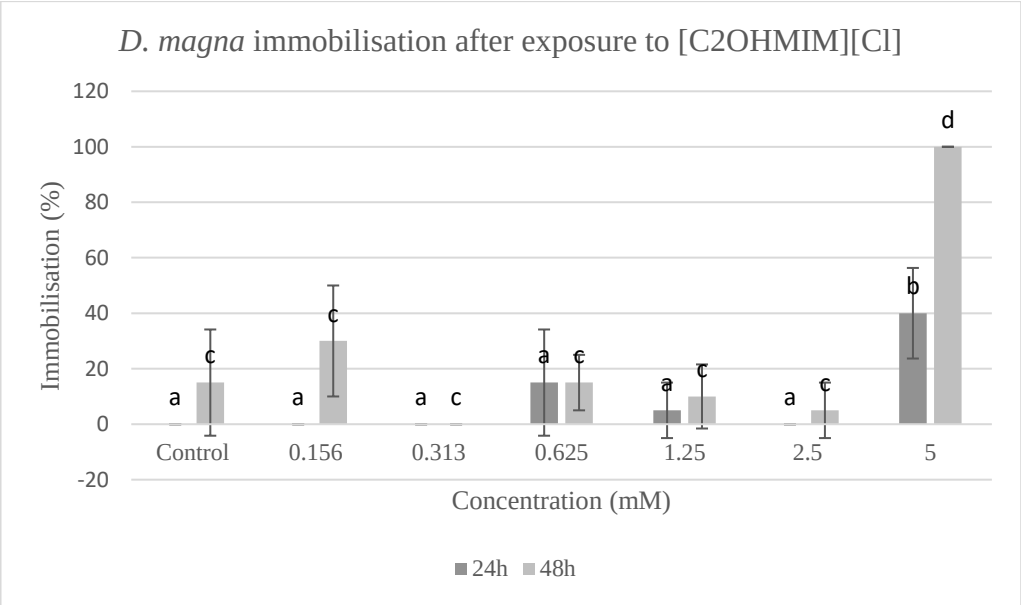

Figure 17 - *D. magna* immobilisation data after 24h and 48h exposure to [C2OHMIM][Cl]. Error bars represent SD. a, b, c, d: no statistically significant differences found between groups.

The Kruskal-Wallis test showed that there was a statistically significant difference in the immobilisation between the different concentrations after 24 hours (H=119.115, p=0.001) and after 48 hours (H=16.787, p=0.002) of exposure to [C2OHMIM][Cl]. The Mann-Whitney test indicated that, after 24 and 48 hours of exposure, there were no significant differences found between the control group and the immobilisation percentage of daphnids exposed to the four lowest concentration. Although, there were significant differences between the control and the highest concentration (5 mM) (U=0.0, p=0.029). All the other groups had no significative differences found among them (p<0.005) (groups marked as “a” and “c” in Figure 17).

The 24h EC50 and 48h EC50 values obtained by the probit test are shown in Table 10. The 24h EC50 and 48h EC50 of [C2OHMIM][Cl] was 5.257 and 3.635 mM, respectively, which classifies it as practically nontoxic to *D. magna*. As seen in Table 11, the NOEC and LOEC values obtained after 24 hours of exposure are the same for the 48 hours exposure period. Thus, the NOEC and LOEC results from the present study, for 24 and 48 hours exposure period, were 2.5 and 5.0 mM, respectively.

Table 10 - The median effect concentration (EC50), 95% confidence intervals and the hazard ranking to *D. magna* of [C16Pyr][Cl] and [C2OHMIM][Cl] in the 24 and 48 hours acute immobilisation assay.

ILs	**EC50 (95% confidence interval) (mM)**		**EC50 (mg L^{-1})**		**Hazard ranking***
	24h	**48h**	**24h[a]**	**48h[b]**	
[C16Pyr][Cl]	0.0000449 (0.000025–0.00007)	0.0000205 (0.000009–0.000029)	0.016	0.007	+++++ [a] ++++++ [b]
[C2OHMIM][Cl]	5.257 (nd)	3.635 (2.289–8.905)	854.90	591.12	+ [a, b]

The values in parentheses are the 95% confidence intervals. *: The following standard was used for the EC50 (mg L^{-1}) to evaluate the toxicity of the ILs to *D. magna* according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987). nd: not possible to determine. "a" is related to the 24h results and "b" to the 48h results.

Table 11 - *D. magna* NOEC and LOEC results after 24 and 48 hours of exposure to [C16Pyr][Cl] and [C2OHMIM][Cl] .

ILs	**24h**		**48h**	
	NOEC (mM)	**LOEC (mM)**	**NOEC (mM)**	**LOEC (mM)**
[C16Pyr][Cl]	0.000025	0.00005	0.0000125	0.000025
[C2OHMIM][Cl]	2.5	5.0	2.5	5.0

4.5. Discussion and Conclusion

Daphnia magna are filter-feeding planktonic freshwater crustaceans, broadly distributed in freshwater bodies (Bekker *et al.*, 2018), often used as model organisms in standard toxicity bioassays because they are easily cultured in the laboratory and are sensitive to a variety of pollutants (Guilhermino *et al.*, 2000). Moreover, they reproduce by parthenogenesis, so individuals derived from a single animal are genotypically identical (Hebert & Ward, 1972).

Because of their trophodynamic role, small invertebrates are often critical components of ecosystems. These animals are often the dominant herbivores in lakes and ponds. Thus, they play a key role in determining water quality by feeding on algae and, because of their value on the diet of fish, they plays an important role in the ecosystem dynamic (Dodson & Hanazato, 1995).

Guilhermino *et al.* (2000) have validated the use of *Daphnia spp.* as model organism for toxicological studies of several toxicants. They found a high correlation between the

acute toxicity of a variety of chemical compounds to the crustacean *D. magna* and the corresponding LC50 values (effective concentration that causes 50% mortality) for rat, a superior vertebrate commonly used as model organism. The results showed that *D. magna* test seems to have a predictive capacity comparable to that of mammalian cytotoxicity tests, which provides good evidence of the applicability of using invertebrate tests as pre-screening methods (Guilhermino *et al.*, 2000).

In the present study *D. magna* was used as a representative organism, from the freshwater ecosystem and the primary consumers trophic level, to assess the potential toxic effect of the pyridinium based IL, [C16Pyr][Cl]. For the populations of daphnids exposed to the [C16Pyr][Cl] solutions, it was observed a dose-response relation in the organisms' immobilisation. It was also demonstrated that the immobilisation percentage increases over time. The 100% immobilisation was reached after 24 hours of exposure to the 0.0002 mM and after 48 hours exposure to the 0.00005 mM. The EC50 results were also lower after 48 hours of exposure, comparing with the 24 hours observations. These results could be caused by the cumulative effect of the compound, which may have enhanced the acute toxic effects on the organisms.

There is not much literature data about [C16Pyr][Cl] toxic effect towards *D. magna*. The REACH regulation estimated a 24h EC50 of 0.027 mM and a 48h EC50 of 0.026 mM, which are higher values than the one resulting from the present study (Environmental Chemicals Agency, n.d.a). However, the testing methodology is not provided.

Although there is not many research about [C16Pyr][Cl] ecotoxicity, some authors have studied the effects of some pyridinium based ILs towards the crustacean *D. magna* (Couling *et al.*, 2006; Pretti *et al.*, 2009; Wells & Coombe, 2006). The hazard ranking of results is very wide and depends on each particular compound.

Pretti *et al.* (2009) evaluated the acute toxicities of eighteen ILs, including a pryridinium based IL (butylpyridinium bis(triflimide)), for three representative freshwater organisms, including the crustacean *D. magna*. Their results demonstrated an EC50 of 0.0042 mM for *D. magna* which can be considered slightly toxic (Pretti *et al.*, 2009). Couling *et al.* (2006) used previously published *D. magna* toxicity data as well as a set of experimental results from a 48h acute static test to assess the factors that govern the toxicity of a range of different ILs to *D. magna* by QSAR models. Among the pyridinium based ILs tested, 1-hexyl-4-piperidinopyridinium bromide demonstrated to be the most toxic to *D.*

magna (48h EC50 of 0.0002 mM) while 1-butyl-3,5-dimethylpyridinium bromide was the least toxic (48h EC50 of 0.0977 mM) (Couling *et al.*, 2006). Comparing with our results, [C16Pyr][Cl] (48h EC50 of 0.0000205 mM) seems to be more toxic to *D. magna* than the most toxic pyridinium based IL assessed by Couling. Wells and Coombe (2006) analysed the freshwater ecotoxicity and biodegradation properties of some common ILs. In this study, the pyridinium based IL 1-butylpyridinium chloride was reported to show an 48h EC50 of 0.1165 mM, which classifies it as slightly toxic to *D. magna* (Wells & Coombe, 2006).

The effect on *D. magna* immobilisation of the imidazolium based IL ([C2OHMIM][Cl]) was also evaluated in the present work. In the control group and in the lowest concentration exposure groups, no significant differences were noted. This result could be caused by the presence of insufficiently high concentrations able to cause immobilisation. Only the highest concentration tested (5 mM) demonstrated some significant difference on immobilisation percentage comparatively to the control group, for both observation periods (24 and 48 hours). [C2OHMIM][Cl] presented 100% immobilisation of population for the highest concentration (5 mM) exposure group after 48 hours. The probit analysis demonstrated that the EC50 was lower for the 48 hours observation, which indicates that the exposure time had negative influence on the daphnids' immobilisation, proving to be a factor that could enhance the toxic effects with the tested concentrations.

Several papers have been published concerning the ecotoxic effect of imidazolium based ILs towards *D. magna* (Bernot *et al.*, 2005; Garcia *et al.*, 2005; Pretti *et al.*, 2009; Samorì *et al.*, 2010; Samorì *et al.*, 2007; Wells & Coombe, 2006; Yu *et al.*, 2009; Zhang *et al.*, 2017). From these studies, it is possible to conclude that the toxic effect of imidazolium based ILs towards *D. magna* are wide and related to ILs' molecular structure (Cho & Yun, 2016; Zhang *et al.*, 2017).

Even though the literature data about imidazolium based ILs ecotoxicity to *D. magna* is vast (comparing with other classes of ILs and other organism), none of these have assessed the effect of [C2OHMIM][Cl]. The most similar compound to [C2OHMIM][Cl] being studied towards *D. magna* was [C2OHMIM][Tf2N] and it was evaluated by Pretti et al (2009). It showed a 48h EC50 superior to 0.245 mM, which classifies it as practically harmless to *D. magna.* Even though our results points [C2OHMIM][Cl] also as practically harmless, it is not possible to make a full comparison with our results, since the anion of

both ILs are different and the results from Pretti reflect an interval for the EC50 value. In the same study, Pretti evaluated the [C3OHMIM][Cl] which showed a 48h EC50 of 0.3402 mM and it was classified as moderately toxic. This result is in agreement with our results, if we have in consideration that the toxicity is intrinsically related with alkyl chain length, increasing with the increase of the alkyl chain (Zhang *et al.*, 2017).

Comparing our results with other results from different imidazolium based ILs, we believe that [C2OHMIM][Cl] profit from the assumption that the introduction of an oxygenated side chain in the imidazolium cation can greatly reduce the toxicity of ILs (Samorì *et al.*, 2007).

Based on the present results from the *D. magna* acute immobilisation assay, it was possible to classify [C16Pyr][Cl] as super toxic and [C2OHMIM][Cl] as practically nontoxic to *D. magna*. Thus, the toxic effect is clearly higher in the pyridinium based ILs, which is not in agreement with Couling *et al.* (2006) conclusion that predicts that the cation imidazolium is related with higher toxicity levels towards *D. magna* than pyridinium (Couling *et al.*, 2006). Although, it does not take into consideration the rest of the molecular structure, which is also crucial in the assessment of ILs toxic behaviour, also pointed out by Couling and other authors (Biczak *et al.*, 2014; Cho & Yun, 2016; Couling *et al.*, 2006; Zhang *et al.*, 2017)

V. *Artemia salina* acute mortality assay

5.1. *Artemia salina* assay overview

Artemia spp. is a crustacean that lives in hypersaline aquatic environments and has wide geographic distribution. It feeds primarily on phytoplankton and it is an important primary consumer of these environments. It is also used as food for fish and aquatic invertebrates. *Artemia spp.* plays a key role in the aquatic environment and is frequently used as a saltwater biological model in ecotoxicology (Libralato *et al.*, 2016; Persoone & Sorgeloos, 1980).

Artemia spp. life cycle begins by the hatching of dormant cysts, small spherical-like structures of high physical and chemical resistance. The cysts can remain dormant for long periods, until they are activated, under favourable conditions (Gajardo & Beardmore, 2012).

Some individual characteristics as high adaptability to different testing conditions, like salinity (5-250 g L^{-1}) and temperature (5-40 °C), short life cycle, high adaptability to adverse environmental conditions, possibility of parthenogenetic reproduction strategy in some species and wide geographical distribution make this organism interesting to use in ecotoxicology. The large offspring production and its homogeneity due to parthenogenetic reproduction, the small body size that allows accommodation in small laboratory containers, the simple laboratory handling and the low cost of the tests make this species a suitable model organism for ecotoxicological tests (Gajardo & Beardmore, 2012; Nunes *et al.*, 2006).

Acute endpoints generally investigate the short-term toxic effect of compounds on the organisms. The *Artemia spp.* mortality assay is based on the ability of test compounds to kill laboratory-cultured *Artemia* nauplii. The ratio between dead nauplii (showing no motility) and living nauplii in comparison to a control group is used to estimate the toxicity of the test solutions. The mortality test is usually performed for 24 or 48 hours (Libralato *et al.*, 2016). Besides acute mortality test of hatched nauplii, some sub-lethal endpoints can be evaluated, such as the hatchability of cysts when exposed to toxicants (Carballo *et al.*, 2002) or the behaviour evaluation, especially swimming speed and pattern alteration (Morgana *et al.*, 2018).

Long-term toxicity tests are performer for longer periods of time, perhaps up to the time of death, and analyse growth, reproduction and immobilisation (Libralato *et al.*, 2016).

5.2. Objectives

The main goals of the *A. salina* acute mortality assay were:

i. to determine the EC50 (concentration that causes mortality of 50% of the nauplii) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 24 and 48 hours of exposure;
ii. to determine the NOEC and LOEC values of [C16Pyr][Cl] and [C2OHMIM][Cl] after 24 and 48 hours of exposure.

5.3. Materials and Methods

5.3.1. Assay

The test was adapted from Manfra *et al.* (2015). The purpose of this test was to determine the effects of [C16Pyr][Cl] and [C2OHMIM][Cl] on the survival of the crustacean *A. salina*. Nauplii were exposed to the test solutions at a range of concentrations for a period of 48 hours. Mortality was recorded in order to calculate the EC50 (effective concentration that causes 50% mortality of the population) after 24 and 48 hours exposure to the test solutions.

5.3.2. *Artemia salina* cysts hatching

The *A. salina* nauplii were obtained from commercially available dehydrated cysts (Prodac, Italy) hatched in a 35 g L^{-1} saltwater solution (35g of sea salt (Marmoto, Portugal) dissolved in 1L of distilled water) at 25 ± 1 ºC, under continuous illumination and aeration. The cysts were incubated in a conical container with 1L of saltwater solution.

After approximately 48 hours of incubation, the phototropic nauplii were collected with a pipette from the lighted side of the container and concentrated in a small beaker containing fresh saltwater solution.

5.3.3. Test solutions

The [C16Pyr][Cl] test solutions were prepared by serial dilutions of the stock solution in 35 g L^{-1} saltwater solution, arranged in a geometric series with a separation factor of 10.0 in the range-finding preliminary test and 2.2 in the definitive test. Five test solutions were tested in the definitive test (described in Table 12).

Because the preliminary test performed with the [C2OHMIM][Cl] concentrations demonstrated that the highest concentration tested (10 mM or 1626.2 mg L^{-1}) showed 1.2% mortality at the end of the test (Figure 18), a limit test was performed, where only a 100 mg L^{-1} solution was tested. This solution was prepared dissolving 5 mg of [C2OHMIM][Cl] in 50 mL of saltwater solution.

Saltwater (35 g L^{-1}) was used both as a control group and as a dilution solution to prepare test solutions (Rajabi *et al.*, 2015).

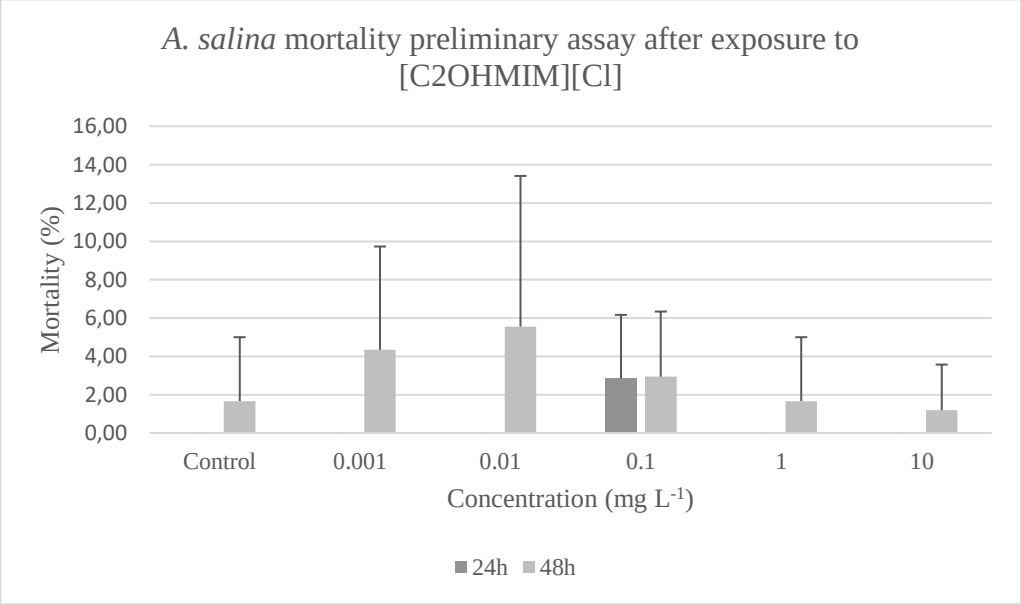

Figure 18 - Mortality percentage of *A. salina* when exposed to [C2OHMIM][Cl] and control group . Results obtained from the preliminary range-finding test.

Table 12 - Concentrations range tested in the *A. salina* definitive tests.

Test organism	**IL**	**Concentrations range tested (mM)**
A. salina	[C16Pyr][Cl]	0.01; 0.022; 0.048; 0.106; 0.234
	[C2OHMIM][Cl]	100 mg L^{-1}

5.3.4. Test procedure

The *A. salina* acute mortality assays were performed in 24-well plates (Orange Scientific, Belgium).

For the [C16Pyr][Cl] assay, 2 mL of each test solution (or saltwater for the control group) and about 15 nauplli were transferred into each well. Four replicates were performed for each treatment.

For the [C2OHMIM][Cl] ecotoxicity assessment, a limit test was performed. The limit test was adapted from OECD (2004). For the [C2OHMIM][Cl] assay, 2 mL of each treatment (100 mg L^{-1} test solution or saltwater for the control group) and about 15 nauplli were transferred into each well. Eight replicates were performed for each treatment. The plates were incubated for 48 hours in the dark at 21 ± 1 ° C. If the percentage of mortality exceeds 10% at the end of the test, a full study should be conducted. If the percentage of mortality does not exceed 10% at the end of the test, the compound can be considered practically harmless to the test organism (OECD, 2004).

Mortality values, recognized by the total absence of movement (i.e. swimming activity or movement of appendices) for approximately 10 seconds of observation (Manfra *et al.*, 2015), were determined by observation using the magnifying glass after 24 and 48 hours of exposure to test solutions. At the end of the test, 4 drops of lugol solution were added to each well with the purpose to immobilise all the individuals in order to allow counting. The total number of individuals in each well was determined. The assay was performed three times.

5.3.5. Statistical analysis

The test acceptability criteria was defined as control mortality ≤ 10% (Manfra *et al.*, 2015).

The percentage of dead nauplii at 24 and 48 hours of exposure were plotted against test concentrations. Data was analysed by probit analysis to calculate the EC50 with 95% confidence limits (α = 0.05) (Finney, 1952) using *IBM SPSS Statistics 25* software.

5.4. Results

A. salina was used to test [C16Pyr][Cl] and [C2OHMIM][Cl] acute toxic effect on crustacean from hypersaline environments. The 24 h and 48 h mortality data is shown in Figure 19 and Figure 20, respectively. Data in the graphics are expressed as the mean of three independent experiments, with four replicates in each run of the [C16Pyr][Cl] evaluation, and eight replicates in each run of the [C2OHMIM][Cl] evaluation. Error bars represent standard deviation (SD). Statistically significant differences between groups are pointed out in each graphic. Each letter (a, b, c, …) corresponds to a set of groups with no significant differences found among them. Only the relevant significant differences for the results interpretation are pointed out.

5.4.1. Acute toxic effects of [C16Pyr][Cl] to *Artemia salina*

The results demonstrated that the mortality percentage of *A. salina* when exposed to different [C16Pyr][Cl] test concentrations increased over time for each treatment. In addition, mortality percentage tends to increase with the increase of the test concentration. From the analysis of Figure 19, it is possible to note a lower mortality percentage for the 0.106 and 0.234 mM comparing to the 0.0484 mM group. However, statistical analysis demonstrated that there were no significant differences found between these groups.

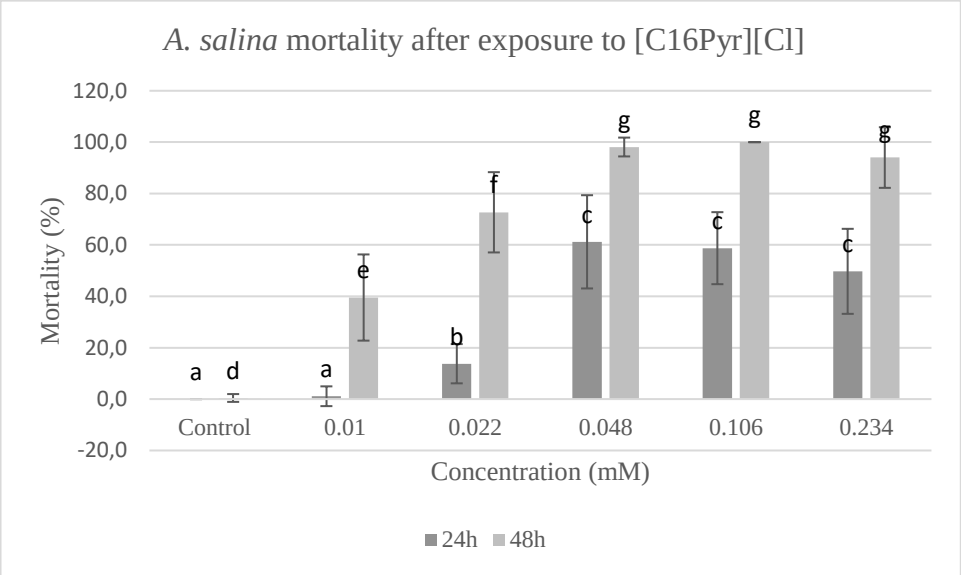

Figure 19 - *A. salina* mortality data after 24h and 48h exposure to [C16Pyr][Cl]. Error bars represent SD. a, b, c, d, e, f, g: no statistically significant differences found between groups.

The Kruskal-Wallis test showed that there was a statistically significant difference in the mortality percentage between the different concentrations after 24 hours (H=60.463, p<0.001) and after 48 hours (H=64.319, p<0.001) of exposure to the [C16Pyr][Cl] concentration. After 24 hours of exposure, the Mann-Whitney test indicated that there were no significant differences found between the control group and the immobilisation percentage of nauplii exposed to the lowest concentration (0.01 mM) (U=66.0, p=1.0) (groups marked as "a" in Figure 19. After 48 hours of exposure, there were significant differences in the mortality percentage between the control and all the treatments groups.

The 24h EC50 and 48h EC50 values obtained by the probit test are shown in Table 13. The mean 24h EC50 and 48h EC50 of [C16Pyr][Cl] was 0.1699 and 0.014 mM, respectively. Based upon these results, it is possible to classify it as slightly toxic after 24 hours of exposure and moderately toxic after a 48 hours period of exposure. As seen in Table 14, the

24h NOEC and 24h LOEC values obtained were 0.01 and 0.022 mM, respectively. The 48h NOEC was not possible to determine and the 48h LOEC value was 0.01 mM.

Table 13 - The median effect concentration (EC50), 95% confidence intervals and the hazard ranking of [C16Pyr][Cl] in the *A. salina* 24 and 48 hours acute mortality assay.

ILs	**EC50 (95% confidence interval) (mM)**		**Mean EC50 (mM)**		**Hazard ranking***
	24h	**48h**	**24h[a]**	**48h[b]**	
[C16Pyr][Cl]	0.18 (-0.409 – 0.792)	0.017 (0.014 – 0.02)	0.169 (65.504 mg L^{-1})	0.014 (5.012 mg L^{-1})	++ [a]
	0.129 (0.007 – 0.248)	0.016 (0.009 – 0.023)			+++ [b]
	0.198 (-0.226 – 0.699)	0.009 (-0.6 – 0.199)			

The values in parentheses are the 95% confidence intervals. *: The following standard was used for the EC50 (mg L^{-1}) to evaluate the toxicity of the ILs to A. *salina* according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987). "a" is related to the 24h and "b" to the 48h results.

Table 14 - *A. salina* NOEC and LOEC results after 24 and 48 hours of exposure to [C16Pyr][Cl].

ILs	**24h**		**48h**	
	NOEC (mM)	**LOEC (mM)**	**NOEC (mM)**	**LOEC (mM)**
[C16Pyr][Cl]	0.01	0.022	nd	0.01

nd: not possible to determine.

5.4.2. Acute toxic effects of [C2OHMIM][Cl] to *Artemia salina*

For the [C2OHMIM][Cl] limit test, the *A. salina* mortality percentage was between 0 and 3% in all the three trials performed at 24 hours and between 0 and 7% at 48 hours observation, as described in Table 15. Because the mortality percentage was inferior to the acceptability criteria (10% mortality) (OECD, 2004), the limit test results were acceptable and a statistical analyses was performed. The Mann-Whitney test showed that no statistically significant differences were found in mortality percentage between control group and the 100 mg L^{-1} [C_2OHMIM][Cl] treatment group after 24 hours (U=275.0, p=0.739) (groups marked as "a" in Figure 20) and after 48 hours of exposure (U=268.5, p=0.626) (groups marked as "b" in Figure 20).

Based on these results, [C2OHMIM][Cl] was considered practically or relatively harmless to *A. salina*.

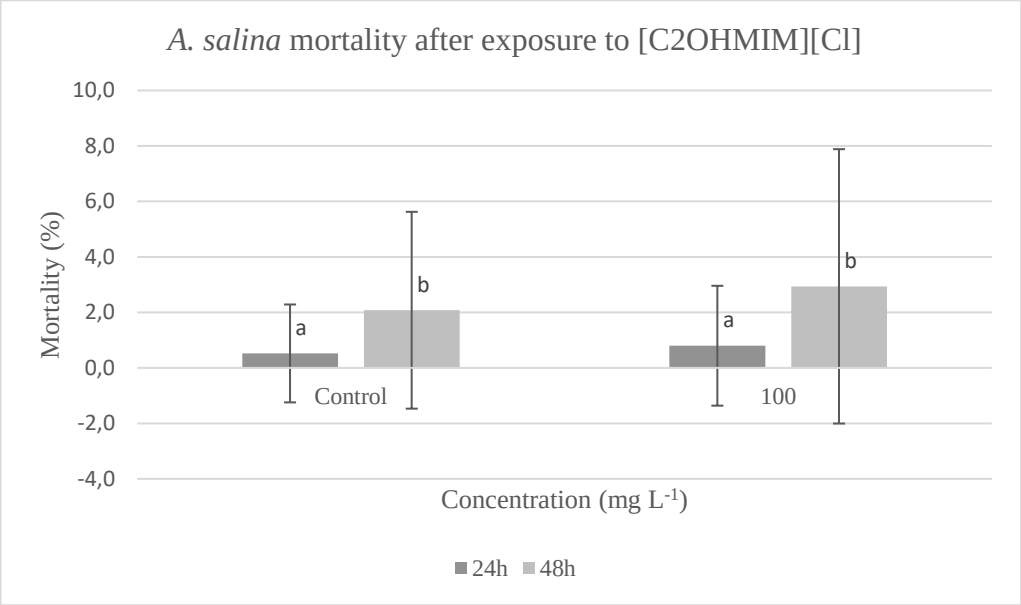

Figure 20 - *A. salina* mortality data after 24h and 48h exposure to [C2OHMIM][Cl] in the limit test. Error bars represent SD. a, b: no statistically significant differences found between groups.

Table 15 – *A. salina* mortality percentage in the limit test after 24 h and 48 h exposure to 100 mg L^{-1} [C2OHMIM][Cl].

ILs	**Mortality (%)**		**Mean mortality (%)**		**Hazard ranking***
	24h	**48h**	**24h**	**24h**	
[C2OHMIM][Cl]	2.4	6.6	0.8	3	-
	0	2.3			
	0	0			

*: The following standard was used for the EC50 (mg L^{-1}) to evaluate the toxicity of the ILs to *A. salina* according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987).

5.5. Discussion and Conclusion

The use of the crustacean *Artemia spp.* is widely spread in ecotoxicology as a model organism (Nunes *et al.*, 2006). However, standard methods that provide protocol guidelines are still unavailable. In order to provide a new step in standardization process, some inter-calibration studies have been performed using *A. franciscana.* The outcomes from the inter-laboratory exercises suggested a good repeatability and reproducibility of all protocols (Manfra *et al.*, 2015). The reliability, replicability and validity of toxicity bioassay with

nauplii of *Artemia spp.* has also been confirmed over time by several ecotoxicological studies performed with different stressors, including marine natural extracts (Carballo *et al.*, 2002), water effluents (Guerra, 2001), dental materials (Pelka *et al.*, 2000), nanoparticles (Rajabi *et al.*, 2015), heavy metals (Hadjispyrou *et al.*, 2001) or pharmaceuticals (Nunes *et al.*, 2005).

Rajabi *et al.* (2015) also demonstrated that the *A. salina* test may speed toxicity experiments and decrease costs and, therefore, may be considered an alternative to the *in vitro* cell culture assay (Rajabi *et al.*, 2015). The microwell cytotoxicity assay using *A. salina* has also been established by different authors (Rajabi *et al.*, 2015; Solis *et al.*, 1993).

Artemia mortality presented various definitions generating potential misunderstanding about the observation of moribund or dead individuals. Therefore, 'immobility' may be an easier state to define the test endpoint (Libralato *et al.*, 2016). Nevertheless, differences in how the immobility state is defined exist. Morgana *et al.* (2018) considered nauplii as dead when they are motionless, or no appendage movement occurs for 5 seconds. Manfra *et al.* (2015) reported that nauplii are dead if they do not show any movement after 10 seconds observation. Nunes *et al.* (2006) considered the nauplii dead if immobilization continued for 10 seconds after gentle shaking (Manfra *et al.*, 2015; Morgana *et al.*, 2018; Nunes *et al.*, 2006). In the present study, nauplii were considered to be dead if they did not show any movement after 10 seconds observation, as suggested by Manfra (Manfra *et al.*, 2015).

Some authors have researched the toxic effects of different classes of ILs towards saltwater organisms (Latała *et al.*, 2005; Ventura *et al.*, 2012, 2014), but not many studies focus on the toxicity to saltwater crustacean (Deese *et al.*, 2016). Due to its high importance in the food chain, we believe that it is important to understand the impact of these compounds on these organisms.

The results demonstrated that the mortality percentage of *A. salina* when exposed to different [C16Pyr][Cl] test concentrations was negatively affected by time of exposure and increasing concentration. The 48h EC50 was lower than the 24h EC50, which clearly indicates the exposure time negative influence, which may be due to the cumulative effect of the compound on the organism. Because it was not possible to determine the NOEC value for the 48 hours observations, it would be necessary to perform an assay with lower test concentration.

Deese *et al.* (2016) evaluated the toxicity of [C16Pyr][Cl] to the hatchability of *A. franciscana*. They found out that it had significant effects on the hatching percentage of *A. franciscana* at 3.5 ppm and above, but did not affect the mortality percentage (Deese *et al.*, 2016).

Because the literature about the effects of pyridinium based ILs on marine crustacean is scarce, it is not possible to compare the present results with previous works. Thus, we believe our results could be a good foundation for future works in the assessment of ILs ecotoxicity to saltwater organisms.

For the ecotoxicity assessment of [C2OHMIM][Cl] towards saltwater environment, we performed a limit test based on the preliminary test, since it showed that [C2OHMIM][Cl] could possibly demonstrate low level of toxicity towards *A. salina*.

According to Passino and Smith (1987), a chemical is considered as practically harmless to organisms if it has an EC50 greater than 100 mg L^{-1} (Passino & Smith, 1987). The limit test performed demonstrated that the EC50 of [C2OHMIM][Cl] towards *A. salina* is greater than 100 mg L^{-1}, since the mortality percentage was found to be under 10% when the organisms were exposed to a 100 mg L^{-1} test solution. Therefore, based on our results, it is possible to classify [C2OHMIM][Cl] as practically harmless to *A. salina*.

Having in consideration the present results, it was possible to recognise that [C16Pyr][Cl] presents a much higher risk to the saltwater ecosystem organisms than [C2OHMIM][Cl]. For a better knowledge about the potential impact of both compounds to saltwater environment, we suggest extending the test to different marine organisms.

VI. *Lactuca sativa* germination and root and shoot growth inhibition assay

6.1. *Lactuca sativa* assay overview

Soil ecotoxicity is assessed by the effects caused by toxicants on terrestrial organisms, such as plants. Several variables can be measured to evaluate phytotoxicity on early stages of development, being germination rate, root growth, shoot growth and photosynthetic activity some of the most commonly studied (Priac *et al.*, 2017; Valerio *et al.*, 2007; Visioli *et al.*, 2016). Such bioassays are simple, reproducible, rapid and inexpensive methods that only require a relatively small amount of sample (Priac *et al.*, 2017).

Apart from being an important indicator of the environment condition, testing the effects of chemicals on plants are also significant because some plant species are introduced in animals and humans' diet.

Lactuca sativa (lettuce) is among the most common plant species recommended by international organizations, such as USEPA (USEPA, 2012). *L. sativa* seeds have been used in several works for germination assays involving different materials because the seeds are easy to handle and grow quickly. It also demonstrated to be a very sensitive organism for phytotoxicity bioassays (Pan & Chu, 2016).

6.2. Objectives

The main goals of the *L. sativa* germination and root and shoot growth inhibition assay were:

i. to determine the EC50 (concentration that causes 50% inhibition) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 96 hours of exposure (96h EC50) for:
   a. germination;
   b. root growth;
   c. shoot growth;

ii. to determine the NOEC and LOEC values of [C16Pyr][Cl] and [C2OHMIM][Cl] after 96 hours of exposure (96h NOEC and 96h LOEC) for all the three endpoints evaluated.

6.3. Materials and Methods

6.3.1. Assay

The methodology used in the experiment was adapted from OECD and Valerio *et al.* (2007) (OECD, 2006a; Valerio *et al.*, 2007). The purpose of this test was to determine the effects of the tested ILs on the seed germination and root and shoot growth of *L. sativa*. Seeds were exposed to the test solutions at a range of concentrations for a period of 96 hours. Seed germination and root and shoot length were recorded and compared with the control group in order to calculate the concentration that causes 50% of germination inhibition and the concentration that causes 50% of root or shoot growth inhibition after 96 hours of exposure to the test solutions.

6.3.2. *Lactuca sativa* seed disinfection

Previously to the beginning of the test, seeds followed a disinfection protocol adapted from Sauer and Burroughs (1986). The seeds were washed in a 5% NaClO solution for 1 minute and this step was repeated. After disinfection, seeds were washed in running tap water for 1 minute, followed by distilled water for 1 minute (Sauer & Burroughs, 1986).

6.3.3. Test solutions

Test solutions were prepared by serial dilutions of the stock solution in distilled water, arranged in a geometric series with a separation factor of 10.0 in the range-finding preliminary test. In the definitive test, the separation factor was 2.0 for the [C16Pyr][Cl] solutions and 1.3 for [C2OHMIM][Cl] test concentrations..

Five test solutions, arranged in a geometric series, were tested in the final definitive test. The concentrations tested in the definitive test are described in Table 16 and were based on the results from the preliminary test.

Table 16 – Concentrations range tested in the *L. sativa* definitive tests.

Test organism	**IL**	**Concentrations range tested (mM)**
L. sativa	[C16Pyr][Cl]	0.313; 0.625; 1.25; 2.5; 5
	[C2OHMIM][Cl]	14.01; 18.21; 23.67; 30.77; 40

6.3.4. Test procedure

The assay was carried out on filter paper disks embedded in 3 ml of the test solution (or distilled water in the control group) arranged in 9 cm diameter Petri dishes. 10 undamaged *L. sativa* (*Natura Sementes, Portugal*) seeds were placed on each filter paper disk equidistantly distributed. Three replicates were performed for each treatment. The Petri dishes were sealed with parafilm to avoid evaporation and incubated in the dark at 21 ± 1 °C for 96 hours. Three independent tests were performed.

6.3.5. Germination assessment

The germination rates were determined by observation with the magnifying glass after 24, 48, 72 and 96 hours of exposure to the test solutions. Germination was considered only when a 5 mm root sprouts was observed. Germination percentage was evaluated as number of germinated seeds of total number of seeds, as described by the equation (4):

$$G = \frac{\mathrm{GS}}{\mathrm{TS}} \quad (4)$$

where G is the germination percentage, GS the number of germinated seed and TS the total number of seeds.

6.3.6. Root and shoot length assessment

After 96 hours of exposure to the test solutions, the roots and shoots of all germinated seeds were measured using millimetre paper by observation with the magnifying glass, according to the scheme in Figure 21. The total length (root plus shoot) was also measured.

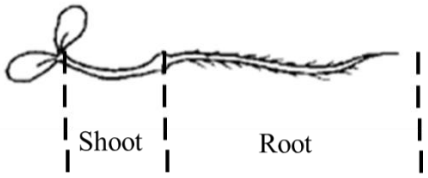

Figure 21 – Exemplified figure of how to identify and measure the root and shoot of *L. sativa*

6.3.7. Statistical analysis

The germination rate (equation (5)) was calculated by dividing the mean value of germinated seeds at each test solution by the mean value of germinated seeds in the control with distilled water.

$$(5) \qquad GER = \frac{\mathrm{GST}}{\mathrm{GSC}} \times 100$$

where GER is the germination rate, GST the number of germinated seeds in the treatment and GSC the average number of germinated seeds in the control.

The germination rate inhibition (GERI) was calculated by the equation (6):

$$(6) \qquad GERI = 100 - \frac{GST}{GSC}\ X\ 100$$

where GERI is the germination rate inhibition, GST the number of germinated seeds in the treatment and GSC the average number of germinated seeds in the control. For determination of the GERI, it was considered that there was 100% germination in the control group.

The growth rate (GR) was calculated by the equation (7):

$$(7) \qquad GR = \frac{LT}{LC}\ X\ 100$$

where GR is the growth rate, LT the mean root/shoot length in the treatment and LC the mean root/shoot length in the control.

The growth rate inhibition (GRI) was calculated by the equation (8):

$$(8) \qquad GRI = 100 - \frac{LT}{LC}\ X\ 100$$

where GRI is the growth rate inhibition, LT the mean root/shoot length in the treatment and LC the mean root/shoot length in the control. For determination of the GRI, it was considered that there was 100% growth in the control group.

The germination and growth rate inhibition after 96 hours of exposure were plotted against test concentrations. Data was analysed by probit analysis to calculate the EC50 value for germination rate inhibition and the EC50 value for root and shoot growth inhibition with 95% confidence limits (α = 0.05) (Finney, 1952) using *IBM SPSS Statistics 25* software.

6.4. Results

Seed vitality of the control group (seed germinated in distilled water) was evaluated by germination rates, which were >90 % in all the three tests performed.

Because no germination occurred in the first 24 hours of the test period, just the results after 48 hours of exposure were considered for analysis.

The following graphics data are expressed as the mean of three independent experiments, with three replicates in each run. Error bars represent standard deviation (SD).

Statistically significant differences between groups are pointed out in each graphic. Each letter (a, b, c, …) corresponds to a set of groups with no significant differences found among them. Only the relevant significant differences for the results interpretation are pointed out.

6.4.1. Acute toxic effects of [C16Pyr][Cl] to *Lactuca sativa*

When exposed to [C16Pyr][Cl], *L. sativa* germination was significantly reduced comparing to control group (p<0.05) for the highest test concentrations. Only for the lowest concentration (0.313 mM) no significant differences were found comparing to the control after 48 hours (group marked as "a") (U=38.0, p=0.83), 72 hours (group marked as "d") (U=39.0, p=0.935) and 96 hours (groups marked as "g") (U=26.0, p=0.23), as seen in Figure 22. For the two highest concentrations (2.5 and 5 mM) it was observed 100% germination inhibition (Figure 22). It was noted that the germination rate inhibition increased with the increasing of the concentration (Figure 23). After 96h, for the 0.313 mM treatment, it was recorded a higher germination percentage than the control. Although, we cannot say that these groups are statistically different (groups marked as "g") (U=26.0, p=0.23).

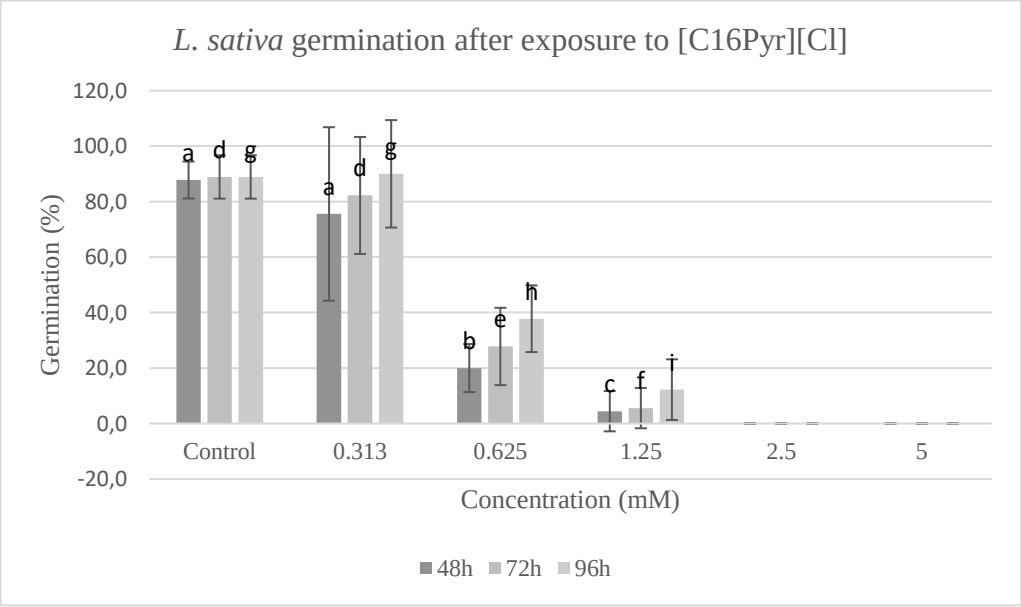

Figure 22 - *L. sativa* germination percentage after exposure to [C16Pyr][Cl] test concentrations and control group. Error bars represent SD. a, b, c, d, e, f, g, h, i: no statistically significant differences found between groups.

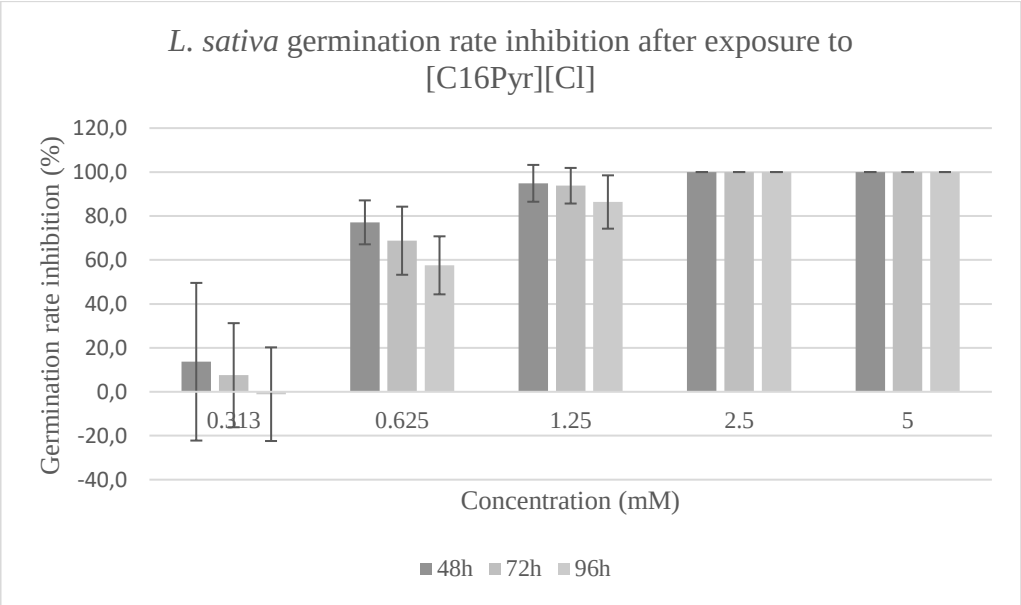

Figure 23 - *L. sativa* germination rate inhibition when exposed to the [C16Pyr][Cl] test concentrations. Error bars represent SD.

The 96h EC50 values for germination inhibition obtained in the three tests performed are shown in Table 17. The 96h EC50 values of [C16Pyr][Cl] varied between 0.54 and 0.82 mM with a mean 96h EC50 value of 0.68 mM, being classified as practically nontoxic to *L. sativa* germination. As seen in Table 19, the NOEC and LOEC values obtained were 0.313 and 0.625 mM, respectively.

When exposed to [C16Pyr][Cl], both root and shoot growth were severely affected. Root and shoot length were significantly reduced at all concentrations tested ($p < 0.05$). A marked fall in root growth (~94 %) and shoot (~85 %) was already observed at 1.25 mM. Because there was no germination occurring at 2.5 and 5 mM, the inhibition is shown as 100% in the graphics (Figure 24 and Figure 25).

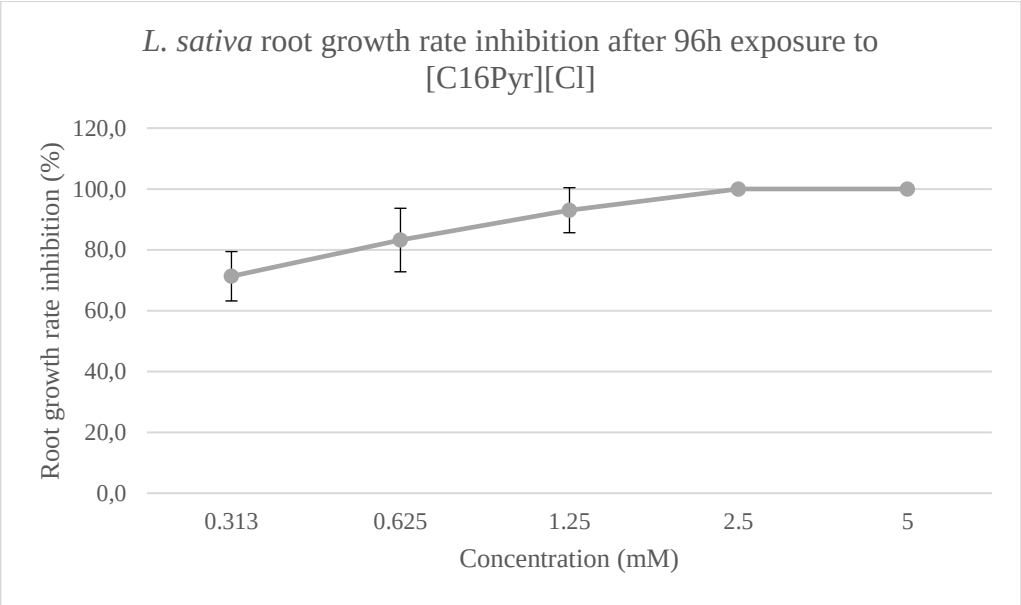

Figure 24 - *L. sativa* root growth rate inhibition after exposure to the [C16Pyr][Cl] test concentrations. Error bars represent SD.

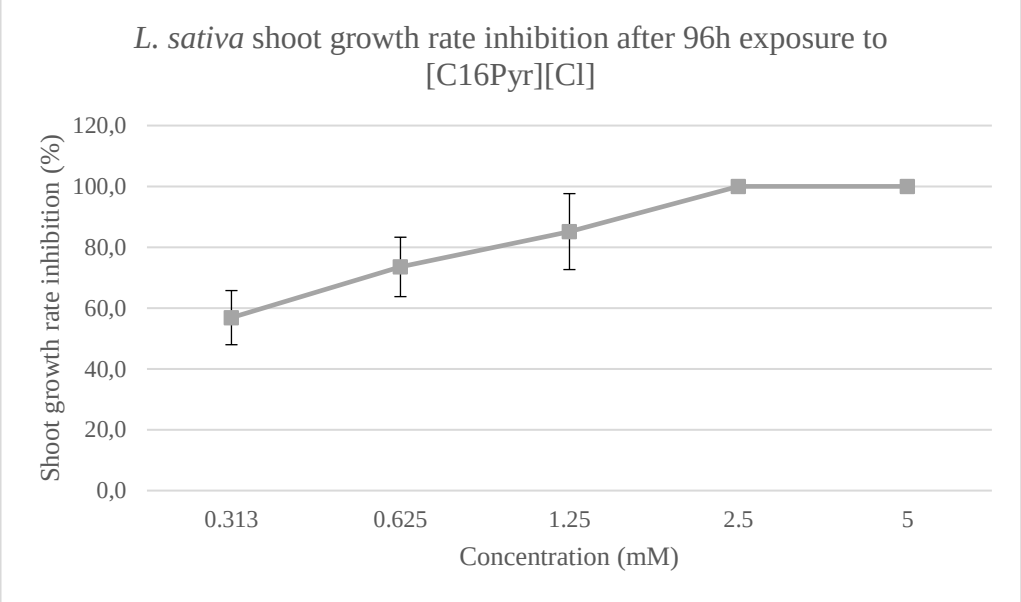

Figure 25 - *L. sativa* shoot growth rate inhibition after exposure to the [C16Pyr][Cl] test concentrations. Error bars represent SD.

The Kruskal-Wallis test showed that there was a statistically significant difference in the root growth between the different treatments after 96 hours of exposure (H=47.89, p<0.001). The Mann-Whitney test indicated that there were significant differences between the control group and the root growth of seeds exposed to all the tested concentrations (p<0.05). In what concerns the shoot growth, it was also demonstrated a statistically significant difference in the shoot growth between the different treatments after 96 hours of

exposure (H=48.12, p<0.001). The Mann-Whitney test indicated that there were significant differences in the shoot growth between the control group and the seeds exposed to all the tested concentrations (p<0.05). It was also determined that no significant differences were found in the growth of root and shoot (p>0.05).

The 96h EC50 values for root and shoot growth inhibition obtained in the three tests performed are shown in Table 18. The 96h EC50 values of [C16Pyr][Cl] for root growth inhibition varied between 0.30 and 0.39 mM with a mean 96h EC50 value of 0.34 mM, being classified as practically nontoxic to *L. sativa* root growth inhibition. The 96h EC50 values for shoot growth inhibition varied between 0.45 and 0.50 mM with a mean 96h EC50 value of 0.47 mM, being classified also as practically nontoxic to *L. sativa* shoot growth inhibition. It was not possible to determine the NOEC and the LOEC value was 0.313 mM for both root and shoot growth inhibition (Table 19).

Apart from germination and root and shoot growth perturbation, it was observed abnormal appearance in seeds exposed to [C16Pyr][Cl]. The root tip of the seeds exposed to all the concentrations tested presented a brownish coloration (Figure 26).

Figure 26 - Macroscopic aspect of roots from seeds germinated in a 0.313 mM [C16Pyr][Cl] test solution, after a 96h period of exposure. The root tips showed a brown coloration.

6.4.2. Acute toxic effects of [C2OHMIM][Cl] to *Lactuca sativa*

The results for the [C2OHMIM][Cl] toxic assessment to *L. sativa* germination are shown in Figure 27 and Figure 28. The Kruskal-Wallis test showed that there was not a statistically significant difference found in the *L. sativa* germination percentage of the seeds exposed to the control and to the different treatments after 48, 72 or 96 hours of exposure to [C2OHMIM][Cl] (p>0.05) (groups marked as “a”, “b” and “c” in Figure 27). Although there was a higher germination percentage for the 18.21 and 23.67 mM treatment, this difference is not statistically significant (p>0.05).

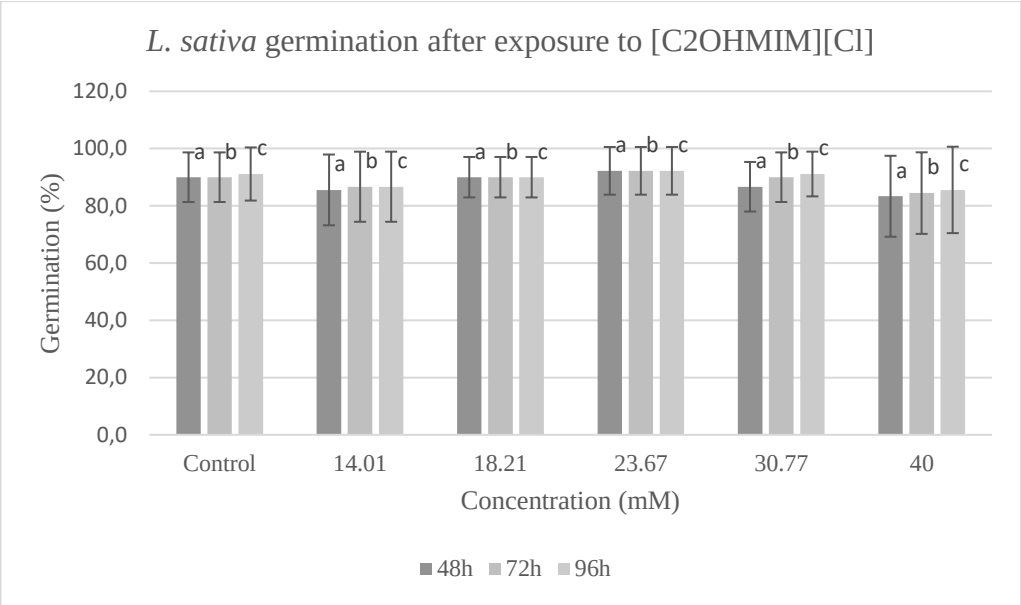

Figure 27 - *L. sativa* germination percentage after exposure to [C2OHMIM][Cl] test concentrations and control group. Error bars represent SD. a, b and c: no statistically significant differences found between groups.

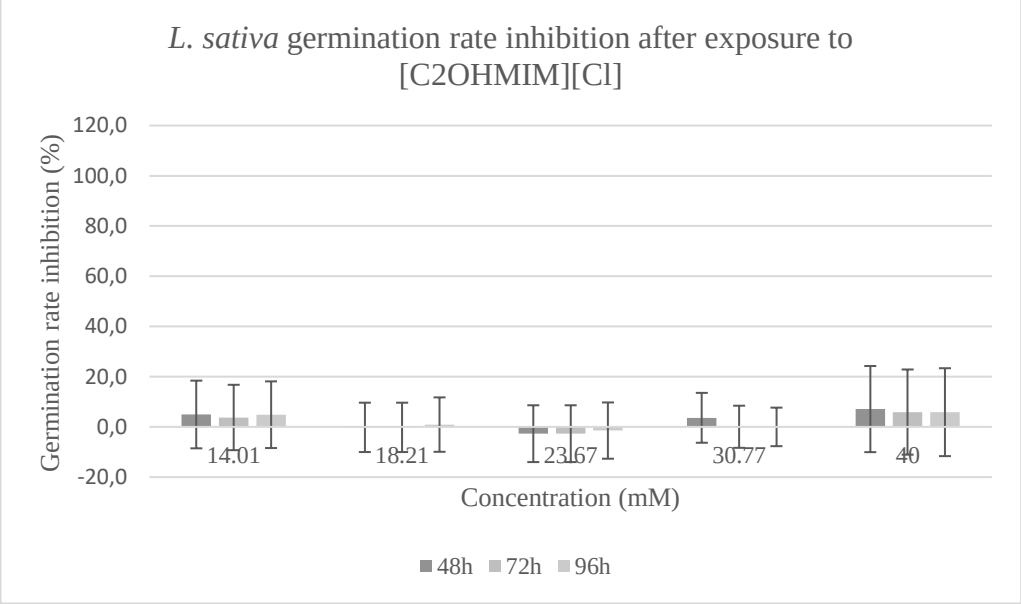

Figure 28 - *L. sativa* germination rate inhibition after exposure to the [C2OHMIM][Cl] test concentrations. Error bars represent SD.

The 96h EC50 values of [C2OHMIM][Cl] for germination inhibition varied between 60.82 and 171.87 mM with a mean 96h EC50 value of 111.97 mM. Based on these results, it is possible to classify [C2OHMIM][Cl] as relatively harmless to *L. sativa* germination (Table 17). It was not possible to determine NOEC and LOEC values for the germination inhibition (Table 19).

When exposed to [C2OHMIM][Cl], both root and shoot growth were affected. Root and shoot length were significantly reduced at all concentrations tested ($p < 0.05$), comparing to the control group (Figure 29 and Figure 30).

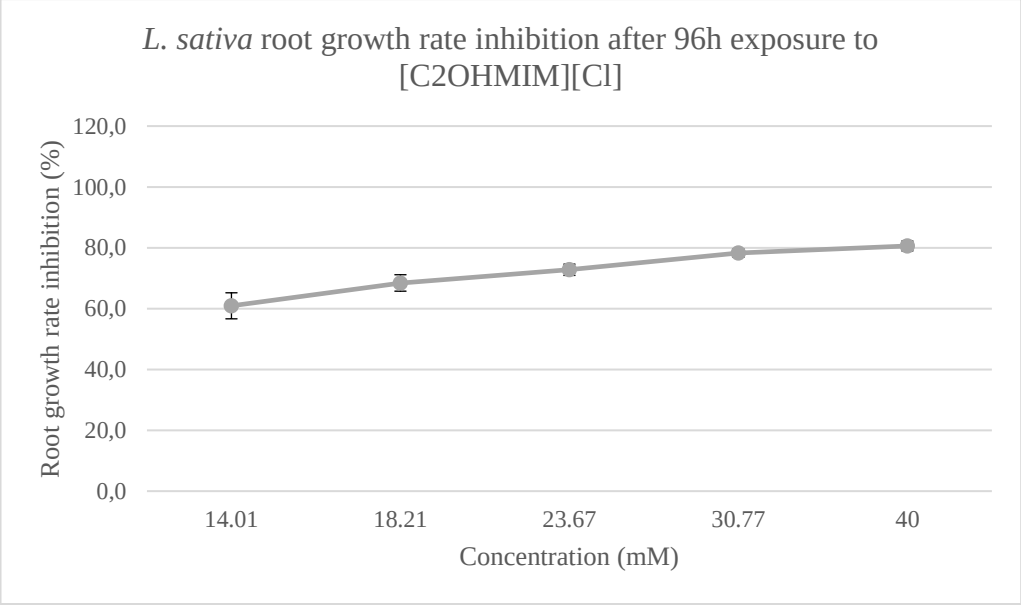

Figure 29 - *L. sativa* root growth rate inhibition after exposure to the [C2OHMIM][Cl] test concentrations. Error bars represent SD.

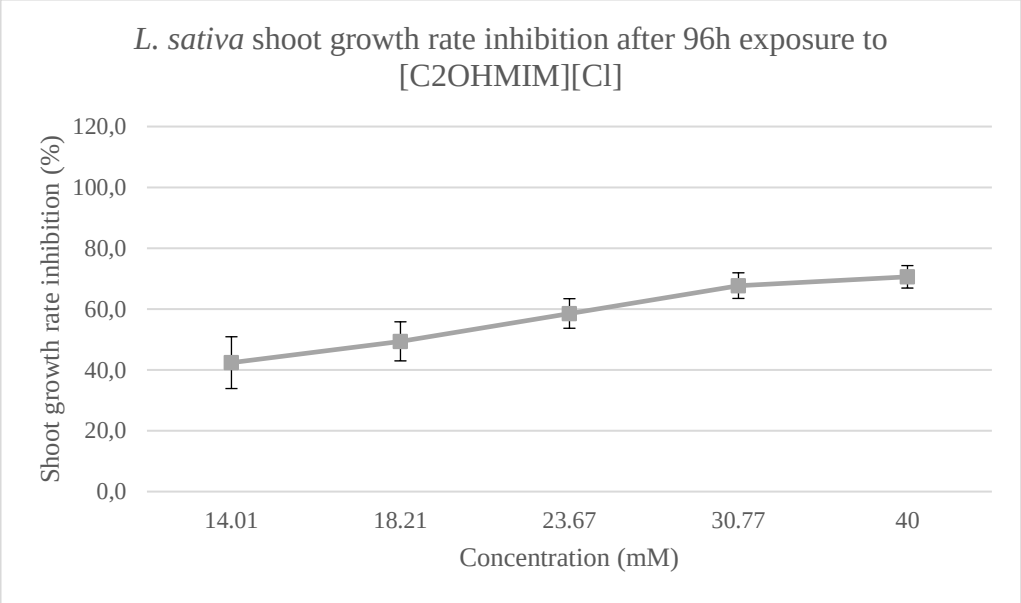

Figure 30 - *L. sativa* shoot growth rate inhibition after exposure to the [C2OHMIM][Cl] test concentrations. Error bars represent SD.

In what concerns the root growth evaluation, the Kruskal-Wallis test showed that there was a statistically significant difference in the root growth between the different treatments

after 96 hours of exposure (H=50.22, p<0.001). The Mann-Whitney test indicated that there were significant differences in the root growth of seeds exposed to the control group and to all the tested concentrations (p<0.05). With respect to the shoot growth evaluation, it was observed that there was also a statistically significant difference in the shoot growth between the different treatments after 96 hours of exposure (H=47.68, p<0.001) and the Mann-Whitney test indicated that there were significant differences between the control group and the shoot growth of seeds exposed to all the tested concentrations (p<0.05). It was also determined that significant differences were found in the root and shoot growth rate (p<0.05). The inhibition of root growth was superior to the inhibition of shoot growth.

The 96h EC50 values of [C2OHMIM][Cl] for root and shoot growth inhibition obtained in the three tests performed are shown in Table 18. The 96h EC50 values of [C2OHMIM][Cl] for root growth inhibition varied between 15.58 and 16.83 mM with a mean 96h EC50 value of 16.12 mM, being classified as relatively harmless to *L. sativa* root growth. The 96h EC50 values for shoot growth inhibition varied between 21.70 and 24.35 mM with a mean 96h EC50 value of 22.69 mM, being classified also as relatively harmless to *L. sativa* shoot growth. It was not possible to determine the NOEC and the LOEC value was 14.01 mM for both root and shoot growth inhibition (Table 19).

Table 17 – *L. sativa* germination inhibition after exposure to [C16Pyr][Cl] and [C2OHMIM][Cl]. 96h EC50 values for the three assays performed are shown in mM.

ILs	**96h EC50 (mM)**	**95% Confidence interval**	**Mean 96h EC50 (mM)**	**Hazard Ranking***
[C16Pyr][Cl]	0.82 0.54 0.70	0.57 – 1.06 0.31 - 0.74 0.48 – 0.93	0.68 (244.9 mg L^{-1})	+
[C2OHMIM][Cl]	103.22 60.82 171.87	nd nd nd	111.97 (18208.6 mg L^{-1})	-

*: The following standard was used for the 96h EC50 (mg L^{-1}) to evaluate the toxicity of the ILs to *L. sativa* according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987). nd: not possible to determine.

Table 18 - *L. sativa* root and shoot growth inhibition after exposure to [C16Pyr][Cl] and [C2OHMIM][Cl]. 96h EC50 values for the three assays performed are shown in mM.

ILs		**96h EC50 (mM)**	**95% Confidence interval**	**Mean EC50 96h (mM)**	**Hazard Ranking***
[C16Pyr][Cl]	*Root*	0.30	-0.11 – 0.70	0.34 (122.8 mg L^{-1})	+
		0.39	-0.12 – 0.82		
		0.33	-0.12 – 0.74		
	Shoot	0.48	-0.13 – 0.94	0.47 (167.9 mg L^{-1})	+
		0.50	-0.04 – 0.91		
		0.45	0.03 – 0.81		
[C2OHMIM][Cl]	*Root*	16.83	5.61 – 25.97	16.12 (2621.4 mg L^{-1})	-
		15.94	3.95 – 25.54		
		15.58	3.84 – 24.96		
	Shoot	24.35	13.12 – 33.84	22.69 (3689.2 mg L^{-1})	-
		21.70	11.50 – 30.23		
		22.01	12.63 – 29.94		

*: The following standard was used for the 96h EC50 (mg L^{-1}) to evaluate the toxicity of the ILs to *L. sativa* according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987).

Table 19 - *L. sativa* NOEC and LOEC results after 96 hours exposure to [C16Pyr][Cl] and [C2OHMIM][Cl] . Results are shown in mM.

ILs	**Germination inhibition**		**Root growth inhibition**		**Shoot growth inhibition**	
	NOEC	**LOEC**	**NOEC**	**LOEC**	**NOEC**	**LOEC**
[C16Pyr][Cl]	0.313	0.625	nd	0.313	nd	0.313
[C2OHMIM][Cl]	nd	nd	nd	14.01	nd	14.01

nd: not determined

6.5. Discussion and Conclusion

Sensitive and simple ecotoxicological bioassays like seed germination and root growth tests are commonly used to evaluate the phytotoxicity of chemicals from different natures, such as biochar (Visioli *et al.*, 2016), phytotoxicity of veterinary antibiotics (Pan & Chu, 2016), wastewater (Priac *et al.*, 2017) and water extracts (Valerio *et al.*, 2007).

L. sativa is a crop species and was selected as the target organism since it showed high sensitivity to toxicants and, because it has edible parts, can be introduced in animals and humans' diet (Pan & Chu, 2016). It also has shown to be very sensitive for the aprotic ILs ecotoxicity assessment (Peric *et al.*, 2014).

The bioassay with *L. sativa* seeds was performed as static acute toxicity, where the ILs phytotoxic effects in the germination of seeds and in the development of the seedlings was evaluated during the first few days of growth (96 hours of exposure).

In the [C16Pyr][Cl] toxic evaluation, the seed germination results showed a dose-response relation. It was noted that the germination rate inhibition increased with the increased of the concentration. The growth of the root and shoot were also compromised by the increase of concentration. It was also determined that no significant differences were found in the growth of root and shoot. This could mean that both structures were similarly affected by the exposure to [C16Pyr][Cl].

Apart from the inhibitory effect on germination and root and shoot growth, it was also observed phenotypic abnormal aspect in seeds exposed to [C16Pyr][Cl]. The root of the seeds exposed to all the concentrations tested showed a brown color, which may be due to cellular necrosis. To clarify the process behind the abnormal phenotype, future cell orientated analysis needs to be performed, such as biochemical or mitotic index analysis.

The toxicity evaluation of pyridinium based ILs to terrestrial plants is very rare in literature. Peric *et al.* (2014) analysed the toxicity of N-butylpyridinium chloride to three terrestrial plant species. In their work, N-butylpyridinium chloride was showed some level of toxicity and was classified into the Acute 3 category of the Globally Harmonized System of Classification and Labelling of Chemicals with a EC50 value of 588 mg kg^{-1} (Peric *et al.*, 2014).

In the evaluation of [C2OHMIM][Cl] effect towards *L. sativa* germination, it was demonstrated that the concentrations tested did not affect the germination percentage. Thus, it is not possible to establish a dose-response relation for germination inhibition with the present results. Results from Biczak *et al.* (2014) also demonstrated that none of the investigated ILs in their study exhibited any adverse effect on the germination capacity of the seeds (Biczak *et al.*, 2014).

When exposed to [C2OHMIM][Cl], both root and shoot growth were also affected. With the increase of the test concentration, it was observed an increase of the growth inhibition, which demonstrates a dose-response relation of the exposure of seeds to [C2OHMIM][Cl]. It was also noted that the inhibition of root growth was superior to the inhibition of shoot growth, which could mean that both structures are not affected in the same way by the exposure to [C2OHMIM][Cl]. This result is in agreement with previous results (Tot *et al.*, 2018).

Some authors have already assessed the effect of imidazolium based ILs towards higher plants. Biczak *et al.* (2014) evaluated the effect of ILs containing 1-ethyl-3-methylimidazolium cation core coupled with five different anions on the growth and development of barley and radish. Results have proved the negative impact of ILs on the tested plants exhibiting a potential slight toxicity to the growth and development of the early developmental stages of higher terrestrial plants. (Biczak *et al.*, 2014). In a different study, Matzke *et al.* (2007) evaluated toxicity of 1-alkyl-3-methylimidazolium ILs on wheat, cress and duckweed. The trend for a higher toxicity with the increasing alkyl side chain length was observed and the anion toxic influence studied (Matzke *et al.*, 2007). Wang *et al.* (2009) also studied the effect of 1-butyl-3-methylimidazolium tetrafluoroborate on wheat seedlings. It was shown that wheat germination was reduced in the presence of the compound. Similarly, the root and shoot length of wheat seedlings decreased with increasing concentrations of the IL. It also decreased the chlorophyll content, thereby reducing photosynthesis and plant growth (Wang *et al.*, 2009). Peric *et al.* (2014) analysed the toxicity of 1-butyl-3-methylimidazolium chloride to three terrestrial plant species and it was classified into the Acute 3 category of the Globally Harmonized System of Classification and Labelling of Chemicals with a EC50 value of 930.0 mg kg^{-1}.

The biochemical toxicity and DNA damage of imidazolium based ILs with different anions in soil on *Vicia faba* seedlings was assessed by Liu *et al.* (2015). The results showed that even at low concentration, the growth of *V. faba* seedlings was inhibited after exposure to the tested ILs and the inhibitory effect was enhanced with increasing concentrations. The level of reactive oxygen species (ROS) was increased after exposure to the three tested ILs, which resulted in lipid peroxidation, DNA damage and oxidative damage in the cells of the *V. faba* seedlings. The authors also pointed the oxidative damage as the primary mechanism by which ILs exert toxic effects on crops (Liu *et al.*, 2015).

Tot *et al.* (2018) evaluated the effect of imidazolium based ILs on wheat and barley germination and growth, with particular focus on the influence of length and oxygen functionalization of alkyl side chain. The [C2OHMIM][Cl] was part of the set of tested ILs. Among all the tested ILs, [C2OHMIM][Cl] demonstrated to be the least toxic for both plant species. Introduction of the polar groups (in the form of hydroxide and/or ether group) in the alkyl side chain of the imidazolium cation and their influence on the reduction of the ILs' toxicity was also demonstrated. It was also shown that alkylation in the position N-3 atom of the imidazole significantly reduces toxicity of the cation (Tot *et al.*, 2018).

In the present work, it was also demonstrated that root growth was shown to be the most sensitive endpoint in the phytotoxicity test rather than germination rate, which is in agreement with literature (Pan & Chu, 2016; Visioli *et al.*, 2016).

Comparing the two tested compounds, [C16Pyr][Cl] proved to be the IL that exhibits higher levels of toxicity to *L. sativa* germination.

Some authors suggested that the ILs inhibitory effects to plants may be due to amylase decreased, which further inhibited degradation of stored starch in seeds and decreased the energy supply to seedlings (Wang *et al.*, 2009) or phytohormones dysregulation (Matzke *et al.*, 2007). The lipophilic behaviour of molecules is also related to higher toxic effect to germination plants developments (Pan & Chu, 2016), which can explain the higher toxic behaviour of [C16Pyr][Cl].

Although the tests were performed following published protocols, various parameters such as the number of seeds per dish, the test duration or the type of support used remain variable among authors. Although, no significant differences on either germination rate or root growth endpoints were shown when different seed densities were tested (Priac *et al.*, 2017), thus we believe that the test conditions used were not a cause of bias to the results.

We consider that the present results are a hint of the ILs' ecotoxicity assessment towards terrestrial organisms. However, future studies are necessary for a better understanding of the mode of action and toxic effect of the tested ILs on terrestrial ecosystems. Thus, we suggest evaluating the terrestrial ecotoxicity by assessing the chlorophyll content and mitotic index of root and shoot cells.

VII. *Allium cepa* root growth inhibition assay

7.1. *Allium cepa* assay overview

Being primary producers and, in some cases, directly consumed by animals or humans, plants are an important link in the food chain. Ecotoxicity studies using plants, such as *Allium sativum* (garlic) and *Allium cepa* (onion) have been used to assess the effects of a variety of compounds that may induce alteration in its normal development (Herrero *et al.*, 2012; Kumari *et al.*, 2009; Rank & Nielsen, 1998; Saxena *et al.*, 2010).

The ability of plants to respond appropriately to the surrounding environment is crucial for their adaptation, including nutrient uptake, anchorage to the soil and the establishment of biotic interactions, and it is highly dependent of the root welfare. The external signals trigger internal molecular mechanisms that modify cell division and cell differentiation processes within the root and have a profound impact on root system architecture and capacity (Harashima & Schnittger, 2010).

The *A. cepa* test is a cost-effective short-term test and has been approved as a standard assay for biomonitoring of environmental pollutants (Fiskesjö, 1985). Fiskesjö (1985) also highlights that the test demonstrates a high sensitivity and shows good correlation to other test systems. To conduct the *A. cepa* test, small bulbs are exposed to test solutions and the macroscopic and microscopic effects on root development are studied.

Plant systems have a variety of measurable endpoints, such as root growth inhibition (Rank & Nielsen, 1998), turgescence and change of colour of the root tips (Fiskesjö, 1985). Also, genetic endpoints, including alterations in ploidy, chromosomal aberrations and micronuclei formation are often studied (Ghodake *et al.*, 2011; Herrero *et al.*, 2012; Kumari *et al.*, 2009; Saxena *et al.*, 2010). Therefore, the *A. cepa* test combines different important test targets, since toxicity can be evaluated by the observation of root growth, number of emerging roots and abnormal aspect, but also by the study of chromosome changes, like chromosome abnormalities and alterations in mitotic index induced by the exposure to toxicants (Fiskesjö, 1985).

7.2. Objectives

The main goals of the *A. cepa* root growth inhibition assay were:

i. to determine the EC50 (concentration that causes 50% inhibition) of [C16Pyr][Cl] and [C2OHMIM][Cl] after 96 hours of exposure (96h EC50) for:

a. number of emerging roots;
b. root growth;

ii. to determine the NOEC and LOEC values of [C16Pyr][Cl] and [C2OHMIM][Cl] after 96 hours of exposure (96h NOEC and 96h LOEC) for the two endpoints tested.

7.3. Materials and Methods

7.3.1. Assay

The procedure of the root growth inhibition test was adapted from Fiskesjö (1985). The purpose of this test was to determine the effects of the tested ILs on the root growth of *A. cepa*. Bulbs were exposed to the test solutions at a range of concentrations for a period of 96 hours. Roots length were recorded and compared with the control group in order to calculate the concentration that causes 50% of root growth inhibition after 96 hours of exposure to the test solutions and the concentration that causes 50% inhibition of the number of emerging root in each bulb (Fiskesjö, 1985).

7.3.2. *Allium cepa* bulbs preparation

A. cepa bulbs were obtained from a local market. The bulbs were washed under running tap water and the dried outer scales were carefully removed. The old roots were removed with scissors. The bulbs were inspected and only bulbs with no signs of contamination and apparently viable were selected to undertake the experiment.

7.3.3. Test solutions

Test solutions were prepared by serial dilutions of the stock solution in distilled water, arranged in a geometric series with a separation factor of 2.0 in definitive test. No preliminary test was performed. The selection of test concentrations was based on the concentrations tested in the *L. sativa* germination and root and shoot inhibition assay.

Five test solutions, arranged in a geometric series, were tested. The concentrations tested in the definitive test are described in Table 20.

Table 20 – Concentrations range tested in the *A. cepa* definitive test.

Test organism	IL	Concentrations range tested (mM)
A. cepa	[C16Pyr][Cl]	0.31; 0.63; 1.25; 2.5; 5
	[C2OHMIM][Cl]	2.5; 5; 10; 20; 40

7.3.4. Test procedure

A. cepa bulbs of similar size and weight were selected and used for the study. Small cylindrical beakers were filled with 25 mL of the test solution or distilled water for the control group and 1 bulb was placed suspended into each beaker. It was assured that the meristematic root area (bulb's area from where the roots emerge) was in contact with the solution, but not the entire bulb was immersed. Four replicates were performed for each treatment. The bulbs were incubated in the dark at 21 ± 1 ° C for 96 hours.

7.3.5. Macroscopic parameters assessment

After 96 hours of exposure to the test solutions, the number of roots on each bulb was counted and five random roots were measured by observation with the magnifying glass. Five other roots were excised and fixed in a 3:1 ethanol acetic acid solution for 48 hours. After this period, the roots were kept in 70% ethanol for future experiments. Other signs of toxicity such as changes in root consistency and colour, and the presence of tumours, hook roots and twisted roots were also examined.

7.3.6. Statistical analysis

The percentage of roots for each bulb was calculated by dividing the mean value of all roots on the four replicates of each test solution by the mean value of roots in the control group (equation (9)).

$$MNR = \frac{MNT}{\mathrm{MNC}} \times 100 \qquad (9)$$

where MNR is the root number rate, MNT the mean number of roots on each treatment and MNC the mean number of roots on the control group.

The roots number rate inhibition (MNRI) was calculated by the equation (10):

$$MNRI = 100 - MNR \qquad (10)$$

where MNRI is the roots number rate inhibition and MNR is the root number rate (see equation 9).

The growth rate (GR) was calculated by the equation (11):

$$GR = \frac{LT}{LC}\ X\ 100 \quad (11)$$

where GR is the growth rate, LT the mean root length in the treatment and LC the mean root length in the control.

The growth rate inhibition (GRI) was calculated by the equation (12):

$$GRI = 100 - \frac{LT}{LC}\ X\ 100 \quad (12)$$

where GRI is the growth rate inhibition, LT the mean root length in the treatment and LC the mean root length in the control. For determination of the GRI, it was considered that there was 100% growth in the control group.

The number of emerging root and growth rate inhibition percentage after 96 hours of exposure were plotted against test concentrations. Data was analysed by probit analysis to calculate the EC50 value for root number rate inhibition and the EC50 value for growth rate inhibition with 95% confidence limits (α = 0.05) (Finney, 1952) using *IBM SPSS Statistics 25* software.

7.4. Results

Statistically significant differences between groups are pointed out in each graphic. Each letter (a, b, c, …) corresponds to a set of groups with no significant differences found among them. Only the relevant significant differences for the results interpretation are pointed out.

7.4.1. Acute toxic effects of [C16Pyr][Cl] to *Allium cepa*

The results for the number of emerging roots after exposure to [C16Pyr][Cl] are shown in Figure 31. The Kruskal-Wallis test showed that no statistically significant differences were found in the number of emerging roots of bulbs exposed to the different treatments after 96 hours of exposure (H=3.563, p=0.614) (groups marked as “a”, in Figure 31).

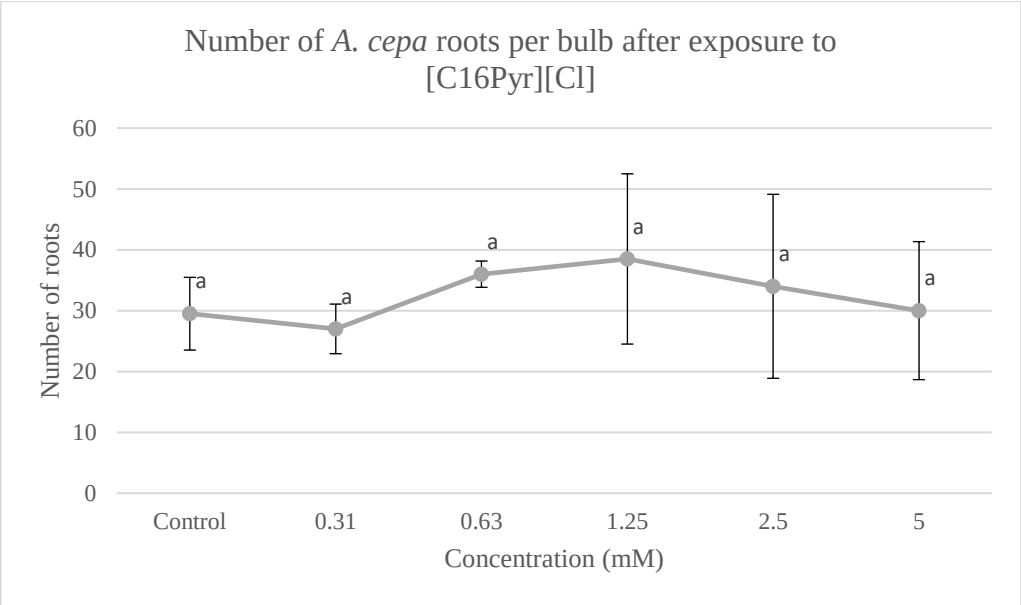

Figure 31 - Number of roots per bulb after exposure to the test concentrations of [C16Pyr][Cl] and control group. Error bars represent SD. a: no statistically significant differences found between groups.

The [C16Pyr][Cl] 96h EC50 values for the number of emerging roots was 35.404 mM (Table 21). Based on these results, it is possible to classify [C16Pyr][Cl] as relatively harmless to *A. cepa* in what concerns to the number of emerging roots. It was not possible to determine NOEC and LOEC values.

The results obtained also demonstrated that root growth was affected by the exposure to [C16Pyr][Cl]. Root length was reduced at all concentrations tested, comparing to the control, as seen in Figure 32. The Kruskal-Wallis test showed that no statistically significant difference in the root growth were found between the different treatments after 96 hours of exposure (H=12.75, p=0.026), which is also demonstrated in Figure 33 by the similar root growth rate inhibition in all the tested concentrations. The Mann-Whitney test indicated that there were significant differences in the root growth of bulbs exposed to the control group and to all the tested concentrations (p<0.05). No significant differences were found in the length of roots of bulbs exposed to all the [C16Pyr][Cl] test concentrations (groups marked as “b” in Figure 32).

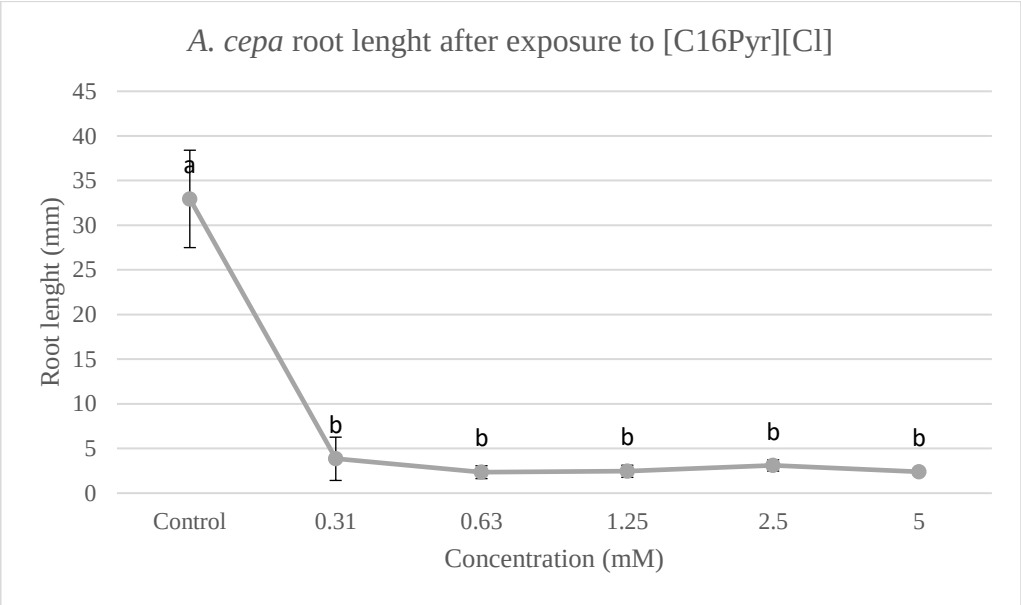

Figure 32 - *A. cepa* root length after 96 hours of exposure to the [C16Pyr][Cl] test concentrations and to the control group. Error bars represent SD. a, b: no statistically significant differences found between groups.

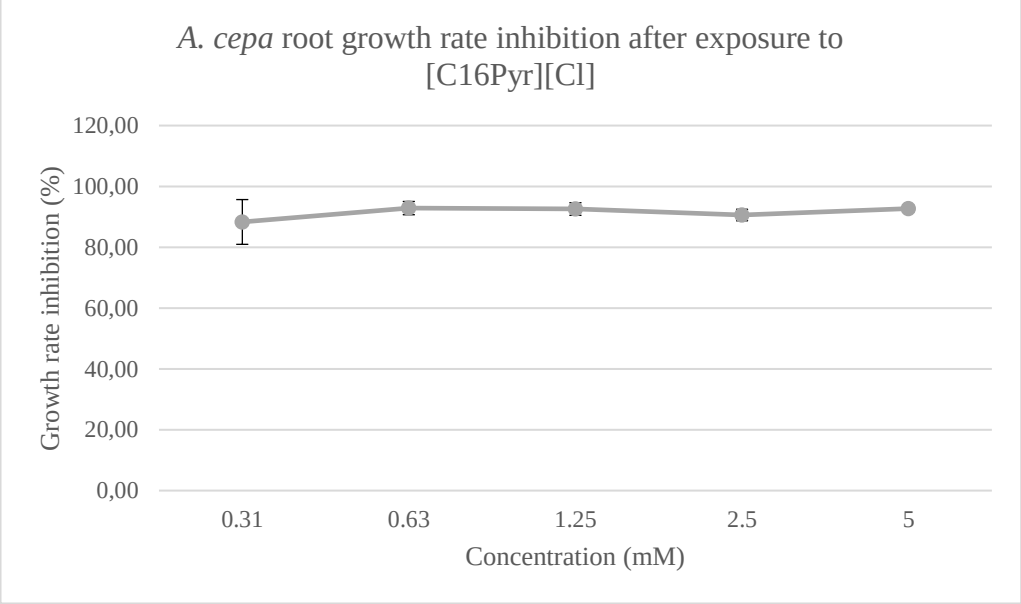

Figure 33 - *A. cepa* root growth rate inhibition after 96 hours exposure to [C16Pyr][Cl] test solutions. Error bars represent SD.

Due to the high levels of growth inhibition observed in the bulbs exposed to the [C16Pyr][Cl] test solutions, it was not possible to determine the 96h EC50 result for the root growth inhibition. Thus, we conclude that the [C16Pyr][Cl] 96h EC50 value was below 0.31 mM (Table 22). Thus, we may classify [C16Pyr][Cl] as super toxic to *A. cepa* root growth. It was also not possible to determine the NOEC and LOEC results for root growth inhibition (Table 23).

It was also observed some macroscopic changes in all the bulbs exposed to the [C16Pyr][Cl] treatments. Figure 34 shows the general aspect of one bulb from the control group (exposed to distilled water) and one bulb exposed to the 0.31 mM [C16Pyr][Cl] treatment. The bulbs demonstrated a brownish coloration of the meristematic root area, with some level of extension upwards the bulbs body. The roots of the bulbs exposed to the [C16Pyr][Cl] solutions also demonstrated a yellow coloration, while the control shows white glossy roots.

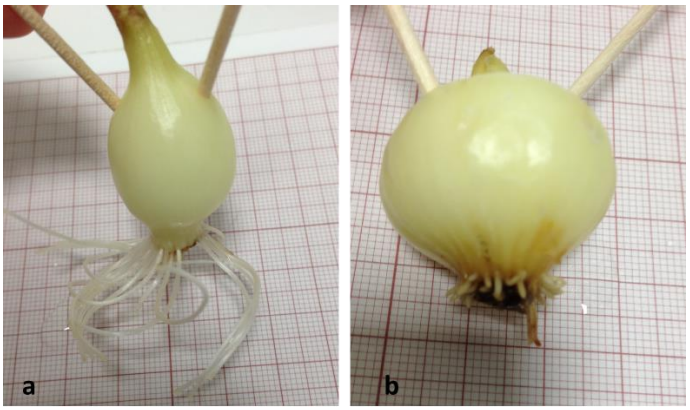

Figure 34 - General macroscopic aspect of bulbs exposed to the control (34a) and bulbs exposed to the [C16Pyr][Cl] treatment (34b).

7.4.2. Acute toxic effects of [C2OHMIM][Cl] to *Allium cepa*

The results for the number of emerging roots after exposure to [C2OHMIM][Cl] are shown in Figure 35. The Mann-Whitnney test showed that no statistically significant differences were found in the number of emerging roots of bulbs exposed to the control and to each of the different treatments after 96 hours of exposure to [C2OHMIM][Cl] (p>0.05) (groups marked as “a”, in Figure 35). Even though it was seen a higher number of roots for the 2.5 mM treatment, comparing with the control, we cannot say that this difference is statistically significant (p>0.05).

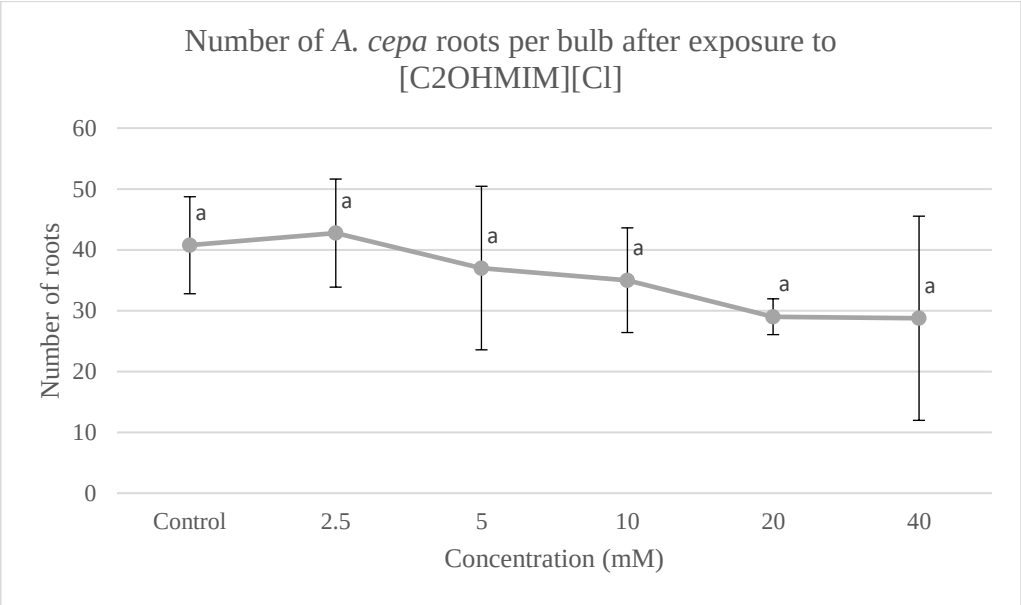

Figure 35 - Number of roots per bulb after exposure to the test concentrations of [C2OHMIM][Cl] and control group. Error bars represent SD. a: no statistically significant differences found between groups.

The [C2OHMIM][Cl] 96h EC50 values for the number of emerging roots was 38.687 mM (Table 21). Based on these results, it was possible to classify [C2OHMIM][Cl] as relatively harmless to *A. cepa* in what concerns to the number of emerging roots. It was not possible to determine NOEC and LOEC values (Table 23).

The results obtained also demonstrated that root growth was affected by the exposure to [C2OHMIM][Cl]. Root length was reduced at all concentrations tested, except for the bulbs exposed to 2.5 mM treatment, where it was observed a superior length comparing to the control. Although, no significant differences were found between the control and the 2.5 mM effect on root growth. Root length was significantly reduced at the 40 mM concentrations tested ($p < 0.05$), comparing to the control group (Figure 36). No other significant differences were found between the control and the tested concentrations. It was also visible that the root growth rate inhibition increased with the increase of the [C2OHMIM][Cl] concentration, as seen in Figure 37.

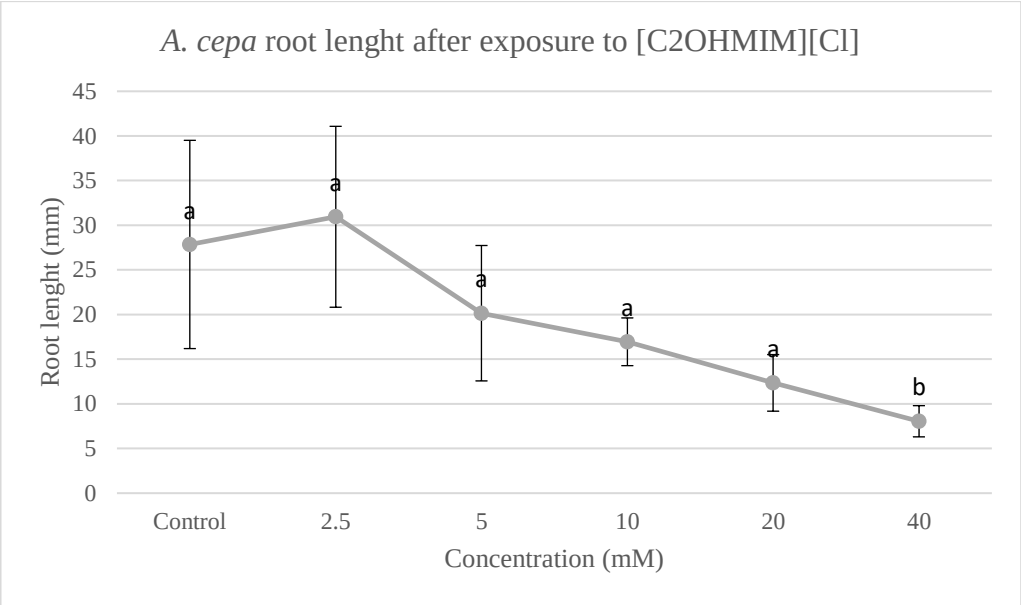

Figure 36 – *A. cepa* root length after 96 hours of exposure to the [C2OHMIM][Cl] test concentrations and to the control group. Error bars represent SD. a, b: no statistically significant differences found between groups.

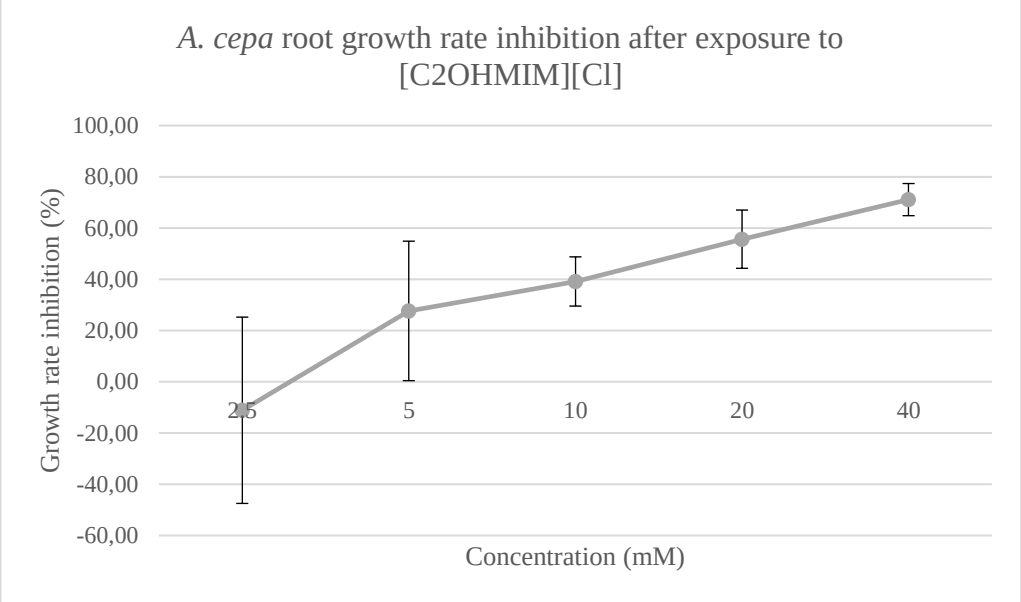

Figure 37 – *A. cepa* root growth rate inhibition after 96 hours exposure to [C2OHMIM][Cl] test solutions. Error bars represent SD.

The [C2OHMIM][Cl] 96h EC50 values for the root growth inhibition was 21.311 mM (Table 22). Thus, it was possible to classify [C2OHMIM][Cl] as relatively harmless to *A. cepa* root growth. The NOEC and LOEC values for the root growth inhibition were 20 and 40 mM, respectively, as seen in Table 23.

Table 21 - [C16Pyr][Cl] and [C2OHMIM][Cl] 96h EC50 results for the number of emerging roots endpoint. 96h EC50 values are shown in mM.

ILs	96h EC50 (mM)	95% Confidence interval	Hazard Ranking*
[C16Pyr][Cl]	35.404 (12675.0 mg L^{-1})	nd	-
[C2OHMIM][Cl]	38.687 (6291.3 mg L^{-1})	nd	-

*: The following standard was used for the 96h EC50 (mg L^{-1}) to evaluate the toxicity of the ILs to *A. cepa* according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987). nd: not possible to determine.

Table 22 - [C16Pyr][Cl] and [C2OHMIM][Cl] 96h EC50 results to the root growth rate inhibition. 96h EC50 values are shown in mM.

ILs	96h EC50 (mM)	95% Confidence interval	Hazard Ranking*
[C16Pyr][Cl]	<< 0.31 (<< 0.111 mg L^{-1})	nd	++++++
[C2OHMIM][Cl]	21.311 (3465.6 mg L^{-1})	-10.501 – 37.509	-

*: The following standard was used for the 96h EC50 (mg L^{-1}) to evaluate the toxicity of the ILs to *A. cepa* according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987). nd: not possible to determine.

Table 23 - *A. cepa* NOEC and LOEC results after 96 hours exposure to [C16Pyr][Cl] and [C2OHMIM][Cl]. Results are shown in mM.

ILs	Number of emerging roots inhibition		Root growth inhibition	
	NOEC	LOEC	NOEC	LOEC
[C16Pyr][Cl]	nd	**n**d	nd	nd
[C2OHMIM][Cl]	nd	nd	20	40

nd: not possible to determine.

7.5. Discussion and Conclusion

Ecotoxicity assays using plants proved to be useful in the risk assessment of water effluents (Rank & Nielsen, 1998), nanoparticles (Ghodake *et al.*, 2011), pesticides (Saxena

et al., 2010), new emerging pollutants (Herrero *et al.*, 2012), radiofrequency electromagnetic fields (Tkalec *et al.*, 2009) and of ILs (Silveira *et al.*, 2017; Thamke *et al.*, 2017).

Plants, as ecotoxicological models, stand out for their simplicity, cost-effectiveness, good chromosome availability and conditions, the possibility to easily study macroscopic and microscopic toxic and genotoxic effects and the good correlation of its outcome with the results of mammalian test systems (Fiskesjö, 1985). Among the plants used for this purpose, *A. cepa* is one of the most commonly applied models for phytotoxic and genotoxic tests.

Herrero *et al.* (2012) suggested that the *A. cepa* bioassay may be a useful and complementary tool to assess the toxic potential of unregulated substances and chemical mixtures found in the environment (Herrero *et al.*, 2012) and Silveira *et al.* (2017) confirms that *A. cepa* model is efficient to evaluate toxicological risks of environmental pollutants (Silveira *et al.*, 2017).

Thamke *et al.* (2017) evaluated the phytotoxicity and genotoxicity of some ILs, including imidazolium and pyridinium based ILs, on *A. cepa*. The results showed that high concentrations of ILs inhibited cell division and ultimately the plant growth. In addition, all the tested ILs induced chromosome abnormalities. Results clearly indicated that some of the ILs were extremely toxic to *A. cepa* (Thamke *et al.*, 2017).

Despite the results from Thamke *et al.* (2017), data on potential toxicity of ILs to terrestrial test species is very limited. And, as suggested by Fiskesjö, positive results in the *A. cepa* test should be regarded as an indication that the tested chemical may be a biological hazard in other organisms as well (Fiskesjö, 1985). Thus, the present study aimed to assess the ILs macroscopic effects on *A. cepa* bulbs by the evaluation of emerging root number and root growth.

In the [C16Pyr][Cl] toxic evaluation towards *A. cepa*, it was noted that [C16Pyr][Cl] did not influence the number of emerging roots. In the other hand, the results obtained demonstrated that root growth was affected by the exposure to [C16Pyr][Cl], since root length was reduced at all concentrations tested, comparing to the control. It was also observed a change in colour of the root tips, from glossy white to brownish, and the bulbs body, which may have happen due to the toxic effects causing cell death, which can influence the uptake ability of the roots (Fiskesjö, 1985).

In the phytotoxicity evaluation of [C2OHMIM][Cl] towards *A. cepa*, the results demonstrated that the number of emerging roots was also not affected by the exposure to the test concentrations. And, although it was observed a higher number of roots for the 2.5 mM treatment comparing with the control, we cannot say that this difference is statistically significant, thus, it does not represent a positive effect. Our results also point to a negative effect of [C2OHMIM][Cl], since it inhibited root growth in a dose-dependent manner.

Both ILs demonstrated that the concentrations tested did not have an influence in the number of emerging roots of bulbs exposed to the test concentrations. This may be due to the insufficiently high concentrations able to induce alteration in the number of emerging roots. Future studies, with higher concentration would be able to clarify it. However, both ILs had some influence in the development and growth of the emerged roots. In the lowest [C16Pyr][Cl] concentrations (0.31 Mm) it was already noted a 88% growth inhibition and for [C2OHMIM][Cl] it was observed a root growth inhibitory effect up to 71%, comparing with the control. These results points to some level of toxicity, since a decrease in root growth over 45% indicates the presence of toxic nature of substances (Fiskesjö, 1985).

It is known that the decrease in the root length observed may be due to disturbance in the cell division, (e.g. decrease of the mitotic index or chromosomal aberrations) or disturbance of the elongation process of the root meristematic cells. That happens into the elongation zone, where they stop dividing and start to rapidly increase in length, and is dependent on enzyme action (Bizet *et al.*, 2014; Harashima & Schnittger, 2010). These are the two meristematic root growth paths and one or both could be affected by the exposure to the tested ILs. Because root growth is regulated by the combined activities of cell division in the mitotically active root zone and cell elongation that occurs subsequently in the more proximal regions of the root tip (Perilli *et al.*, 2012). Even in the cases where there is no apparent disturbance in the root growth, it is important to clarify if the roots possibly continue to grow due to elongation of pre-existing cells. As so, the possibility of disturbed mitosis of *A. cep*a root meristematic cells is not discarded.

On the basis of these results, it is rational to believe that the ILs are able to permeate *A. cepa* roots and affect the roots elongation, metabolism, and genetic materials (Kumari *et al.*, 2009). The molecular mechanism of the toxicity due to ILs in the plant roots is not clear and requires further investigation. Further studies of the exposure consequences on the cellular level, such as the possible effects on cell division or chromosome disturbances are

important for a better understanding of the action of chemicals on biological systems (Fiskesjö, 1985). With this in mind, the root that were excised and fixed during this experiment will be used to investigate genotoxic impacts of the studied ILs on *A. cepa* in a near future.

VIII. GENERAL DISCUSSION

In the present work, we evaluated the ecotoxicity profile of one pyridinium ([C16Pyr][Cl]) and one imidazolium based IL ([C2OHMIM][Cl]) that are currently involved in chemical research and development of industrial applications (Ferraz *et al.*, 2012; Lee *et al.*, 2017; Parveen *et al.*, 2016; Teng *et al.*, 2016). Up to date, due to the low levels of annual tonnage, [C2OHMIM][Cl] does not require registration under REACH regulation. On the other hand, [C16Pyr][Cl] is currently under REACH registration conditions (EC number: 204-593-9) (Environmental Chemicals Agency, n.d.a).

Having in consideration that a single bioassay cannot provide a full picture of the ecotoxicity profile of compounds, a representative evaluation should be developed with a battery of test organisms and indicators (Repetto, 2001). For our investigations, we used an ecotoxicological test battery considering aquatic and terrestrial compartments as well as different trophic levels including producers and primary consumers from different ecosystems. The strategy to check toxicities within a flexible ecotoxicological test battery has been proven to be effective for uncovering hazard potentials of ILs (Matzke *et al.*, 2007).

[C16Pyr][Cl] EC50 results varied between 0.0000205 and 35.404 mM. [C2OHMIM][Cl] results varied between 3.635 and 111.97 mM, as demonstrated in Table 24. [C16Pyr][Cl] demonstrated to be more toxic to all the test organisms than [C2OHMIM][Cl]. [C16Pyr][Cl] hazard classification varied from relatively harmless for *A. cepa* number of emerging roots to super toxic to *D. magna* 48h acute immobilisation assay. [C2OHMIM][Cl] hazard classification varied from relatively harmless for all test systems to practically nontoxic to *D. magna*. These general results are in agreement with results from Costa *et al.* (2014), who stated that the cetylpyridinium group presented one of the lowest EC50 values in their study, being classified as slightly toxic to aquatic organisms and the imidazolium group was the less toxic being classified as practically harmless to *V. fischeri* (Costa *et al.*, 2014).

For the crustaceans, *D. magna* and *A. salina*, the EC50 values were lower after 48 hours of exposure than after 24 hours, which proves the enhancement of the toxicity over time. In the *A. cepa* test, the root growth demonstrated to be a more sensitive endpoint than the number of emerging roots. The same happened in the *L. sativa* assay results, where the root and shoot growth proved to be a more sensitive endpoint to the ILs effect than seed germination, which is in accordance with other works (Pan & Chu, 2016; Visioli *et al.*, 2016). In general, *D. magna* 48 hours mortality seemed to be the most sensitive endpoint for

both compounds, while *A. cepa* root number and *L. sativa* germination were the least sensitive.

Table 24 - Acute toxicity data of [C16Pyr][Cl] and [C2OHMIM][Cl]. Results summary from the present work.

	Assay	**[C16Pyr][Cl]**		**[C2OHMIM][Cl]**	
Organism	**Endpoint**	**EC50 (mM)**	**Hazard**	**EC50 (mM)**	**Hazard***
C. vulgaris	**Growth 96h**	0.011	+++	34.03	-
D. magna	**Mortality 24h**	0.0000449	+++++	5.257	+
	Mortality 48h	0.0000205	++++++	3.635	+
A. salina	**Mortality 24h**	0.169	++	>100 mg L^{-1}	-
	Mortality 48h	0.014	+++	>100 mg L^{-1}	-
L. sativa	**Germination 96h**	0.68	+	111.97	-
	Root growth 96h	0.34	+	16.12	-
	Shoot growth 96h	0.47	+	22.69	-
A. cepa	**Root quantity 96h**	35.404	-	38.687	-
	Root growth 96h	<<0.31	<<+++	21.311	-

*The following standard was used for the EC50 (mg L^{-1}) to evaluate the toxicity of the ILs according to the acute toxicity rating scale by Passino and Smith (1987): less than 0.01, super toxic (++++++); 0.01-0.1, extremely toxic (+++++); 0.1-1, highly toxic (++++); 1-10, moderately toxic (+++); 10 to 100, slightly toxic (++); 100-1000, practically nontoxic (+); greater than 1000, relatively harmless (-) (Passino & Smith, 1987).

Based on the results, *D. magna* demonstrated the highest sensitivity for both ILs, [C16Pyr][Cl] and [C2OHMIM][Cl]. This is in agreement with Egorova and Ananikov (2014) who have stated that *D. magna* is one of the most sensitive organisms to ILs (Egorova & Ananikov, 2014). Zhang *et al.* (2017) also found out that *D. magna* are much more sensitive than *C. vulgaris* to the imidazolium based ILs tested in their study (Zhang *et al.*, 2017).

Our results suggest the following decreasing order of sensitivity to [C16Pyr][Cl]: *D. magna* > *C. vulgaris* > *A. salina* > *L. sativa* > *A. cepa*. The organisms sensitivity to [C2OHMIM][Cl] decreased in the following order: *D. magna* > *L. sativa* > *A. cepa* > *C. vulgaris* > *A. salina*.

Since different anions leads to a diverse pattern for the observed toxicities in the different test systems (Matzke *et al.*, 2007), it is possible that the same cation core combined

with different anions can show different results in what concerns the organisms sensitivity. Because both compounds had the same anion composition (the halide chloride), we assign the different toxicity to the cation core and its interaction mode with the individual organisms´ structure.

It is well known that the ILs toxicity is dependent on the molecular structure of the compounds. Couling *et al.* (2006) demonstrated that the descriptors of the QSAR for *D. magna* are similar to those given for *V. fischeri*, which suggests that there may be similar indicators of toxicity found in many different species (Couling *et al.*, 2006). However, the different EC50 values found in the present work indicate that the individual characteristics of the organisms are also important to the effect of ILs on the living organisms.

There are still some uncertainties about the ILs toxic mode of action, however some recent studies focused on revealing correlations between ILs' structure and its toxicity towards different classes of organisms. A large number of ILs with different anions and cations bearing side chains of variable length were tested.

Costa et al (2017) summarizes in a recent review the recently published data on ILs toxicity on living organisms of different complexity. The data points that, in general, ILs demonstrated negative effects on the tested organisms. ILs biological activity is dependent of the chemical structure, with cation alkyl chain length and composition, cation core and nature of anion moiety as main contributors (Costa *et al.*, 2017). Egorova and Ananikov (2014) pointed in their review the following principal components as major factors modulating the toxicity of ILs: length of the alkyl chain in the cation; degree and nature of functionalization in the alkyl chain of the cation; nature of the anion; nature of the cation; and mutual influence of anion and cation (Egorova & Ananikov, 2014). Matzke *et al.* (2007) also deduced that ILs toxic nature were mainly caused by the cationic species rather than the anionic ones (Matzke *et al.*, 2007).

Some other works directed their efforts to the formulation of ILs toxicity QSAR models. The prediction models for toxicological effects of ILs proved to be useful to understand ILs' toxicological interactions and to design environmentally benign ILs structures (Cho & Yun, 2016; Das & Roy, 2014; Grzonkowska *et al.*, 2016).

Although the toxicity mechanism of ILs is not fully understood, it has been proposed that the mode of toxic action is related with membrane disruption. Specifically, the long alkyl chain of ILs increases the lipophilic nature of the compound which increases possible

interaction with the phospholipid bilayer of the cell membrane leading to disruption of membrane physiological function and ultimately exhibiting greater toxicity (Gal *et al.*, 2012).

Because both compounds tested in the present work have the same anion, we can exclude its influence on the toxic behaviour of the compounds. In addition, the halides (such as the chloride anion present in the compounds tested in the present work) demonstrated that they show none or very little toxic effect (Liu *et al.*, 2015; Montalbán *et al.*, 2016; Stolte *et al.*, 2007). Thus, we may conclude that the cation core was the main contributor to the observed effects of the tested ILs. Also, as the anion was shown to be less significant than the cation core when assessing the impact of ILs on the viability of the organisms (Couling *et al.*, 2006; Grzonkowska *et al.*, 2016; Ruokonen *et al.*, 2016), it is important to focus on the cation core structure when assessing ILs toxicity.

Costa *et al.* (2014) demonstrated that the cetylpyridinium group was one of the most toxic cations to *V. fisheri* and that the imidazolium group was the less toxic (Costa *et al.*, 2014). In the same work, the toxicity of the cation head groups tested showed the trend: cetylpyridinium > benzalkonium > benzethonium > imidazolium (Costa *et al.*, 2014). Couling *et al.* (2006) also reported similar results, with pyridinium head group showing higher toxicity than imidazolium (Couling *et al.*, 2006). Grzonkowska *et al.* (2016) also studied the toxicity of some ILs and found out that it depends mainly on their cations' structure: larger, more branched cations with long alkyl chains are more toxic than the smaller, linear ones (Grzonkowska *et al.*, 2016).These results are in agreement with the results from the present work, since [C16Pyr][Cl] showed greater toxicity when compared with [C2OHMIM][Cl]. In addition, toxicity is expected to decrease with ring methylation (Couling *et al.*, 2006), which also justify the lower toxicity of [C2OHMIM][Cl].

Some authors demonstrated that the inclusion of the polar groups (in the form of hydroxide, for example) in the alkyl chain of the cation also reduces the toxicity of the ILs (Grzonkowska *et al.*, 2016; Hernández-Fernández *et al.*, 2015; Tot *et al.*, 2018; Ventura *et al.*, 2012). Tot *et al.* (2018) demonstrated that the introduction of the polar groups (in the form of hydroxide and/or ether group) in the alkyl side chain of the imidazolium cation had influence on the reduction of the ILs' toxicity (Tot *et al.*, 2018). The same conclusion was achieved by Ventura *et al.* (2012) (Ventura *et al.*, 2012). And, in a different study, using a QSAR model, it was demonstrated that the increase of the number of oxygen atoms in the

cation results in the decrease of ILs toxicity (Grzonkowska *et al.*, 2016). Hernández *et al.* (2015) also found out that the inclusion of an hydroxyl group in the alkyl chain length of the cation also reduce the toxicity of the ILs (Hernández-Fernández *et al.*, 2015). Based on these results, we believe that the hydroxide group in the alkyl side chain had some role in the low toxic levels demonstrated by [C2OHMIM][Cl].

It has also been demonstrated that the toxic effect of ILs is highly dependent on the alkyl side chain length of the cation. With the increasing of the alkyl chain lengths, the toxicity of the ILs tends to increase (Couling *et al.*, 2006; Gal *et al.*, 2012; Ruokonen *et al.*, 2016; Stolte *et al.*, 2007; Zhang *et al.*, 2017). [C16Pyr][Cl] has a longer alkyl chain (16 carbon atoms) while [C2OHMIM][Cl] has a shorter alkyl chain, with only 2 carbon atoms. Based on the principle that the toxicity increases with the increase of the alkyl chain, it is possible to justify the greater toxic behaviour of [C16Pyr][Cl] when compared with [C2OHMIM][Cl].

The alkyl chain effect on the increase of toxicity could be explained by the increase of lipophilic nature of compounds, since the length of the alkyl chain is the main structural feature of ILs determining their lipophilicity (Freire *et al.*, 2007). Gal *et al.* (2012) demonstrated that both the side chain composition and particularly the head-groups of ILs constitute determinants for membrane activity and consequent cell toxicity (Gal *et al.*, 2012).

A relationship between the toxicity, expressed as Log EC50, and the 1-octanol-water partition coefficient (Log Kow) was established and shows how Log (1/EC50) increases with increasing Log Kow (Montalbán *et al.*, 2016), which means that high Log Kow are related with higher toxicity. The present results are also in accordance with this factor, since [C16Pyr][Cl] log Kow (1.71) is higher than log Kow of [C2OHMIM][Cl] (-2.32), which may explain the higher toxic profile of [C16Pyr][Cl].

Stolte *et al.* (2007) also demonstrate strong interactions of hydrophobic ILs cations with cell membranes, indicating that the membrane system of organisms is probably a primary target site of toxic action (Stolte *et al.*, 2007).

IX. CONCLUSION

The present work showed that [C16Pyr][Cl] demonstrated to be more toxic to all the test organisms than [C2OHMIM][Cl]. [C16Pyr][Cl] hazard classification varied from relatively harmless to super toxic, while [C2OHMIM][Cl] hazard classification varied from relatively harmless to practically nontoxic. The results also shown that *D. magna* demonstrated the highest sensitivity for both ILs tested.

Although the molecular structure of the ILs is a decisive factor in the toxic behaviour of the compounds, the different EC50 values found in the present work indicate that it is important to assess the effect of ILs in different organisms, from different organization levels and environments, to achieve the most complete ecotoxicological profile.

In the present work, two ILs with very different ecotoxicological profiles were evaluated. Under our experimental conditions, [C16Pyr][Cl] may in some way be considered toxic, but different effects were noted depending on the organism and endpoint analysed. In the other hand, [C2OHMIM][Cl] showed some promising results in terms of ecotoxicological profile, which makes it a good candidate for future researches and applications. And, since [C2OHMIM][Cl] is not yet under REACH regulation, the present results could demonstrated useful in the future, if the manufacture levels requires REACH registration. In the other hand, [C16Pyr][Cl] high levels of consumption already require REACH registration. The present results may be useful to evaluate the environmental risk of this compounds if released into the environment from manufactures or consumers.

It is true that the most attractive property of ILs is its design flexibility, which can also apply to their toxicity profile. Thus, ILs ecotoxicological impact must be taken into consideration in the design of ILs, opening to the possibility to create safer ILs which are nontoxic for humans and for the environment. Therefore, the toxicity data here reported can be considered as evidence to help for the appropriate choice of cation and anion structure in the design of ILs with improved physicochemical properties but also to obtain safer compounds.

Information about aquatic and terrestrial toxicity is essential to ecological risk assessments of ILs, and different organisms should be taken into the test battery in order to develop a comprehensive toxicity profile for these pollutants.

In conclusion, and according to literature, it is possible to assign the lower toxicity of [C2OHMIM][Cl] mainly to its cation core structure that aggregates several parameters that

decreases ILs toxicity, such as methylated imidazolium ring, short alkyl chain and hydroxide group incorporation.

In future works, we intend to study the cytogenetic effects of these compounds on the meristematic root cells of *A. cepa* using mitotic index, chromosomal and mitotic aberrations as endpoints. We also believe that effects of chronic exposure of ILs should be considered for further investigation.

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XI. APPENDIX

Appendix I - Z8 Kotai medium

To 1 litre of sterilized water, add 10 mL of stock solutions I, II and III and 1 mL of stock solution IV.

Table 25 – Z8 Kotai medium stock solutions composition.

Stock solution I	**g L^{-1}**
$NaNO_3$	46.7
$Ca(NO_3)_2 \cdot 4H_2O$	5.9
$MgSO_4 \cdot 7H_2O$	2.5
Stock solution II	**g L^{-1}**
K_2HPO_4	3.1
Na_2CO_3	2.1
Stock solution III	**mL L^{-1}**
$FeCl_3$	10.0
EDTA-Na	9.5
Stock solution IV	**mL L^{-1}**
1 to 12*	10.0
13 to 14*	100.0

Table 26 - Solutions 1 to 14 composition. These solutions are used to prepare stock solution IV.

Solutions 1 to 14	**mL L^{-1}**	**Solutions 1 to 14**	**mL L^{-1}**
1 – $Na_2WO_4.2H_2O$	0.33	8 – $CuSO_4.5H_2O$	1.25
2 – $(NH_4)_6Mo_7O_{24}.4H2O$	0.88	9 – $NiSO_4(NH_4)_2SO4.6H_2O$	1.98
3 - KBr	1.2	10 – $Cr(NO_3)_3.9H_2O$	0.41
4 – KI	0.83	11 – V_2O_5	0.089
5 – $ZnSO_4.7H_2O$	2.87	12 – $Al_2(SO_4)_3K_2SO_4.12H_2O$	4.74
6 – $Cd(NO_3).4H_2O$	1.55	13 – $MnSO_4.4H_2O$	3.1
7 – $Co(NO_3)_2.6H_2O$	1.46	14 – $MnSO_4.4H_2O$	2.23

Appendix II - M4 Elendt medium

M4 Elendt medium composition.

Table 27 - M4 Elendt medium composition.

Stock solutions	**mg L^{-1}**
H_3BO_3	2.8595
$MnCl_2.4H_2O$	0.3605
LiCl	0.3060
RbCl	0.0710
$SrCl_2.6H_2O$	0.1520
NaBr	0.0160
$Na_2MoO_4.2H_2O$	0.0630
$CuCl_2.2H_2O$	0.0168
$ZnCl_2$	0.0130
$CoCl_2.6H_2O$	0.0100
KI	0.0033
Na_2SeO_3	0.0022
NH_4VO_3	0.0006
$Na_2EDTA.2H_2O$	2.5000
$FeSO_4.7H_2O$	0.9955
$CaCl_2.2H_2O$	293.80
$MgSO_4.7H_2O$	123.30
KCl	5.8000
$NaHCO_3$	64.800
$Na_2SiO_3.5H_2O$	10.000
$NaNO_3$	0.2740
KH_2PO_4	0.1430
K_2HPO_4	0.1840
Tiamin-HCl	0.0750
Vitamin B12	0.0010
Biotin	0.0008