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The acute toxic effects of 1-alkyl-3-methylimidazolium nitrate ionic liquids on *Chlorella vulgaris* and *Daphnia magna*☆

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ABSTRACT

Given their increasingly widespread application, the toxic effects of ionic liquids (ILs) have become the subject of significant attention in recent years. Therefore, the present study assessed the acute toxic effects of 1-alkyl-3-methylimidazolium nitrate ($[C_n mim]NO_3$ (n = 2, 4, 6, 8, 10, 12)) on *Chlorella vulgaris* and *Daphnia magna*. The sensitivity of the tested organism *Daphnia magna* and the investigated IL concentrations in water using high-performance liquid chromatography (HPLC) were also evaluated to demonstrate the reliability of the present study. The results illustrated that *Daphnia magna* is indeed sensitive to the reference toxicant and the investigated ILs were stable in the aquatic environment. The 50% effect concentration (EC_{50}) was used to represent the acute toxic effects on *Chlorella vulgaris* and *Daphnia magna*. With the increasing alkyl-chain lengths, the toxicity of the investigated ILs increased in both the test organisms. Accordingly, the alkyl-chain lengths can cause significantly toxic effects on aquatic organisms, and *Daphnia magna* are much more sensitive than *Chlorella vulgaris* to the imidazolium-based ILs used in the present study. Furthermore, the present study provides more information on the acute toxic effects of 1-alkyl-3-methylimidazolium nitrate.

1. Introduction

With rapid economic development and the growing environmental protection awareness, the methods to select environmentally friendly and efficient solvents have aroused wide attention. Ionic liquids (ILs), a type of new solvent consisting entirely of various cations and anions (Couling et al., 2006), are considered "green solvents," similar to supercritical carbon dioxide and water due to their unique physicochemical properties compared to traditional solvents. Thus, ionic liquids have been widely used in various physical and electrochemical applications and separation processes in recent years (Ho et al., 2011; Shiddiky and Torriero, 2011; Zhang et al., 2011) due to their exceptional solubility, stability, nonflammability, recyclability and synthetic flexibility (Bado-Nilles et al., 2015; Roy et al., 2014).

Jastorff et al. (2003) suggested that ILs can be harmful in design applications. Few ILs can exist in the air due to their low vapor pressures; they are more likely to enter and accumulate in the soil and water environment and cause pollution due to their exceptional solubility and stability (Liu et al., 2015c; Wilkes, 2004). However, little attention has focused on the acute toxicity of imidazolium-based ILs with different alkyl-chain lengths on aquatic organisms. Thus, it is important to determine whether the acute toxic effects of imidazolium-based ILs on *Chlorella vulgaris* and *Daphnia magna* are related to the alkyl-chain lengths before their widespread application.

Imidazolium-based ILs have been extensively applied and investigated (Couling et al., 2006; Deng et al., 2015; Liu et al., 2013; Ma et al., 2014). While some studies have indicated that ILs have toxic effects on plants, microorganisms and aquatic organisms (Liu et al., 2015a; Pernak et al., 2003; Pretti et al., 2005, 2008; Yu et al., 2009), little data is available on the toxicity of the 1-alkyl-3-methylimidazolium nitrate ILs. Imidazolium-based ILs $[C_n mim]NO_3$ (n = 2, 4, 6, 8, 10, 12), which have all the properties of typical ILs, were investigated in this study to evaluate their acute toxic effects on aquatic organisms.

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The acute toxic effects of ($[C_n mim]NO_3$, n = 2, 4, 6, 8, 10,12) on zebrafish (*Danio rerio*) have been described in another paper (Zhang et al., 2017), while we are still far from comprehensive understanding of possible risks of ILs toward the aquatic environment. Thus, as a widely used strain (Baker and Wallschläger, 2016; Latała et al., 2009; Mu et al., 2009), *Chlorella vulgaris* was used to test exposure to the ILs $[C_n mim]NO_3$ (n = 2, 4, 6, 8, 10, 12) to determine the acute toxic effects on aquatic organisms. The aquatic organism *Daphnia magna*, which was widely utilized by the Organization for Economic Cooperation and Development (OECD) as a bioindicator to test the toxic effects of chemicals (Jeong et al., 2016; Luo et al., 2008; Maselli et al., 2017; Yu et al., 2009; Zimmermann et al., 2017), was also assessed. In the present study, 1-alkyl-3-methylimidazolium nitrate ILs were used to evaluate the toxic effects on *Chlorella vulgaris* using 24, 48, 72, 96 h EC_{50} and on *Daphnia magna* using 24, 48 h EC_{50}, respectively. The present study aimed to investigate the influence of different chemical structures on the acute toxicity of the investigated ILs and to find a more sensitive model organism.

2. Materials and methods

2.1. Chemicals and reagents

Chengjie Chemical Company Limited (Shanghai, China) provided the six ionic liquids (ILs) used in the present study. They were as follows: 1-ethyl-3-methylimidazolium nitrate ($[C_2mim]NO_3$, 99% purity, CAS Nos. 143314-14-1), 1-butyl-3-methylimidazolium nitrate ($[C_4mim]NO_3$, 99% purity, CAS Nos. 179075-88-8), 1-hexyl-3-methylimidazolium-nitrate ($[C_6mim]NO_3$, 99% purity, CAS Nos. 203389-26-8), 1-octyl-3-methylimidazolium nitrate ($[C_8mim]NO_3$, 99% purity, CAS Nos. 203389-27-9), 1-decyl-3-methylimidazolium nitrate ($[C_{10}mim]NO_3$, 99% purity, CAS Nos. 1057409-912), and 1-dodecyl-3-methylimidazolium nitrate ($[C_{12}mim]NO_3$, 99% purity, CAS Nos. 799246-93-8). The other chemicals and reagents used in the present study were obtained from Sigma Chemical (St. Louis, Missouri, USA) and Solarbio Science &Technology Company (Beijing, China) and were all of analytical-grade.

2.2. Experimental designs for the acute toxicity study of the six ILs to Chlorella vulgaris

2.2.1. Batch cultures of Chlorella vulgaris

The *Chlorella vulgaris* samples used in the present study were purchased from the Institute of Hydrobiology at the Chinese Academy of Sciences (Wuhan, China) and were domesticated until the third generation reached its logarithmic phase. The culture methods for *Chlorella vulgaris* are described in procedure 201 of the OECD (2011). The use of Blue-Green (BG-11) medium and the test algae were the main modifications of the culture conditions in the present study. The BG-11 medium is a mixture of 10 mL 15 g/100 mL $NaNO_3$, 10 mL 2 g/500 mL K_2HPO_4, 10 mL 3.75 g/500 mL $MgSO_4 \cdot 7H_2O$, 10 mL 1.8 g/500 mL $CaCl_2 \cdot 2H_2O$, 10 mL 0.3 g/500 mL Citric acid, 10 mL 0.3 g/500 mL Ferric ammonium citrate, 10 mL 0.05 g/500 mL $EDTANa_2$, 10 mL 1.0 g/500 mL Na_2C and 1 mL A_5 (a trace metal solution) diluted with distilled water to 1 L. A_5 is a mixture of 2.86 g H_3BO_3, 1.86 g $MnCl_2 \cdot 4H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$ and 0.05 g $Co(NO_3)_2 \cdot 6H_2O$ diluted with distilled water to 1 L; the pH of A_5 was adjusted to 7.1 with 1 M NaOH or HCl. The prepared BG-11 medium and all the glassware used in the present study were high-temperature sterilized for 20 min using a high-pressure steam sterilizer (TOMY SX-500, TOMY digital biology, Japan). After cooling, the clean bench (SJ-CJ-1FD, Suzhou Sujie purification devices co., LTD, China) was used to sterilize the BG-11 medium and glassware for 30 min using ultraviolet radiation to prevent the test *Chlorella vulgaris* from contamination with the other algae and bacteria strains. The 100 mL solutions of the test algae were batch-cultured in 1 L glass conical flasks with 400 mL sterilized BG-11 medium. The aforementioned culture solutions of *Chlorella vulgaris* were acclimatized for 10 day at 22 ± 1 °C, with an illumination intensity of 3000 lx and a photoperiod 16 h light: 8 h dark in an artificial climate chest (RXZ–500B–LED, Ningbo Jiangnan Instrument Factory, China) for the entire domestication period. The culture solutions were replaced every three days. The glass conical flasks were shaken at regular intervals four or five times every day to prevent precipitates from forming.

2.2.2. Preliminary and formal experimental designs for Chlorella vulgaris

According to Mu et al. (2009), the concentrations used for the acute tests of the formal experiment are shown in Table 1.

Other than the experimental group, one control group without ILs was utilized in each test. Three experimental replicates were applied to each experiment and to each control group. The methods used to determine the acute toxicity of *Chlorella vulgaris* after exposure to six ILs in a static test followed the OECD 201 procedure (2011). The batch-cultured *Chlorella vulgaris* was observed under a microscope (Nikon YS100, Jiangnan photoelectric co., LTD, China) with a hemocytometer at regular intervals. Then, normal cells from the final batch-cultured *Chlorella vulgaris* in the logarithmic phase were used in the present study, with concentrations in cells per mL ranging from 10^5 to 10^6. The 20 mL solutions of the test algae were placed into 250 mL glass conical flasks with 80 mL sterilized BG-11 medium. The ILs were dissolved at the selected concentrations in dechlorinated tap water. Then, 20 μL test IL solutions were added to the glass conical flasks. The aforementioned solutions of *Chlorella vulgaris* were acclimatized for 96 h at 22 ± 1 °C, with an illumination intensity of 3000 lx and a photoperiod of 16:8-h in an artificial climate chest (RXZ–500B–LED, Ningbo Jiangnan Instrument Factory, China) for the entire domestication period. The aforementioned culture solutions were not renewed during the entire environmental period. The glass conical flasks were shaken at regular intervals four or five times every day to prevent the formation of precipitates. The cell numbers of *Chlorella vulgaris* were observed under the microscope with a hemocytometer and were recorded at 0, 24, 48, 72 and 96 h from the initiation time of the tests after the exposure to the tested ILs used in the present study for the duration of the entire experiment. The median effects concentration (EC_{50}) is the concentration at which half of the *Chlorella vulgaris* growth is inhibited. The 24 h, 48 h, 72 h and 96 h EC_{50}s were used to describe the acute toxic effects of the six ILs on *Chlorella vulgaris*.

Table 1
The concentrations of ionic liquids (ILs) for *Chlorella vulgaris* in the acute toxicity study.

ILs	The concentrations of the formal experiment (mg/L)
$[C_2mim]NO_3$	200, 400, 600, 800, 1000, 1200
$[C_4mim]NO_3$	50, 100, 150, 200, 250, 300
$[C_6mim]NO_3$	40, 60, 80, 100, 120, 140
$[C_8mim]NO_3$	5, 10, 15, 20, 25, 30
$[C_{10}mim]NO_3$	1.0, 1.4, 1.8, 2.2, 2.6, 3.0
$[C_{12}mim]NO_3$	0.1, 0.2, 0.3, 0.4, 0.5, 0.6