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Toxicity of 1-alkyl-3-methyl imidazolium nitrate ionic liquids to earthworms: The effects of carbon chains of different lengths

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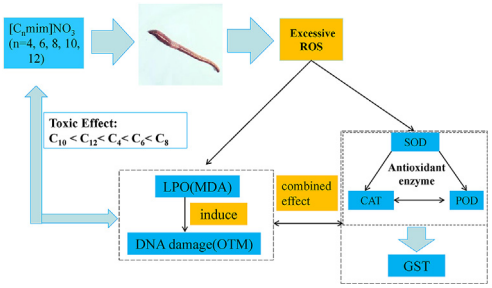
HIGHLIGHTS

- Effects of ILs $[C_nmim]NO_3$ on earthworms were systematically investigated first.
- Alkyl chain effect and cut-off effect were both observed in the present study.
- $[C_nmim]NO_3$ can cause oxidative stress and oxidative damage in earthworms.

GRAPHICAL ABSTRACT



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ABSTRACT

Ionic liquids (ILs), which are alternatives to traditional organic solvents, have the potential to enter soil and cause negative effects on the soil micro-environment, especially soil organisms. The objective of this study was to determine the "alkyl chain effect" and "cut-off effect" mechanisms underlying the toxicity of ILs. The assessment for subchronic toxicity toward earthworms (*Eisenia fetida*) by five common imidazole nitrate ILs ($[C_nmim]NO_3$ (n = 4, 6, 8, 10, and 12)) was conducted on day 28 after exposure to five concentrations (0, 5, 10, 20, and 40 mg kg^{-1}) of ILs. Earthworms showed oxidative stress and oxidative damage, and both "alkyl chain effect" and "cut-off effect" (occurred in C_{10}) were observed. In addition, the toxicity of ILs increased with the increase in concentration. Analysis of imidazolium ILs in artificial soil at the end of the experiment indicated that these selected ILs remained relatively stable, with a rate of change of less than 7.39%. The present study provides theoretical support for decisions regarding IL use and helps to establish a friendly IL structure database.

1. Introduction

Ionic liquids (ILs) are widely known as alternative solvents due to their excellent properties such as negligible vapor pressure, good thermal stability, nonflammability, and a wide electrochemical (conductivity) window (Ho et al., 2014; Made et al., 2015). ILs have been considered as "green solvents" for a long time; however, concerns over their ecological effects on plant and soil health have been reported by recent studies (Ghanem et al., 2018). Ławniczak

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et al. (2016) studied the effects of herbicidal ILs (HILs) on the bacterial community structure of soil microorganisms, and their study proved that HILs affected the structure of autochthonic soil bacteria. Sydow et al. (2018) studied the influence of six phosphonium-based ILs at sublethal concentrations on the structure of the microbial community present in urban park soil and found that ILs can reduce the biodiversity of soil. With regard to soil plants, by the evaluation of the effects of imidazolium-based ILs with different anions on wheat seedlings, Chen et al. (2018) reported that ILs impeded plant growth by disrupting the metabolic physiology and changing the cellular structures. Xu et al. (2018) found that imidazolium-based ILs with different anions caused oxidative stress and oxidative damage in wheat (*Triticum aestivum* L.) seedlings. Regarding toxicity research in soil animals (consider earthworms as an example), Luo et al. (2009) observed the acute toxicity of ILs toward earthworms, and they found that the toxicity of ILs increased with the increase in carbon chain length. Liu et al. (2016) proved that ILs [omim]PF_6 cause inevitable oxidative stress and oxidative damage in earthworms.

Earthworms are an important part of soil ecosystems (Chen et al., 2017), and *Eisenia fetida* is widely used as a model organism to detect contaminants (Zhao et al., 2014; Li et al., 2017).

In the present study, *E. fetida* was infected with 1-alkyl-3-methyl imidazolium nitrate ILs [$C_n$mim]NO_3 (n = 4, 6, 8, 10, and 12) to comprehensively examine the toxicity of ILs with carbon chains of different lengths and the same anions toward earthworms. After 14 and 28 days of exposure, levels of reactive oxygen species (ROS), antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (POD)), detoxification enzymes (glutathione S-transferase (GST)), lipid damage markers (malondialdehyde (MDA)), and DNA damage (olive tail moment (OTM)) were measured to determine the extent of oxidative stress and oxidative damage. Finally, we compared the toxicity of these ILs by objectively evaluating the toxicity of ILs with different chain lengths toward earthworms, with the goal of providing theoretical support for their future use and to help in establishing an IL structure database.

2. Materials and methods

2.1. Materials

Five ILs [$C_n$mim]NO_3 (n = 4, 6, 8, 10, and 12) with 99.0% purity were obtained from Shanghai Cheng Jie Chemical Co., Ltd. (Shanghai, China). All biochemical reagents required for the experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

E. fetida was purchased from the Shandong Agricultural University. Earthworms were cultivated using cow manure as the feeding medium (20–25 °C); mature and healthy earthworms (0.3–0.6 g) were chosen and precultured for 24 h before being used for experiments.

The artificial soil (10% sphagnum peat moss+20% kaolin clay+70% industrial sand, with the soil moisture content of 35%) was prepared in accordance with the Organisation for Economic Co-operation and Development (OECD, 1984) guidelines.

2.2. Methods

2.2.1. Exposure

ILs were dissolved in distilled water with concentrations of 0, 5, 10, 20, and 40 mg kg^{-1} which cover possible concentrations occurring in the environment. Each IL was thoroughly mixed with 500 g of dry artificial soil in a beaker, and the final soil moisture was about 35%. Then, 10 earthworms (after preculturing for 24 h) were placed into each beaker and cultivated in a growth chamber (20 ± 2 °C). Each concentration was replicated three times. On days 14 and 28, nine earthworms from each concentration (three from each beaker) were removed, and three earthworms of each were used for determining ROS levels, the comet assay, and the levels of other biomarkers.

2.2.2. Measurement of the protein content

The protein content in the earthworms was determined using the Bradford method (Bradford, 1976), and bovine serum albumin was used for the standard curve.

2.2.3. Measurement of the ROS content

The ROS content was measured by the 2′,7′-dichlorohydrofluorescein-diacetate (DCFH-DA) method described by Liu et al. (2014). Three earthworms were homogenized with phosphate-buffered saline (PBS) and then centrifuged (4 °C) twice: first at 3000 × g for 20 min and second at 20,000 × g for 20 min (Eppendorf, Centrifuge 5804, GER). Next, the precipitate was resuspended in PBS, and the protein content was determined. Thereafter, DCFH-DA was mixed with the enzyme solution and PBS, and the mixture was quickly transferred to a 37 °C water bath and incubated for 20 min. The fluorescence was then measured using a fluorescence spectrophotometer (Shimadzu, RF-5301PC).

2.2.4. Measurements of SOD, CAT, POD, and GST activities and the MDA content

Three earthworms were homogenized in 10 vol of PBS relative to their bodyweights and centrifuged at 10,000 rpm for 10 min to extract the enzymes.

SOD activity was measured according to the inhibition of SOD by the reduction of nitroblue tetrazolium chloride (NBT) in light, as described by Han et al. (2014). The reaction solution included PBS, L-methionine, NBT, ethylenediamine tetraacetic acid disodium (Na_2-EDTA), riboflavin, and deionized water. The reaction solution was added to the enzyme extract (sample) and PBS (reference), the solutions were illuminated under a fluorescent lamp (4000 lx) for 30 min, and then, the ultraviolet absorbance of the mixture was measured at 560 nm.

CAT activity was measured based on the decomposition of hydrogen peroxide (H_2O_2), which caused a change in the absorbance of the mixture, using the method described by Han et al. (2014). The enzyme extract was added to PBS-I (reference) and PBS-II (sample, consisting of PBS-I and 30% H_2O_2). Then, the ultraviolet absorbance at 250 nm was measured every 5 s for a total of 1 min.

POD activity was measured based on its ability to oxidize guaiacol and produce the brown material using the method described by Han et al. (2014). The reaction solution comprised PBS, guaiacol, and H_2O_2. Next, the reaction solution was added to the enzyme extract (sample) and PBS (reference). Then, the ultraviolet absorbance at 470 nm was measured every 30 s for a total of 3 min.

GST catalyzes the binding of reduced glutathione (GSH) to 1-chloro-2,4-dinitro-benzene (CDNB), and the light absorption peak of the bound product was measured at 340 nm. GST activity was measured by determining the increase in absorbance at 340 nm (Han et al., 2014). The buffer comprised Na_2HPO_4, NaH_2PO_4, glycerine, phenylmethylsulfonyl fluoride (PMSF), EDTA, and dithiothreitol (DTT). Buffer, CNDB, and GSH were added to the reference cell; furthermore, buffer, CNDB, the enzyme extract, and GSH were added to the sample cell. Then, the ultraviolet absorbance at 340 nm was measured every 30 s for a total of 3 min.

MDA reacts with thiobarbituric acid (TBA). The light absorption