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Investigation of toxic effects of imidazolium ionic liquids, [bmim][BF_4] and [omim][BF_4], on marine mussel *Mytilus galloprovincialis* with or without the presence of conventional solvents, such as acetone

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ABSTRACT

This study investigated the cytotoxic, oxidative and genotoxic effects of two commonly used imidazolium ionic liquids (ILs), [bmim][BF_4] (1-butyl-3-methylimidazolium) and [omim][BF_4] (1-methyl-3-octylimidazolium tetrafluoroborate), on the marine mussel *Mytilus galloprovincialis*, as well as whether acetone could mediate their toxic profile. In this context, mussels were firstly exposed to different concentrations of [bmim][BF_4] or [omim][BF_4], with or without the presence of acetone (at a final concentration of 0.06% v/v), for a period of 96 h, in order to determine the concentration that causes 50% mussel mortality (LC_{50} values) in each case. Thereafter, mussels were exposed to sub- and non-lethal concentrations of ILs for investigating their ability to cause lysosomal membrane impairment (with the use of neutral red retention assay/NRRT), superoxide anion and lipid peroxidation byproduct (malondialdehyde/MDA) formation, as well as DNA damage and formation of nuclear abnormalities in hemocytes. The results showed that [omim][BF_4] was more toxic than [bmim][BF_4] in all cases, while the presence of acetone resulted in a slight attenuation of its toxicity. The different toxic behavior of ILs was further revealed by the significantly lower levels of NRRT values observed in [omim][BF_4]-treated mussels, compared to those occurring in [bmim][BF_4] in all cases. Similarly, [bmim][BF_4]-mediated oxidative and genotoxic effects were observed only in the highest concentration tested (10 mg L^{-1}), while [omim][BF_4]-mediated effects were enhanced at lower concentrations (0.01–0.05 mg L^{-1}). Overall, the present study showed that [bmim][BF_4] and [omim][BF_4] could induce not only lethal but also nonlethal effects on mussel *M. galloprovincialis*. The extent of [bmim][BF_4] and/or [omim][BF_4]-mediated effects could be ascribed to the length of each IL alkyl chain, as well as to their lipophilicity. Moreover, the role of acetone on the obtained toxic effects of the specific ILs was reported for the first time, giving evidence for its interaction with the ILs and the modulation of their toxicity.

1. Introduction

Ionic liquids (ILs) are considered to be the ideal alternative to conventional organic solvents, due to their unique properties (reviewed in Earle and Seddon, 2000; Plechkova and Seddon, 2008). Among ILs, imidazolium-based ILs such as 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4] and 1-methyl-3-octylimidazolium tetrafluoroborate [omim][BF_4] are the most widely used, either alone or in combination with organic solvents such as acetone, in a wide range of industrial and chemical applications (Grishina et al., 2013; Keskin et al., 2007; Ruiz et al., 2013; Stepnowski and Zaleska, 2005). Specifically, ILs/acetone mixtures, being one of the most attractive mixtures of ILs with polar organic compounds, have been used in a variety of applications, such as acetone removal from water (Izak et al., 2008), improving the kinetic and selectivity effects in heterogeneous hydrogenation of acetone (Khodadadi-Moghaddam et al., 2009), analyzing the presence of acetone by microextraction-gas chromatography (Carda-Broch et al., 2010), in SCFs technology and many others (Ruiz et al., 2013 Zhang et al., 2007). However, despite the fact that IL mixtures with either aqueous media or acetone could alter their structural, thermodynamic and dynamic properties (Endres and Zein El Abedin, 2006; Wasserscheid and Welton, 2002), their toxicity remains still unknown.

The increasing interest in expanding their applications has raised great concerns about their environmental risk, since their

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entrance into the water via accidental spills, leaching of landfill sites or via effluents is one of the most questioned issues. In fact, although ILs can lessen the risk of air pollution, their water solubility, as well as their low degree of environmental degradation (Modelli et al., 2008) and biodegradability (Docherty et al., 2007; Garcia et al., 2005; Gathergood et al., 2006; Romero et al., 2008) may pose an environmental threat for aquatic organisms. In addition, ILs can easily undergo structural and properties modifications at the presence of even low concentrations of water, medium or solvent (Freire et al., 2010; Thomazeau et al., 2003), thus leading to unpredictable ILs-induced effects on aquatic species.

Although the toxic effects of ILs have been studied in different organisms, including aquatic species and cell lines (Bernot et al., 2005; Costello et al., 2009; Du et al., 2012; Kumar et al., 2011; Matzke et al., 2007; Pretti et al., 2009; Radošević et al., 2013; Ranke et al., 2004; Samori et al., 2010), the majority of these studies were based on the estimation of ILs-mediated lethal endpoints. Furthermore, taking under consideration recently published data, regarding the susceptibility of marine bivalve mollusks such as *Mytilus galloprovincialis* to ILs, as well as the important role of acetone to IL-mediated toxicity (Tsarpali and Dailianis, 2015), it is of great interest to investigate both the ability of ILs to promote nonlethal effects and the role of acetone in the obtained toxic profile of ILs.

Species of the genus *Mytilus sp*. are systematically used as biological models for assessing the adverse effects of xenobiotic compounds on the marine biota, due to their well-known physiology and ability to respond rapidly to environmental stress (Livingstone, 1991; UNEP/RAMOGE, 1999). Specifically, the exposure of mussels to xenobiotics could lead to alterations in their homeostasis, as a net result of exposure and toxicity. Those alterations are measurable endpoints, called biomarkers or stress indices and their investigation is systematically used both in field and laboratory studies for determining pre-pathological alterations, before mortality and/or other irreversible conditions occur (for more details see Dailianis, 2011; Moore et al., 2004). Among mussel tissues, hemocytes of hemolymph represent the main immune defense of mollusks (Cheng, 2001), while their viability and functional integrity could affect the health status of the organism (Alvarez and Friedl, 1992; Matozzo et al., 2001). For this reason, they have been widely used for assessing the adverse effects of xenobiotic compounds (Dailianis, 2009; Dailianis et al., 2014, 2011, 2009; Danellakis et al., 2011; Giannapas et al., 2012; Olabarrieta et al., 2001; Patetsini et al., 2013; Toufexi et al., 2013; Tsarpali and Dailianis, 2012; Tsiaka et al., 2013).

Since the knowledge of IL-mediated adverse effects on aquatic biota is of great importance for their development, usage/application and safety (European, 2006), the present study investigated the adverse effects of [bmim][BF_4] and [omim][BF_4] on the mussel *M. galloprovincialis*, as well as whether conventional solvents, such as acetone, could mediate their toxic potency. Given that lethal endpoints (in terms of LC_{50} values) represent an important index for determining the degree of IL toxicity, mussel mortality test (96 h) was primarily performed. Thereafter, mussels were exposed to sub- and nonlethal concentrations of each IL (with or without the presence of acetone) for investigating IL-mediated cytotoxic (currently tested with the use of neutral red retention time/NRRT assay), oxidative (superoxide anions and lipid peroxidation enhancement) and genotoxic (with the use of Comet and MN assays) effects on their hemocytes. To our knowledge, the present study is the first to show adverse effects of [bmim][BF_4] and [omim][BF_4] on marine mussels, as well as the potential role of conventional organic solvents, such as acetone, in the observed IL-mediated toxic potency.

2. Materials and methods

2.1. Chemicals and reagent

The ILs [bmim][BF_4] and [omim][BF_4] (Cas No. 174501-65-6 and 244193-52-0, respectively), purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA), were of analytical grade (≥97%, HPLC) (SI Fig. 1) and were used without further purification. Acetone was purchased from Applichem (Darmstadt, Germany). All reagents and solvents used were of the highest analytical grade and purity.

2.2. IL stock solution preparation

IL stock solutions (1 g L^{-1}) were prepared in filtered artificial seawater (ASW) with or without acetone in each case. Specifically, each stock solution was freshly prepared by diluting appropriate volumes of analytical grade [bmim][BF_4] or [omim][BF_4] in ASW ([bmim][BF_4]/ASW and [omim][BF_4]/ASW, respectively). In the case of acetone, an appropriate volume of [bmim][BF_4] or [omim][BF_4] was added to acetone and thereafter, an appropriate quantity of the IL-acetone stock solution was further diluted with ASW in order to yield a stock solution of each IL, containing 1 g L^{-1} IL. The final concentration of the carrier solvent (acetone) within the aforementioned stock solutions (acetone-[bmim][BF_4]/ASW and acetone-[omim][BF_4]/ASW, respectively) was 0.06% v/v. All stock solutions were kept in the dark at 4 °C, before being used for further analysis.

2.3. UV–vis spectrophotometric determination of final concentration of ILs tested

The range of each IL concentration currently used was based on initial experimental studies with the use of known calibration curves, commonly performed with the use of UV–vis spectrophotometric analysis (Díaz-Rodríguez et al., 2015) (for more details see SI 3, SI Fig. 2A,B).

2.4. Mussel collection and handling

Mussels (almost 500 individuals, 5–6 cm long, approximately 1-year old), collected from a mussel farm located to the north side of Korinthiakos Gulf (Gulf of Kontinova, Galaxidi, Greece), were transferred to the laboratory and acclimated without feeding in static tanks for 7 days prior to their use. The collection, handling, transfer and acclimation of mussels were appropriately carried out in order to minimize animal suffering (for more details see SI 2.4).

2.5. Determination of IL-mediated lethal endpoints in mussels (96 h mortality test)

Since there are no data concerning the levels of ILs in the receiving waters, thus making the prediction of the environmental risk posed by these compounds difficult, mortality test (96 h) was first performed in order to estimate the range of each IL concentration where no mortality occurs. In brief, laboratory-acclimated mussels were placed in static glass tanks (10 L; 1 mussel L^{-1}) under conditions mentioned above, and exposed for 96 h to different concentrations of (a) [bmim][BF_4]/ASW and/or acetone-[bmim][BF_4]/ASW (0.5, 1, 10, 100, 200 and 500 mg L^{-1}), (b) [omim][BF_4]/ASW and/or acetone-[omim][BF_4]/ASW (0.01, 0.1, 0.5, 10, 100, 200 and 500 mg L^{-1}). Moreover, in order to investigate any interference of the carrier solvent (acetone) with the obtained results, one group of mussels (acetone-treated group of mussels) was exposed to 0.06% v/v of acetone (which corresponds to the final concentration of acetone in each IL stock solution tested). In parallel, an acetone-free group of mussels were kept as control mussels