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Research article

Subcellular effects of imidazolium-based ionic liquids with varying anions on the marine bivalve *Mytilus galloprovincialis*

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ABSTRACT

Green Chemistry involves applying a set of principles aimed at minimizing the use of hazardous substances in the design, production, and application of chemical products. In recent decades, Ionic Liquids (ILs) have emerged as more environmentally friendly substitutes for traditional organic solvents. This preference is primarily due to their low vapor pressure, which results in minimal atmospheric pollution and enhanced industrial safety. However, existing literature highlights the toxicity of ILs towards aquatic invertebrates. Consequently, this study points to assess the biochemical effects of a selection of ILs through an *in vitro* approach. Specifically, digestive gland and gill cellular fractions (S9) of the marine bivalve *Mytilus galloprovincialis* were exposed to varying concentrations (0.05–2 μM) of three ILs featuring identical cations but different anions. The ILs tested were 1-ethyl-3-methylimidazolium octanoate ([EMIM][Oct]), 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]), and 1-ethyl-3-methylimidazolium ethyl sulfate ([EMIM][$EtSO_4$]). The results indicate that [EMIM][Oct] induces higher toxicity in both S9 tissues, highlighting a strong effect of the anion. Overall, antioxidant and biotransformation defenses were significantly altered for all three ILs assessed. While acetylcholinesterase activity was significantly inhibited of about half of control activity, indicating neurotoxic damage as part of the toxicity mode of action of these ILs, neither lipid peroxidation nor alterations to DNA integrity were observed (≥100 %). This study supports the use of *in vitro* techniques as important tools capable of generating reliable ecotoxicological data, which can be further considered as a screening before *in vivo* testing and used for *in silico* modeling.

1. Introduction

Until recently, Ionic Liquids (ILs), organic salts with low melting points (<100 °C), were considered green solvents and plausible substitutes for organic solvents in industrial contexts [1,2]. One of the primary motivations for their use, besides their low vapor pressure, was that these eco-friendly solvents would minimize waste streams, aiming for atom-efficiency and thereby providing environmental and economic benefits [3,4]. In fact, it has been shown that ILs can be easily isolated from reaction systems, making

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them reusable [5].

Ionic Liquids result from the combination of organic, bulky, asymmetric cations with organic or inorganic anions, allowing the development of task-specific molecules [6,7]. These solvents are interesting for their unique features, such as good solubility, electrical conductivity, catalytic action, non-flammability, low vapor pressure and high chemical and thermal stability [8–12]. Imidazolium, phosphonium, pyridinium, and ammonium-based ILs are used in a variety of applications, such as organic synthesis, biocatalysis, antimicrobial formulations, biomass transformation, and in the food and pharmaceutical industries [7,13–16].

While low vapor pressures reduce the hazard potential to operators and the risk of atmospheric pollution, the high solubility of these solvents in water and their environmental stability raise concerns about aquatic biota health [17–20]. To assess the potential environmental impact of ILs, it is essential to consider the life cycle of these compounds, from their synthesis process to their release into the environment [21,22]. Additionally, many ILs are derived from petroleum-based products and non-renewable sources, with synthesis processes that are often energy-consuming and involve large volumes of organic solvents, which are also concerning [23]. At wastewater treatment facilities, the mineralization of several ILs by microbial communities in activated sludge has been reported to be low, sometimes lower than 5 %, emphasizing the risk of salt-rich wastewater being released into the environment [24].

Regarding chemical structures, longer alkyl chain ILs tend to exhibit higher toxicities [25,26]. Moreover, ILs acting in a way similar to surfactants may cause significant damage to biological membranes [20,27]. Published literature indicates that the combination of imidazole-based cations with longer alkyl chains induces the strongest effects to marine invertebrate species [25,26,28–31]. At the sub-cellular level, studying the interactions between chemicals and lipid bilayers or proteins associated to cell membranes can enhance the understanding of a toxicant's mode of action, making *in vitro* methodologies highly relevant [27,32].

Among possible structures, three paramount examples of ILs used in industrial or pre-industrial applications, which share the same cation and vary in the nature of the anion, were selected. Specifically, 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) represents the benchmark for dissolving and transforming polysaccharides [33]. Rogers and colleagues founded the company 525 Solutions, and more recently Mari Signum, using [EMIM][OAc] for the extraction and valorization of chitin from marine biomass [34]. Additionally, 1-ethyl-3-methylimidazolium octanoate ([EMIM][Oct]) is one of the ILs used in the HighPerCell® spinning process to produce hemp-based cellulose fibers, a process developed by the German Institute for Textile and Fiber Research Denkendorf (DITF), which is nearing industrialization (DITF, 2023). Finally, 1-ethyl-3-methylimidazolium ethyl sulfate ([EMIM][$EtSO_4$]) was selected as it is one of the standards for evaluating ILs' performances in rheological and tribological applications [35].

Accordingly, the aim of the present research work was to evaluate biochemical impairments induced in the marine bivalve *Mytilus galloprovincialis* digestive gland and gill cellular fractions after *in vitro* exposure to a selection of three ILs, in terms of neurotoxic damage, DNA damage (strand breaks - DNAssb), potential damage to cell membranes, and metabolism and antioxidant defense impairments. The present work might constitute a support to the use of *in vitro* techniques as a tool able to generate data which can screen the need of investigating further using *in vivo* experimental set ups. In particular, it may represent a quick and easy method for preliminary evaluations of in-development new-generation chemicals, such as ionic liquids, deep eutectic solvents, green solvents, drugs, etc.

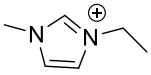
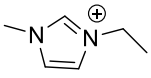
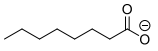
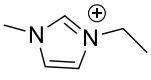
2. Material and methods

2.1. Chemicals

Table 1 shows the three Ionic Liquids (ILs) selected to assess the response of different anions when combined with the same cationic base: IL1 - 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]), IL2 - 1-ethyl-3-methylimidazolium octanoate ([EMIM][Oct]), and IL3 - 1-ethyl-3-methylimidazolium ethyl sulfate ([EMIM][$EtSO_4$]).

1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) and 1-ethyl-3-methylimidazolium octanoate ([EMIM][Oct]) were synthesized from methylcarbonate precursors following previously reported procedure [36]. The synthesis of these two ILs involved the addition of either acetic acid or octanoic acid (1 equivalent) to a commercial methanolic solution of EMIM methylcarbonate IL. Methylcarbonate IL concentrations in methanol solutions were pre-determined by volumetric titration using a standard 0.1 M HCl

Table 1
Structure of the selected ILs.

	CAS n.	Name	Abv.	Cation	Anion
IL1	143314-17-4	1-ethyl-3-methylimidazolium acetate	[EMIM][OAc]		
IL2	1154003-55-0	1-ethyl-3-methylimidazolium octanoate	[EMIM][Oct]		
IL3	342573-75-5	1-ethyl-3-methylimidazolium ethyl sulfate	[EMIM][$EtSO_4$]		

solution (Eutech pH meter, pH 700, calibrated with three standard buffer solutions at pH 4.01, 7.00, and 10.00). The resulting mixture was stirred at room temperature for 1 h, and the solvent was then evaporated at 60 °C for 12 h under reduced pressure to afford a light-yellow liquid in quantitative yield. 1-Ethyl-3-methylimidazolium ethyl sulfate ([EMIM][$EtSO_4$]) was purchased from Iolitec Ionic Liquids Technologies GmbH (Heilbronn, Germany).

Stock solutions of each IL were prepared with a molarity of 2 mM in ultrapure water. Working concentrations for all tested ILs were 0.05, 0.1, 0.5, 1, and 2 μM. The choice of working concentrations was based on published literature concerning ILs' ecotoxicological studies [26,37]. From the respective stock solutions, working concentrations were directly prepared in the exposure tubes by spiking the tissue homogenates with the correct volume of each IL.

2.2. Experimental methods for *M. galloprovincialis* collection and maintenance and biomarker analyses

All information relative to the collection and maintenance of *M. galloprovincialis* individuals and to all biomarker analyses performed are reported in Ref. [37] and in the Supplementary Materials of the present work.

2.3. Statistical analyses

The data resulting from the study was first assessed for normality with a Shapiro-Wilk test and then reported as mean ± standard deviation (SD). Statistical analysis included a one-way ANOVA test with a Dunnett post hoc test to determine significance among various conditions and their respective negative controls. The null hypothesis (H_0) stated that cellular fractions of *M. galloprovincialis*, exposed to different IL concentrations, do not exhibit significant differences compared to controls in their overall biochemical response.

Additionally, a Tukey test was performed to identify significances among all evaluated conditions, with detailed results provided in Supplementary Materials, Tables S1–S7. All statistical analyses were conducted using GraphPad Prism version 9.00 (GraphPad Software, San Diego California USA).

Subsequently, biochemical data was normalized to compute a Euclidean distance similarity matrix, then subjected to ordination analysis using Principal Coordinates Ordination (PCO). In the resulting graphs, variables that strongly influenced the spatial distribution of samples ($r > 0.75$) were depicted as superimposed vectors.

3. Results

3.1. Protein carbonylation (PC)

Protein carbonylation in S9-digestive gland fractions (DG) showed a statistically significant increase compared to the control only when exposed to IL2. Specifically, concentrations of 0.5, 1, and 2 μM of IL2 exhibited a significant effect (Fig. 1A). In gills fractions (G), significant increases were observed under exposure to IL2 (0.1, 0.5, 1, and 2 μM) and IL3 (0.1 and 0.5 μM) compared to the control (Fig. 1A).

Other significant differences among concentrations for each IL are detailed in Table S1. In DG fractions, statistically significant differences were noted between the lowest concentration (0.05 μM) and the three highest concentrations of IL2, with the highest values observed at 1 μM. Regarding IL3, differences were identified between the lowest (0.1 μM) and highest (2 μM) concentrations, with higher protein carbonylation values at 0.05 μM (Table S1). For G, IL2 exhibited significant differences between the lowest

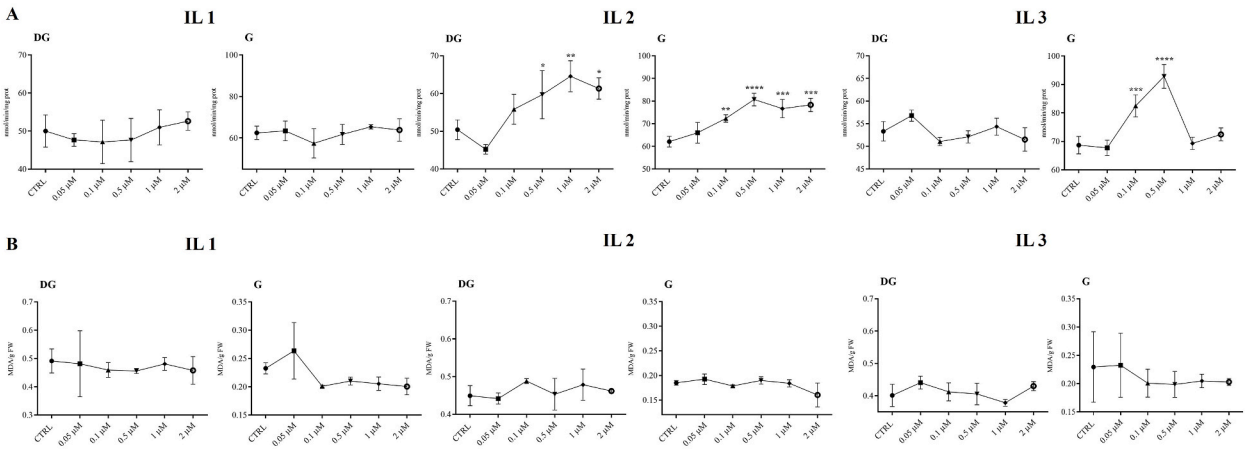


Fig. 1. Protein carbonylation (PC), A - digestive gland (DG) and gills (G): S9-fractions incubated with IL1, IL2 and IL3; Lipid peroxidation (LPO), B - digestive gland (DG) and gills (G): S9-fractions incubated with IL1, IL2 and IL3. Asterisks (*) indicate statistically significant differences between treatment and control (0 μM).

concentration (0.05 μM) and the three highest concentrations, with the peak values observed at 0.5 μM (Table S1).

3.2. Lipid peroxidation (LPO)

None of the three ILs assessed induced lipid peroxidation (LPO) in either DG or G fractions (Fig. 1B).

Additional significant differences among the tested concentrations for each IL are provided in Table S2. Specifically, in G fractions, the lowest concentration of IL1 (0.05 μM) showed significant differences from 0.1 μM to 2 μM, with the highest value observed at 0.05 μM.

3.3. Superoxide dismutase (SOD)

Regarding SOD activity (Fig. 2A), there was a statistically significant increase in S9-DG fractions exposed to IL2 at concentrations of 0.5, 1, and 2 μM compared to the control. Additionally, concentrations of 0.5 and 2 μM of IL3 also resulted in a significant increase in SOD activity respect to the control. In S9-gill fractions, enhanced SOD activity was observed following exposure to IL1 (2 μM), IL2 (1 and 2 μM), and IL3 (0.5 and 2 μM).

Additional significant differences among concentrations for each IL are detailed in Table S3. In DG S9-fractions exposed to IL2, significant differences in SOD activity were observed between the lowest and the three highest concentrations, with the highest activity noted at 2 μM (Table S3).

3.4. Glutathione peroxidase (GPx)

The activity of glutathione peroxidase (GPx) showed a statistically significant increase across all tissue fractions exposed to the three assessed ILs (IL1, IL2, IL3) at concentrations of 0.5, 1, and 2 μM compared to the control (Fig. 2B). Both S9-DG and G fractions exhibited a consistent trend of increased GPx activity, particularly in response to IL2 (Fig. 2B).

Additional significant differences among concentrations for each IL are detailed in Table S4. In both DG and G fractions, all three ILs showed significant differences between the lowest (0.05 μM) and highest (2 μM) concentrations in terms of GPx activity (Table S4). Notably, IL3 concentrations did not exhibit statistically significant differences in the exposed G subcellular fractions (Table S4).

3.5. Glutathione S-transferases (GSTs)

Regarding S9-DG fractions, exposure to IL2 and IL3 resulted in a significant increase in GSTs enzymatic activity compared to the control, particularly at the three highest concentrations of IL2 and all concentrations of IL3, except for 1 μM (Fig. 3A). In S9-gill fractions, a consistent and statistically significant increase in enzyme activity was found following exposure to IL1. Exposure to IL2 led to significant increases at the lowest concentration (0.05 μM) and the highest concentration (2 μM) compared to the control. Conversely, exposure to IL3 induced an almost alternating pattern of increases and decreases in GSTs activity respect to the control (Fig. 3A).

Additional significances among tested concentrations for each IL are detailed in Table S5. In DG fractions, significant differences were noted between the lowest and highest concentrations for all three ILs (Table S5). A congruous pattern was observed in G fractions exposed to IL1 (Table S5).

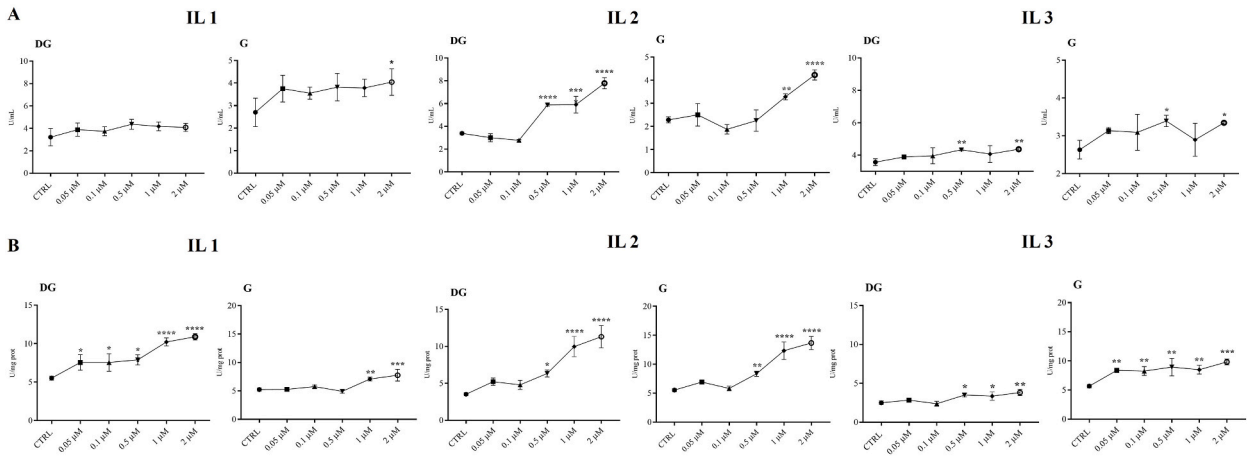


Fig. 2. Superoxide dismutase (SOD), A - digestive gland (DG) and gills (G): S9-fractions incubated with IL1, IL2 and IL3; Glutathione peroxidase (GPx), B - digestive gland (DG) and gills (G): S9-fractions incubated with IL1, IL2 and IL3. Asterisks (*) indicate statistically significant differences between treatment and control (0 μM).

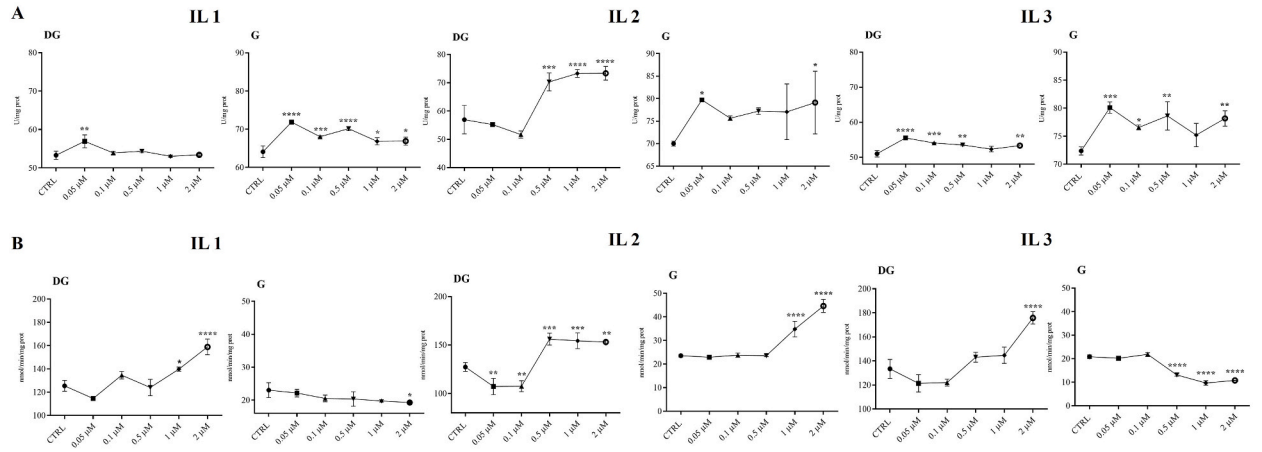


Fig. 3. Glutathione s-transferases (GSTs), A - digestive gland (DG) and gills (G): S9-fractions incubated with IL1, IL2 and IL3; Carboxylesterases (CEs), B - digestive gland (DG) and gills (G): S9-fractions incubated with IL1, IL2 and IL3. Asterisks (*) indicate statistically significant differences between treatment and control (0 µM).

3.6. Carboxylesterases (CEs)

Regarding carboxylesterases (CEs) activity, S9-DG fractions exposed to all three ILs displayed a similar trend (Fig. 3B). There was a significant increase in CEs activity observed at the three highest concentration conditions (0.5, 1, and 2 µM), while a tendency towards decreased activity was noted at the two lowest concentrations (0.05 and 0.1 µM), with statistically significant differences observed for IL2.

In S9-gill fractions, significant increases in CEs activity were observed at 1 and 2 µM of IL2 compared to control values. However, for the other two ILs (IL1 and IL3), the activity was significantly inhibited at 2 µM for IL1 and at the three highest concentrations for IL3.

Additional significant differences among the tested concentrations for each IL are detailed in Table S6. Statistically significant differences were observed between the lowest and highest concentrations in S9-DG fractions exposed to all three ILs (Table S6). A similar pattern was observed in S9-G fractions, except for IL1 (Table S6).

3.7. Acetylcholinesterase (AChE)

Regarding acetylcholinesterase (AChE) results (Fig. 4A), it is evident that this enzyme was inhibited across both tissue fractions and all tested ILs. Both S9-DG and gill fractions exhibited similar inhibition patterns, with statistically significant differences compared to the control.

Additional significant differences among the tested concentrations for each IL are detailed in Table S7. In S9-DG fractions, statistically significant differences were observed between the lowest and highest assessed concentrations for ILs 1 and 2 (Table S7).

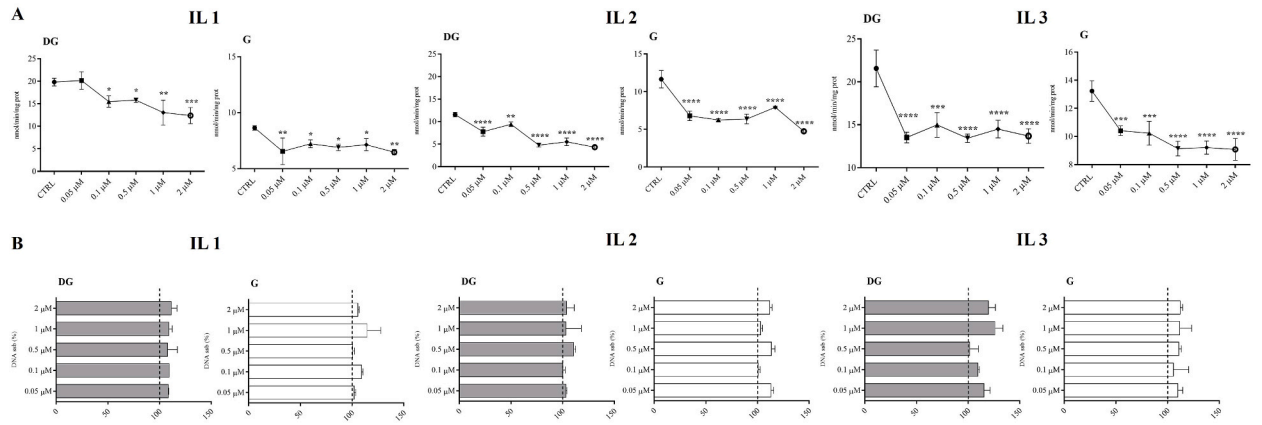


Fig. 4. Acetylcholinesterase (AChE), A - digestive gland (DG) and gills (G): S9-fractions incubated with IL1, IL2 and IL3; DNAssb, B - digestive gland (DG) and gills (G): S9-fractions incubated with IL1, IL2 and IL3. Asterisks (*) indicate statistically significant differences between treatment and control (0 µM).

Similarly, in S9-G fractions, significant differences were found for IL2 between the lowest and highest concentrations (Table S7).

3.8. DNA strand breaks (DNAssb)

Results for DNAssb, depicted in Fig. 4B, clearly show that none of the ILs induced genotoxic damage of this specific kind in either of the studied tissue fractions. In each graph, the results reached the 100 % dashed line, indicating no observable genotoxic effects caused by the IL exposures.

3.9. Multivariate analysis - Principal Coordinates Ordination analysis (PCO)

Results of PCO analysis are shown in Fig. 5 DG – digestive gland and Fig. 5 G – gills.

Regarding the biochemical response of exposed S9-DG fractions, the first principal coordinate axis (PCO1) explained 47.4 % of the total variability, making a distinction between the control and lowest/intermediate concentration conditions, on the negative side, and the highest concentrations of ILs 1 and 2 on the positive side. The second principal coordinate axis (PCO2), while explaining 23.1 % of the variability, separated all IL3 conditions (positive side) from the majority of the remaining ILs respective conditions (negative side). The positive side of PCO1 (see Supplementary Materials Table S8 for Pearson correlation values (*r*)) was best explained by CEs, PC, SOD, GSTs, GPx and LPO variables, being closely associated with the highest concentration conditions of ILs 1 and 2. Its negative side was best correlated with the variables DNAssb and AChE ((*r*): −0,007 and −0.775, respectively), grouping the lowest concentrations of ILs 1 and 2 and all concentrations of IL3. Then, AChE, DNAssb, CEs, PC and SOD were the variables best correlated with the positive side of PCO2 (Supplementary Materials, Table S9), being associated with all IL3 concentrations and 0.5 and 2 μM of ILs 2 and 1, respectively. Regarding its negative side, LPO, GPx and GSTs ((*r*): −0.826, -0.412, and −0.095, respectively) were the variables which best explained it, being in close association with the lowest concentration of ILs 1 and 2, 1 μM of ILs 1 and 2, and 2 μM of IL 2.

Regarding the response of S9-G exposed fractions, the first principal coordinate axis (PCO1) explained 39 % of the total variability, dividing all three control conditions and all IL1 concentrations (positive side) from the remaining two IL concentrations (negative side). In the yy axis, the second coordinate axis (PCO2) explained 20.8 % of the total variability while separating almost all ILs 2 and 3 concentrations (positive side) from all IL1 concentrations (negative side). AChE ((*r*): 0.810) was the variable best explaining the positive quadrants of PCO1. On the other hand, its negative side was best correlated with SOD ((*r):* −0.766), while GPx was the variable that best explained the negative side of PCO2 ((*r):* −0.903).

4. Discussion

The use of ILs is driven by their highly tunable properties [38]. Recent evidence suggests that anions can contribute to toxicity [39]. Considering this, coupled with the expeditious and straightforward nature of *in vitro* testing as a screening tool, the present study aimed to assess the biochemical responses of *M. galloprovincialis'* DG and G cellular fractions exposed to a set of three well-known ILs characterized by the same cation but with different anions (acetate, octanoate and ethyl sulfate). The biomarker assay battery, following *in vitro* exposure of the samples, included the assessment of indicators of biological membrane damage and antioxidant defense/biotransformation mechanisms, neurotoxic damage and DNA strand breaks (loss of DNA integrity).

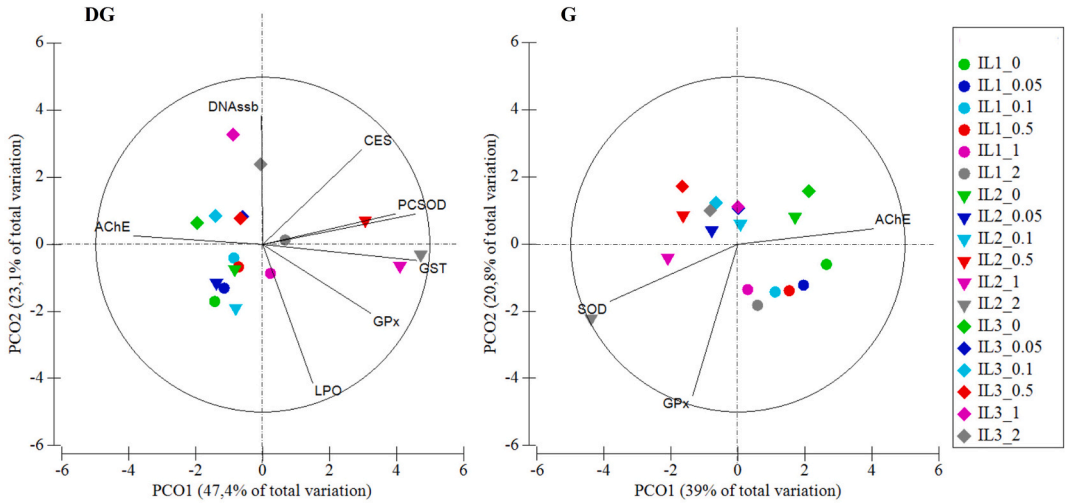


Fig. 5. Principal Coordinates Ordination diagram (PCO) based on biochemical parameters measured in *Mytilus galloprovincialis* S9-DG and S9-G cellular fractions exposed to 0.05, 0.1, 0.5, 1 and 2 μM of ILs 1, 2 and 3. Pearson correlation vectors (r > 0.75) of biochemical descriptors were provided as supplementary variables being superimposed on the top of the PCO graph: AChE, DNAssb, CEs, PC, SOD, GST, GPx and LPO (S9-DG) and SOD, GPx and AChE (S9-G).

Among the obtained results, a hazard ranking may be defined, putting IL2 as the potentially most hazardous compound of the three tested substances, followed by IL3 and, lastly, IL1. To be noted that for some assessed markers (GPx, SOD and PC on DG) IL3 and IL1 showed similar effects, still lower than those of IL2. IL 2 [EMIM][Oct] exhibited a more pronounced toxicity profile, revealing significant effects for all biomarkers except LPO and DNA damage. This observation may be attributed to a longer anionic alkyl chain of this anion compared to those of the other two assessed compounds that share the same cation. The longer alkyl chain is known to be a potential factor in increasing toxicity [40]. Longer alkyl chains are reported to induce entropy changes due to the addition of methylene groups, reducing contact with an aqueous medium and, thus, generating a more lipophilic compound [41]. This phenomenon, observed for cations, appears to operate similarly for certain anion species, such as fatty acid esters like octanoate and palmitate. Ref. [40] demonstrated this when assessing the toxicity of three newly tailored ILs synthesized from fatty acids as anion precursors in *Aliivibrio fischeri* bacteria, observing increased toxic effects with the rise of the alkyl chain length. Lipid peroxidation can occur when biological membranes face oxidative stress, where reactive oxygen species, if not scavenged by enzymatic and non-enzymatic defenses, break down phospholipids containing unsaturated fatty acyl moieties [42]. In the present study, none of the three ILs induced LPO. The S9-fraction encompasses both the endomembrane system and cytosolic components, providing a comprehensive display of phase I and phase II enzymes' metabolic profiles [43]. Although fatty acids from the endomembrane system are susceptible to peroxidation processes [44], it has been reported that cytosolic components may possess the ability to inhibit peroxidative damage to endomembranes [45]. The presence of Ca^{++} in the cytosol, forming a calcium phosphate precipitate in the phosphate-buffered subcellular medium, could act as an inhibitor of lipid peroxidation. Another hypothesis is that antioxidant enzymes eliminated the excess reactive oxygen species (ROS) produced.

Furthermore, all three evaluated ILs influenced the first line of antioxidant defense in both tissues, as superoxide dismutase (SOD) activity exhibited a significant increase. Subsequently, glutathione S-transferases (GSTs) and glutathione peroxidase (GPx) became involved with the resulting substrates and showed significant increases themselves in the two studied tissue S9-fractions. A study by Ref. [46], which assessed growth inhibition and oxidative stress in *Scenedesmus obliquus*, suggested that the increase in ROS production associated with exposure to imidazolium and other ILs might be the main factor responsible for oxidative stress. Similar observations were reported by Ref. [47] after evaluating the toxicity of imidazolium-based ILs towards the plant *Vicia faba*. Observations on both LPO levels and antioxidant/biotransformation enzyme activity reflect the oxidative potential of the evaluated ILs, presumably coped by cellular antioxidant defenses.

Concerning neurotoxic damage, AChE activity was strongly reduced after exposure to all three assessed ILs in both tested tissues. While studies on the *in vivo* neurotoxicity of ionic liquids remain limited [48], other investigations utilizing cell-based *in vitro* and structure-activity relationship studies, conducted with *in silico* approaches [49–52], or purified enzyme assays [53] have identified several ILs as inhibitors of acetylcholinesterase activity. Ref. [52] underpinned the role of the cation and its lipophilicity as the main factors affecting the inhibitory activity towards AChE.

The present study further revealed the absence of DNA strand breaks in all tested subcellular fractions. This aligns with DNA preserving and stabilizing properties reported for several imidazolium based ILs as emphasized by Refs. [54,55]. Interactions between imidazolium cations and the guanine bases in the DNA groove have been noted to lead to more effective stacking between guanine bases [56]. However, given the comprehensive biomarker results, careful consideration should be given to the use of imidazolium based ILs in any potential direct contact with living cells or their contents.

5. Conclusion

The present study could represent an important screening tool and a complement to *in vivo* testing. Obtained results identified IL2 as the one exerting higher toxicity, a fact associated with the presence of a long-chained anion (octanoate), confirming previous observations from other authors (e.g. Ref. [40]). While neurotoxic damage and oxidative stress were observed, all three assessed ILs were not able to induce loss of DNA integrity. Such results might strengthen the application of ILs in DNA preservation and stabilization mediums, making them, eventually and with the needed precautions, important solvents for biomedical and biotechnological use. The hypothetical contribution of anions in IL toxicity should be considered in future IL eco-compatibility assessments. In agreement with the statements of [27], *in vitro* methodology becomes useful in determining mediated biological effects and in exploring specific biochemical pathways which underly the toxicity of this type of compounds. Moreover, this kind of study might help in the preliminary selection of the safest IL cationic and anionic combinations when it comes to potential impacts towards biota and the environment, derived from ILs being released into natural systems. As results obtained *in vitro* cannot be directly extrapolated to what might happen *in vivo*, further investigation needs to focus on the effects of Ionic Liquids on other physiological endpoints, this time at the individual level (mortality, filtration rate, etc.). Moreover, studies of bioaccumulation might give important insight into the lipophilic and accumulation behavior of these chemicals in biological tissues.

Data availability statement

Data will be made available on request.

Ethics statements

Animal experiments

Only invertebrate animals (non-cephalopods) like bivalves (mussels) have been used in this experiment. These animals do not fall within the European Communities Council Directive 2010/63/EU and Italian Legislative Decree March 4, 2014, No. 26 on animal experimentation.

CRediT authorship contribution statement

Matilde Vieira Sanches: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Carlo Pretti:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Andrea Mezzetta:** Writing – review & editing, Validation, Methodology. **Lorenzo Guazzelli:** Writing – review & editing, Validation, Funding acquisition, Conceptualization. **Alessia Cuccaro:** Writing – review & editing, Methodology, Investigation. **Lucia De Marchi:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Rosa Freitas:** Writing – review & editing, Supervision, Funding acquisition. **Matteo Oliva:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36242.

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