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Chronic toxicity of treated and untreated aqueous solutions containing imidazole-based ionic liquids and their oxydized by-products

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Abstract

In the present work, an experimental study is presented aimed at assess the chronic toxicity of three imidazole-based ionic liquids, i.e. imidazole (IM), 1-methylimidazole (1MIM), 1-ethyl-3-methyl-imidazolium chloride (1E3MIM), and 1-butyl-3-methyl-imidazolium chloride (1B3MIM), generally considered as environmentally friendly surrogates of traditional industrial solvents. The study was conducted on *Daphnia magna*, monitoring both the cumulative survival of exposed organisms, and their reproductive parameters. The

intracellular oxidative stress of daphnids was also assessed through the determination of Reactive Oxygen Species (ROS) and Catalase activity (CAT), to better elucidate the toxicity mechanism. The chronic toxicity of their oxidized by-products (BPs), generated by advanced oxidation treatment with UV_{254}/H_2O_2, was also evaluated. Four generations of BPs were considered, each formed at reaction times higher than those required for the complete removal of the parent compounds. Results indicate that IM and 1MIM have a moderate chronic toxicity, which mainly manifests in the alteration of reproductive parameters. On the contrary, 1E3MIM and 1B3MIM showed significantly higher chronic toxicity effects resulting in an important increase of the mortality of exposed organisms compared to the controls. UV/H_2O_2 treatment of the compounds does not always reduce the observed effects, because of the formation of BPs that, in some cases, have chronic toxicity higher than their corresponding parent compounds. Chronic toxic effects remain significant up to the fourth generation of BPs in the cases of 1E3MIM and 1B3MIM, whereas they can be negligible from the second generation of BPs in case of IM and 1MIM. The results of oxidative stress measurements confirm the previous findings, suggesting a potential risk for the aquatic ecosystem induced by the mentioned compounds and their BPs.

1. Introduction

Imidazole-based-compounds (IMB-Cs) have been increasingly adopted in the bulk and fine chemical industry for the production of many different materials, such as pesticides, ion-exchange resins, dyeing auxiliaries, polyurethanes, and curing agent for epoxy resins (Wiley-VCH, 2005; Frizzo 2013; Zhang et al., 2009; Zhang et al., 2013).

Among IMB-Cs, imidazole-based ionic liquids (IMB-ILs) are attracting increasing interest due to their physical characteristics, including their low vapour pressure and flammability,

and the solvent capacity towards organic molecules (Dupont and Suarez, 2006; Heintz and Wertz, 2006; Anderson and Long, 2010; Frizzo et al., 2013). As an example, IMB-ILs are required for the largely diffused patented processes known as BASIL™, DIMERSOL™, and DIFASOL™ (Plechkova and Seddon, 2008; Vega et al., 2010).

Although at present these substances have not yet been found in surface waters or in the effluent of industrial and civil wastewater treatment plants, their presence in the environment cannot be totally ruled out. Indeed, even though IMB-ILs are promoted as green replacements for traditional industrial solvents, recent studies have demonstrated that they are poorly or negligibly biodegradable (Stolte et al., 2008; Jordan and Gathergood, 2015), and exhibit a moderate to high toxicity towards aquatic biota, including bacteria, microalgae, watercress, duckweed and invertebrates (Cho et al., 2008; Pham et al. 2010; Bubalo et al., 2014; Wang et al. 2015).

The detailed toxicity mechanism of IMB-ILS, as representative of the class of ionic liquids (ILs), is far from been completely understood, but it seems to be attributable to the excess generation of reactive oxygen species (ROS), and mainly results in the inhibition of the antioxidant system and in DNA damages of the exposed aquatic organisms or cultured cells (Kumar et al., 2011; Li et al., 2012a; Dong et al., 2013).

For the aforementioned reasons, IMB-ILs, together with other ILs, have been added to the class of new emerging xenobiotic compounds known as “Contaminants on Horizon” (Richardson and Ternes, 2014; Richardson and Kimura, 2016).

To reduce the potential threat of IMB-ILs in the environment, several authors have investigated the possibility of removing them from wastewaters through advanced oxidation processes (AOPs), which are recognised as highly efficient towards recalcitrant organic compounds (Andreozzi et al., 1999).

77 Results obtained using homogeneous and heterogeneous photocatalytic treatments (Bocos et
78 al., 2016; Munoz et al., 2015; Domínguez et al., 2014; Banić et al., 2016; Banić et al., 2014),
79 and UV_{254}/H_2O_2 processes (Czerwicka et al., 2009; Stepnowski and Zaleska, 2005) have been
80 very promising, achieving extremely high removal efficiencies.
81 Unfortunately, treated wastewaters still represent a concern, since they contain oxidized
82 IMB-IL by-products (BPs) that can be even more toxic than their parent compounds
83 (Czerwicka et al., 2000). It has been reported, for example, that oxidized IMB-IL BPs
84 generated by UV_{254}/H_2O_2 processes may exhibit the same acute toxicity of their parent
85 compounds (Spasiano et al., 2016a). Similar results have also been reported in case of AOPs
86 applied to other organic chemicals, such as oxytetracycline, doxycycline, ciprofloxacin,
87 fenofibric acid and benzoylecgonine (Yuan et al., 2011; Santiago et al., 2011; Spasiano et al.,
88 2016b).
89 Whilst a substantial number of studies describes the acute toxicity of several IMB-ILs
90 (Docherty et al.; 2005; Thamke et al., 2016; Shao et al., 2017), the information about their
91 chronic toxicity and the chronic toxicity of their oxidized BPs is very scarce. Such a situation
92 may lead to underestimate the risks, since the ratio between the acute toxicity, measured as
93 effective concentration causing 50% of the maximum response (EC_{50}), and the chronic
94 toxicity, measured as maximum acceptable toxicant concentration (MATC), can be as high as
95 1.8×10^4 (Kenaga, 1982).
96 The present study intends to partially overcome the mentioned lack of information, through a
97 detailed investigation concerning the chronic toxicity of imidazole (IM), 1-methylimidazole
98 (1MIM), 1-ethyl-3-methyl-imidazolium chloride (1E3MIM), 1-butyl-3-methyl-imidazolium
99 chloride (1B3MIM), and of their oxidized BPs generated during UV_{254}/H_2O_2 treatments.
100 The study is carried out on *Daphnia magna*, which is the most widely used model animal in
101 the field of ecotoxicology, genetics, physiology, as well as biomedical toxicology, due to its

small size, short life span, and ease of cultivation (Siciliano et al., 2015). Moreover the sensitivity of *Daphnia magna* to oxidant stress and DNA damage makes it an ideal model organism for mechanistic studies (Galdiero et al., 2016), such as the one presented in this paper.

2. Experimental

2.1 Materials

Imidazole (≥ 99%), 1-methylimidazole (99%), 1-ethyl-3-methylimidizalium chloride (98%), 1-butyl-3-methylimidazolium chloride (≥ 98%), hydrogen peroxide (30% v/v), acetonitrile (≥ 99.9%), ammonium acetate (≥ 98%), 2',7'dichlorodihydrofluorescein diacetate (≥ 97%), and dimethyl sulfoxide anhydrous (≥ 99.9%) were purchased from Sigma-Aldrich and used as received. Solutions of IM, 1MIM, 1E3MIM, and 1B3MIM, were prepared adding solid IMB-Cs and H_2O_2 (36 mM) to bi-distilled water without any further pH adjustment. Considering the results of the previously reported acute toxicity tests (Spasiano et al., 2016a), the following initial concentrations of IMB-ILs were chosen: IM, 2.2 µM; 1MIM, 1.3 µM; 1E3MIM, 1.02 µM; and 1B3MIM, 0.86 µM. No other chemicals were added to the tested solution to avoid the interference of additional compounds.

2.2 Batch photoreactor

Oxidized BPs of the solutions containing IM, 1MIM, 1E3MIM, and 1B3MIM were generated in a cylindrical glass stirred batch photoreactor ($V = 4.8 \cdot 10^{-1}$ L), equipped with a quartz jacketed low-pressure mercury lamp (Helios Italquartz, 17 W, emitting at 254 nm), having the following main characteristics: optical length equal to $2.2 \cdot 10^{-2}$ m; photon flux, estimated

by means of hydrogen peroxide actinometry (Goldstein et al., 2007), equal to $2.9 \cdot 10^{-6}$ ein$\cdot s^{-1}$, i.e. 81.65 J$\cdot min^{-1}$. The previously prepared solutions were fed to the reactor and thermostated at 25 ºC.

Samples containing oxidized BPs were collected at different reaction times established as function of the expected complete substrate conversion time (t_f), evaluated through the kinetic model proposed by Spasiano et al. (2016a). Specifically, samples were collected at the following times: t_f, twice t_f ($2t_f$), four times t_f ($4t_f$), and 8 times t_f ($8t_f$). The last two samples were not collected for those solutions, which did not show a residual toxicity after $2t_f$. The values of tf are reported in Table 1 together with the respective accumulated UV energies per litre of solution (Q_{UV}). The latter has been evaluated as follows:

$$Q_{UV} = t \cdot \frac{P}{V}$$

where P is the power of the lamp at 254 nm expressed in J$\cdot min^{-1}$, V is the solution volume expressed in L and t is the experimental time in min.

Before use, collected samples were analysed by HPLC for the determination of the residual concentration of IMB-ILs to confirm the predictions of the model.

2.3 Chemical determinations

Hydrogen peroxide and IMB-IL concentrations were detected using a HPLC (1100 Agilent) equipped with a Synergy 4u Polar-RP 80A column. The mobile phase consisted of acetonitrile (A) and aqueous ammonium acetate (20 mM) (B) flowing at 1.0 mL/min. For determining IM and 1MIM concentration the gradient was established as follows: 7% (A) constant for 7 min and then up to 27% (A) in 10 min, in case of IM; and 10% (A) constant for

10 min and then up to 17% (A) in 7 min, in case of 1MIM. An isocratic method with 25% (A) was adopted to evaluate 1E3MIM and 1B3MIM concentrations.

2.4 Survival and reproductive tests

The effect of the IMB-ILs and of their oxidized BPs on the reproductive and survival capacity of the organisms was assessed in a semi-static chronic test according to the standard protocol for *Daphnia magna* Reproduction Test (OECD, 2012)

Daphnids (< 24h old) were exposed for a period of 21 days to untreated and oxidized IMB-IL solutions. For each sample, ten beakers, each containing the solution to be tested and a single test organism, were prepared. The solutions were renewed three times weekly and daphnids were fed daily with a feeding rate of $3.0x10^7$ algal cells per animal per day. Survival and offspring production were recorded daily. pH and oxygen were measured too, in order to maintain controlled conditions. Test beakers were incubated at 21±1°C under a photoperiod of 16-h light and 8-h dark.

2.4 Determination of ROS content and Antioxidant Enzyme Activity

ROS level was determined in *Daphnia magna* after 2 and 21 days of exposure considering acute and chronic effects, following the procedure reported in details elsewhere (Galdiero et al., 2016). In short, the 2',7'dichlorodihydrofluorescein diacetate stock solution (25 mM in DMSO) was diluted in the culture medium to a final concentration of 10 mM. After 48 h and 21 days of exposure only alive *Daphnia magna* were collected for ROS determination. Each sample was homogenized with 50 mM phosphate-buffered solution (pH 7.4) and supernatants from each sample were used for the assays.

Catalase (CAT) activity was measured using Sigma-Aldrich kits according to the manufacturer's instructions as already described elsewhere (Galdiero et al. 2016; Russo et al. 2016). The reaction was monitored spectrophotometrically for 2 min at 25 °C at 240 nm using a UV–vis spectrophotometer (Hach Lange DR5000). The protein content in the supernatant was determined according to Lowry et al. (1951).

2.6 Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analyses were carried out to determine the effect of tested solutions on survival, reproductive output and time to the first brood, as well as the induction of ROS and the CAT activity. Data for survival and reproduction were checked for normal distribution with the Shapiro–Wilk method followed by one-way analysis of variance (ANOVA) and Tukey as *post hoc* analysis using Microsoft® Excel 2013/XLSTAT©-Pro (Version 7.2, 2003, Addinsoft, Inc., Brooklyn, NY, USA).

3. Results

3.1 Ecotoxicity of IMB-ILs

The results showing the long-term effects of tested IMB-IL solutions are presented in Figure 1. As it can be seen, the mortality of the control group did not exceed 10% at the end of the 21 days, while the mortality of daphnids exposed to IMB-ILs was generally higher, except the mortality of daphnids exposed to 1MIM, which was found to be equal to the mortality of the control group. In more details, the survival of daphnids exposed to IM was 80% after 6 days ($p > 0.05$ vs. control) and decreased to 70% between day 19 and day 21 ($p < 0.05$ vs.

control). The effect was even more important in case of organisms exposed to 1E3MIM and 1B3MIM. 1E3MIM solutions led gradually to decrease the survival of exposed daphnids within the first 6 days. Only 60% of surviving daphnids was observed after 21 days. Similarly 1B3MIM solutions led to 50% reduced population after exposure.

Fig. 1

Reproductive parameters of daphnids appeared to be even more sensitive to the chronic stress induced by IMB-ILs exposure (Figures 2A and 2A). This was explained considering that the consequences on brood quantity and quality can be a response toxicant-induced to alterations food conditions and/or oxygen consumption (Cleuvers et al. 1997) causing measurable physiological disease at the organism level. Indeed the effect of 1MIM exposure, which was negligible in terms of cumulative survival percentage, was instead one of the most important in terms of variation of number of neonates and delay of the first brood. The number of offspring was reduced by 85%, with respect to the control, while it was only 77% and 64% lower in the presence of IM and 1E3MIM, respectively. Similarly, the first brood was delayed by 11 days with respect to IM and 1B3MIM.

Fig. 2

The effect on reproductive parameters was even worst in presence of 1B3MIM ($p < 0.05$). Total neonates ranged between 3 to 6 per female, and the first brood was delayed by up to 11 days. Results were in agreement with those reported by Bernot (2005) after exposure to 0.2 ppm of 1B3MIM.

Overall reported data, compared with those showing the acute toxicity of the same tested IMB-ILs (Spasiano et al., 2016a), indicated that a too short exposure time represents an important limitation for the evaluation of the real ecotoxicological effects. Therefore, to provide an appropriate risk assessment (Blinova et al. 2018), chronic toxicity studies, based on longer exposure time, should be performed.

3.2 Ecotoxicity of oxidized IMB-IL BPs

Advanced oxidation of IMB-ILs resulted in variable effects in terms of chronic toxicity of exposed daphnids depending on the process reaction time, and was not equal for all tested aqueous solutions (Figures 3 and 4).

Fig. 3 and 4

As previously indicated, *Daphnia magna* was exposed to oxidized IMB-ILs after treatment times of the solutions equal to t_f, $2t_f$, $4t_f$, and $8t_f$. At the selected times the residual concentration of IM, 1MIM, 1E3MIM and 1B3MIM, reported in Table I, were totally negligible. Therefore the treated aqueous solutions only contained IMB-IL BPs, here indicated as: first generation BPs, those corresponding to t_f; second generation BPs, those corresponding to $2t_f$; third generation BPs, those corresponding to $4t_f$, and fourth generation BPs, those corresponding to $8t_f$.

Cumulative survival percentage in the presence of oxidized IM (Figure 3A) showed a significant decrease ($p < 0.05$ vs control) after 9, 12 and 19 days exposure to the first generation BPs (70%, 50% and 30%, respectively), whereas no effects on longevity occurred during exposure to the second generation, indicating the complete oxidation of the parental compound.

Surprisingly enough, the survival percentage strongly decreased also for daphnids exposed to the first generation of 1MIM BPs (Figure 3B), despite the fact that the untreated compound had no effect on the number of deaths. The dramatic decrease in survival within day 16 indicated a strong toxicity to juveniles. However the effect was already reduced in presence of the second generation of BPs, although not yet completely absent (75% survival), as in the case of second generation BPs of IM.

Daphnia magna exposed to the first and to the second generation of 1E3MIM BPs (Figure 3C) was very negatively affected, since mortality was 100%. Compared to the available data referred to acute toxicity (Spasiano et al., 2016b) which revealed almost no measurable effects, such a result highlighted once more the fundamental role of chronic toxicity tests to verify the toxic action occurring just a few days after the exposure, probably due to an inhibition of efflux pumps, as also observed by Georgantzopoulou et al. (2016).

A slightly reduced toxicity was obtained only in the presence of the third generation of BPs. In this case, in fact (Figure 3C), it was obtained a survival of 50% of exposed organisms after 2 days and of 20% of exposed organisms at the end of the exposure ($p < 0.05$). A prolonged oxidation of the parental compound, up to $8t_f$, was required to obtain negligible effects (Figure 3C).

Similar results were obtained in presence of oxidized 1B3MIM solutions (Figure 3D). A 100% mortality was already observed at day 3 and day 6 in the presence of the first and the second generation of BPs. As in the previous case, the toxicity was reduced in presence of the third generation (20% mortality at the end of the exposure), and completely absent in prolonged oxidized 1B3MIM solutions ($8t_f$).

Results of toxicity tests in terms of reproductive parameters partially confirmed the previous observations (Figures 4A and 4A). Once more, as in the case of exposure to untreated IMB-IL solutions, reproduction appeared to be more sensitive to the chronic stress induced by IMB-Il BPs. The delay of offspring as well as the reduction of the number of neonates indicated that even when *Daphnia magna* adapted to the presence of IMB-IL BPs, did not recover enough to reproduce in all cases.

Oxidized IM solution reduced the total number of neonates compared to controls ($p < 0.01$), with a maximum reduction of 86% in presence of the first generation BPs (Figure 4A). Time at first brood, instead (Figure 4B), was mainly delayed in the presence of the second

generation BPs, while was not significantly affected ($p > 0.05$) by the exposure to compounds resulting from a shorter oxidation time (t_f).

The first day of brood production in oxidized 1MIM was day 16 or day 17, indicating that there was an apparent delay in the onset of production in the presence of both first and second generation BPs (Figure 4B). The mean number of neonates per surviving adult in these replicates was less than 25, indicating a reduction of 66-84% with respect to the controls (Figure 4A).

An important alteration of reproductive parameters was observed in *Daphnia magna* exposed to 1E3MIM BPs. No daphnids was able to produce at least one brood when exposed to the third generation BPs, and, the total number of neonates was significantly reduced respect to controls in presence of more oxidized compounds ($8t_f$) ($p < 0.01$) (Figure 4A), being even lower than the total number observed for the exposure to the parent compound. Moreover, the release of the first brood was significantly delayed ($p < 0.01$) in the presence of the solution treated for $8t_f$ (fourth generation BPs) (Figure 4B).

Although the chronic test in presence of oxidized 1B3MIM also showed a significant effect ($p < 0.05$) on reproduction or day to first brood, for both exposures to the third and the fourth generation BPs, this effect was similar to the one observed in presence of the parent compound.

Overall results clearly indicated that formed BPs were responsible for chemical and enzymatic reactions causing chronicle effects more important than the acute ones.

3.3 Oxidative stress response

A further comparison between acute and chronic toxicity caused by IMB-Cs and by their oxidized BPs was carried out analyzing the short and long term intracellular oxidative stress in the daphnids (Figures 5 and 6).

Fig. 5 and 6

After 48h, the ROS levels were sensitively higher compared to the controls for organisms exposed to untreated IMB-IL-containing solutions. The fluorescence increase for daphnids exposed to IM and 1B3MIM was higher than for those exposed to 1MIM and 1E3MIM. Nonetheless, the reported increase was partially recovered after 21 days in the presence of IM, while it was even higher than the previous values in the presence of 1E3MIM and 1B3MIM. No substantial temporal changes were reported for *Daphnia magna* exposed to 1MIM. The observed trend allowed correlating a higher induction of ROS to *Daphnia magna* mortality.

Similarly, in the case of exposure to oxidized BPs, it was observed a very important accumulation of ROS after 48 h, in the presence of the second generation BPs of 1E3MIM, and first and second generation BPs of 1B3MIM. Such an accumulation corresponded to the reported 100% mortality.

Data referred to ROS levels were confirmed by the CAT activity results, summarized in Figures 7 and 8. Indeed, compared to control group, CAT activity in daphnids exposed to both tested IMB-ILs and to their oxidized BPs, showed an increase at 48 h, and a significant reduction after 21 days.

Fig. 7 and 8

As a result, it could be concluded that daphnids were able to overcome the oxidative stress induced by tested compounds, because of a gradual return of antioxidant activities, which indicated a non-negligible adaption capacity. In other words, while an important oxidative stress was present during the first hours of the exposure, similarly significant antioxidant activation was present at the end of the test. It was therefore supposed the existence of a balance between the production and the elimination of ROS, due to the instability of chemical intermediates, corresponding to a homeostatic mechanism.

Such a result, obtained for the first time with IMB-Cs in *Daphnia magna*, was in agreement with previous finding referred to different compounds in different aquatic organisms (Wu et al., 2013), and suggested that the mechanism of ROS production and CAT activity is directly related to the reduced survival capacity, as well as to the reduced fitness of offspring.

4. Conclusions

The potential threat to the environment resulting from the discharge of IMB-ILs, in the aquatic compartments, as representatives of contaminant on Horizon characterized by poor or null biodegradability, is still object of investigation. Even less is known about the potential ecotoxic effects of the BPs generated by the advance oxidation of the mentioned compounds, which is considered to be one of the most efficient ways to remove them from wastewater.

The present study, contributed to partially fill the mentioned gap of knowledge analyzing the chronic toxicity on *Daphnia magna* of IM, 1MIM, 1E3MIM, and 1B3MIM.

The following main conclusions may be derived from obtained results:

- untreated IM, 1MIM, 1E3MIM, and 1B3MIM produce a significant toxic effect on daphnids, mainly highlighted by the variation on reproductive parameters of exposed organisms with respect to the controls;
- the observed effect dramatically increases when the mentioned compounds are oxidized. In particular, the first generation of oxidized BPs resulted in an increased mortality of exposed daphnids, which can be as high as 100%;
- successive BPs generations have a variable toxic effect on exposed daphnids. The toxicity persists in case of 1E3MIM, and 1B3MIM oxidized BPs (up to the third generation in the first case), whereas it tends to disappear in the case of IM, and, with less evidence, in the case of 1MIM;

- data referred to ROS content and antioxidant enzyme activity reveal the existence of a notable correlation among ROS production, anti-oxidant anzymes activation, and biological endpoints.

Overall the obtained results highlight the importance of analyzing long-term effects in the risk assessment of emerging pollutants, being evident that classic acute toxicity tests tend to underestimate the hazard of these chemicals.

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Table I. Values of t_f and the respective accumulated energies per unit of volume (Q_{UV}).

Compound	Initial concentration	t_f (Q_{UV})	$2t_f$ (Q_{UV})	$4t_f$ (Q_{UV})	$8t_f$ (Q_{UV})
IM	150 ppm	41 min (6.97 kJ/L)	82 min (13.9 kJ/L)	164 min (27.9 kJ/L)	328 min (55.8 kJ/L)
1MIM		33 min (5.61 kJ/L)	66 min (11.2 kJ/L)	132 min (22.5 kJ/L)	264 min (44.9 kJ/L)
1E3MIM		27 min (4.59 kJ/L)	54 min (9.19 kJ/L)	108 min (18.4 kJ/L)	216 min (36.7 kJ/L)
1B3MIM		21 min (3.57 kJ/L)	42 min (7.14 kJ/L)	84 min (14.3 kJ/L)	168 min (28.6 kJ/L)

Figure 1. Cumulative percentage survival of *Daphnia magna* after exposure to the untreated IMB-IL aqueous solutions (Ctr = control).

Figure 2. Effect of 21 days exposure to the untreated IMB-IL aqueous solutions on reproductive parameters (Ctr = control): A) cumulative live offspring produced per female; B) day of the first brood.

Figure 3. Cumulative percentage survival of *Daphnia magna* after exposure to the oxidized IMB-IL BPs, varying treatment time: A) IM BPs; B) 1MIM BPs; C) 1E3MIM BPs; D) 1B3MIM BPs.

Figure 4. Effect of 21 days exposure to oxidized IMB-IL BPs on reproductive parameters: A) cumulative live offspring produced per female; B) day of the first brood.

Figure 5. Effect of IMB-ILs on ROS level in *Daphnia magna* after 2 days and 21 days of exposure (Ctr = control).

Figure 6. Effect on ROS level in *Daphnia magna* after 2 days and 21 days of exposure to the oxidized IMB-IL BPs, varying treatment time: A) IM BPs; B) 1MIM BPs; C) 1E3MIM BPs; D) 1B3MIM BPs.

Figure 7. Effect of IMB-ILs on CAT activity in *Daphnia magna* after 2 days and 21 days of exposure (Ctr = control).

Figure 8. Effect on CAT activity in *Daphnia magna* after 2 days and 21 days of exposure to the oxidized IMB-IL BPs, varying treatment time: A) IM BPs; B) 1MIM BPs; C) 1E3MIM BPs; D) 1B3MIM BPs.

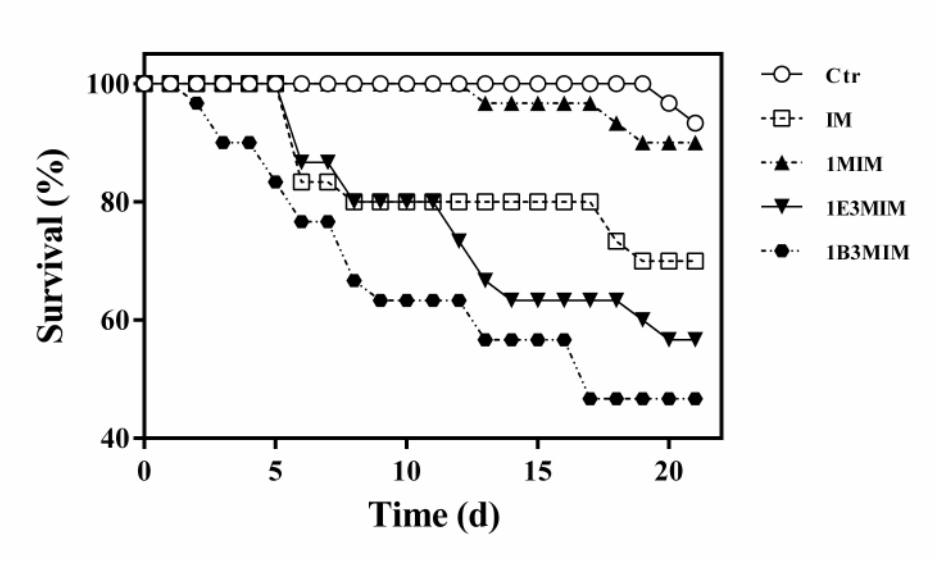

Survival (%)
100
80
60
40
0
5
10
15
20
Time (d)
Ctr
IM
1MIM
1E3MIM
1B3MIM

Fig. 1

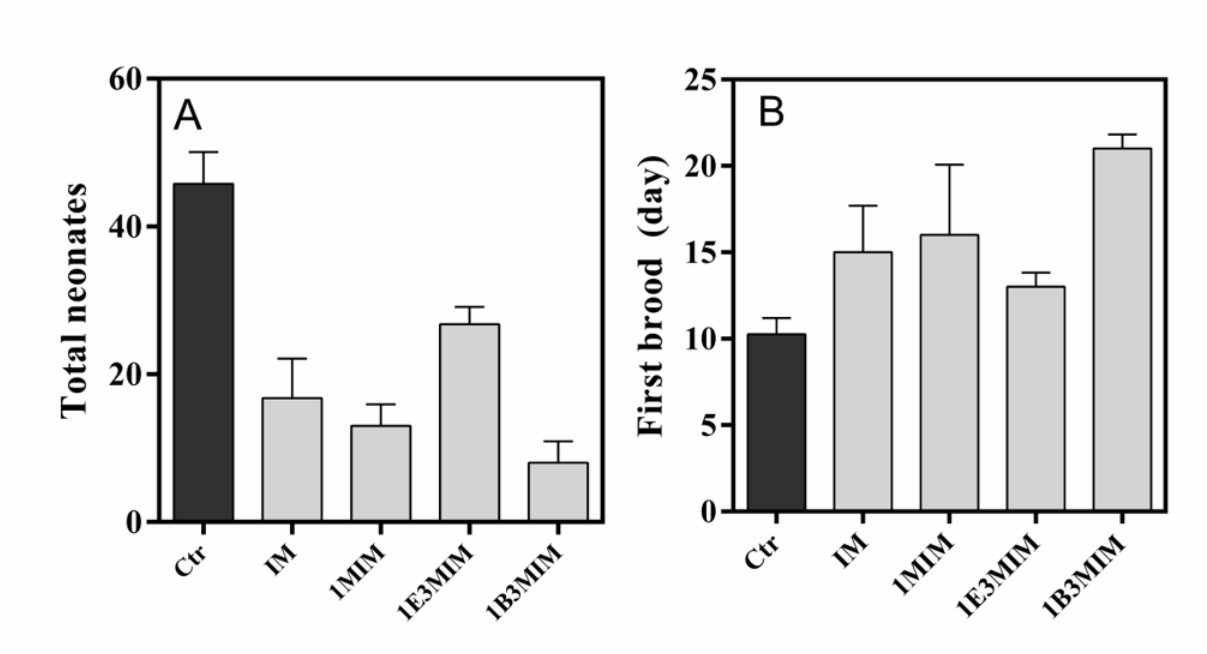

A
Total neonates
60
40
20
0
Ctr
IM
1MIM
1E3MIM
1B3MIM
B
First brood (day)
25
20
15
10
5
0
Ctr
IM
1MIM
1E3MIM
1B3MIM

Fig. 2

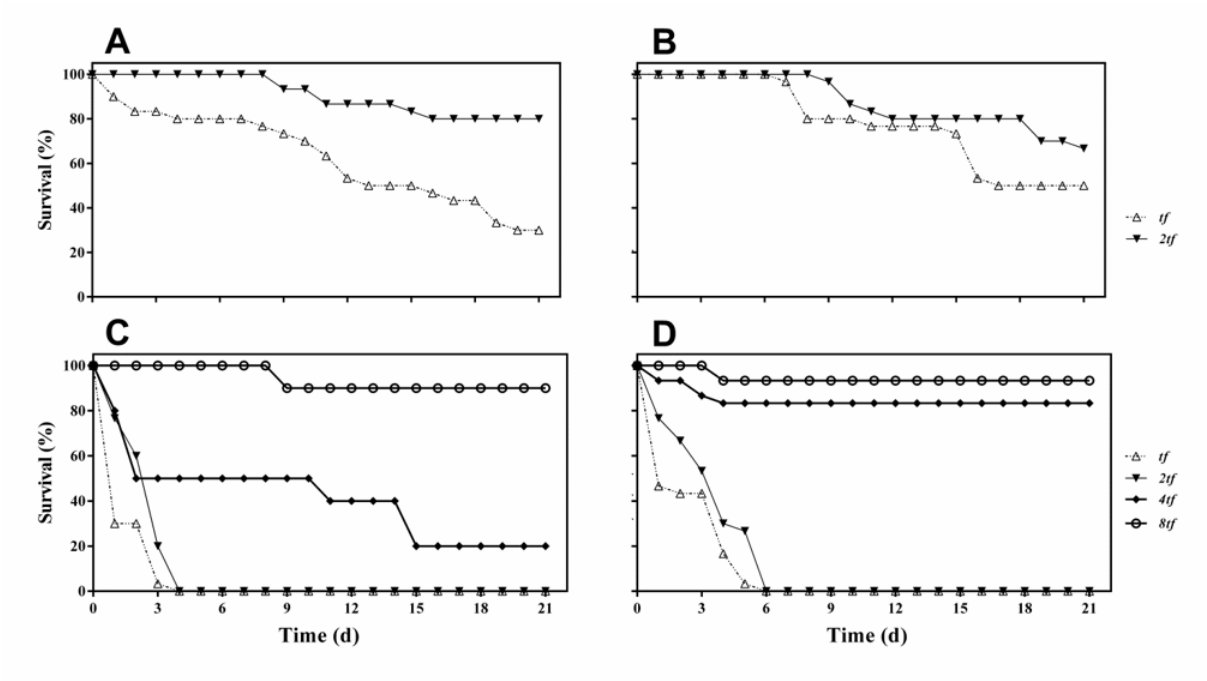


Fig. 3

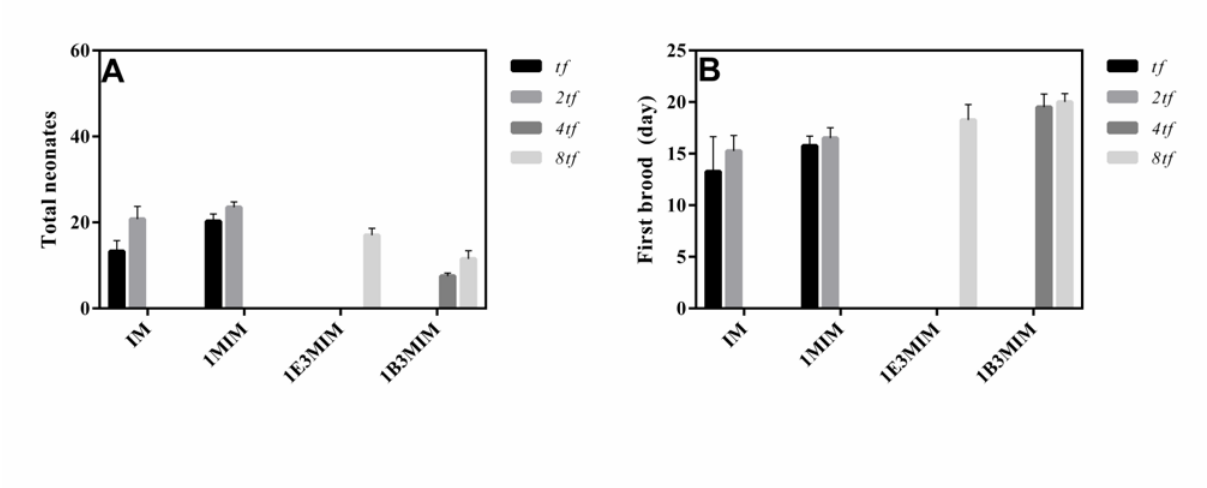
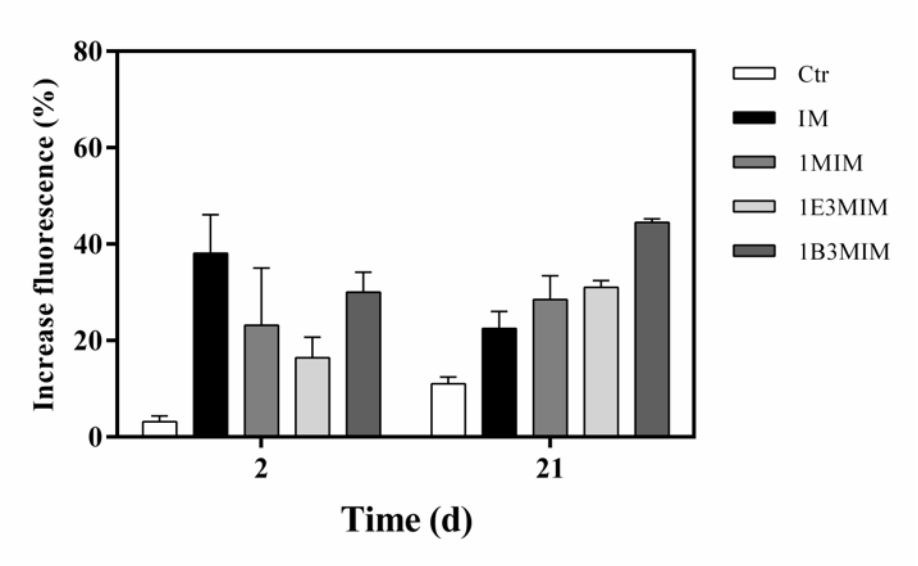

A
Total neonates
60
40
20
0
IM
1MIM
1E3MIM
1B3MIM
tf
2tf
4tf
8tf
B
First brood (day)
25
20
15
10
5
0
IM
1MIM
1E3MIM
1B3MIM
tf
2tf
4tf
8tf

Fig. 4



606

607 Fig. 5

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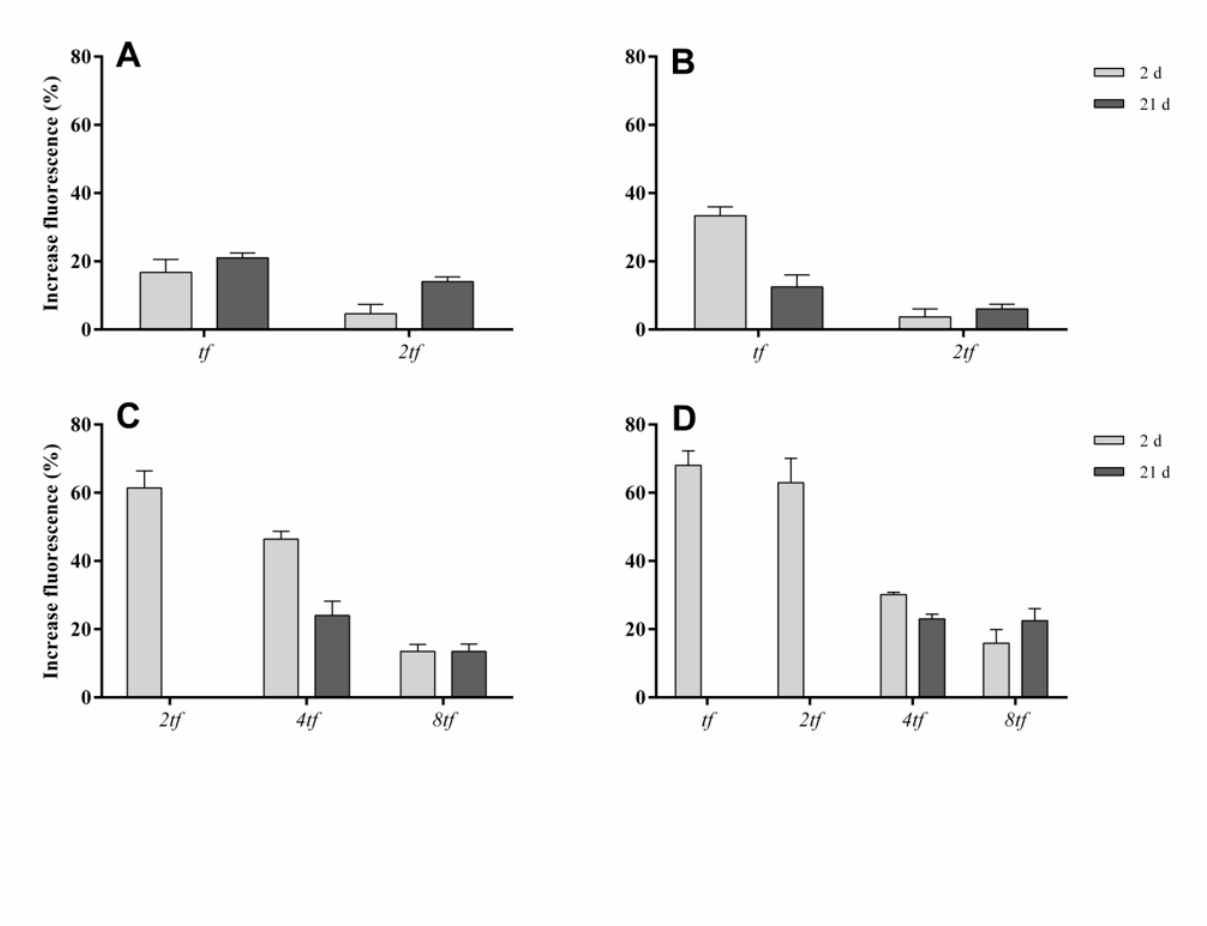


Fig. 6

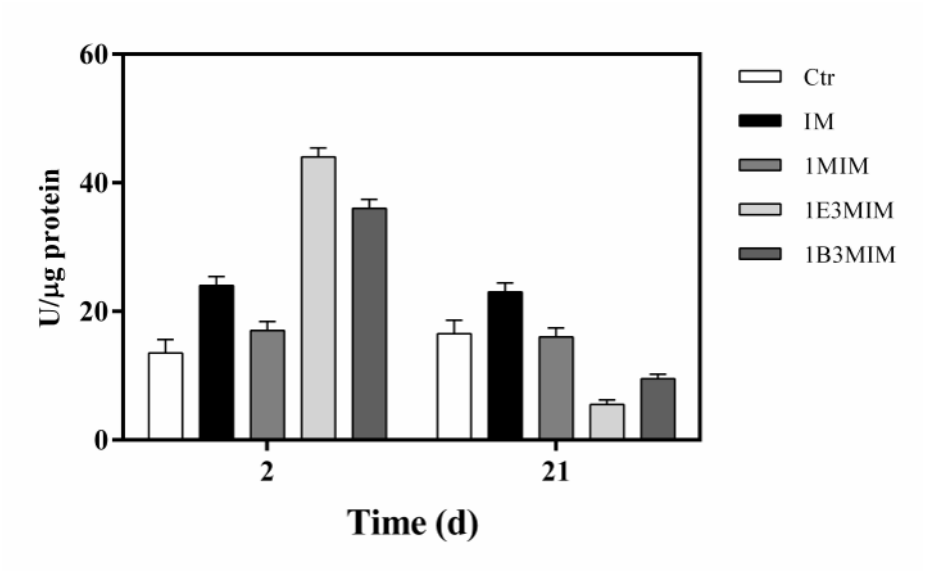


Fig. 7

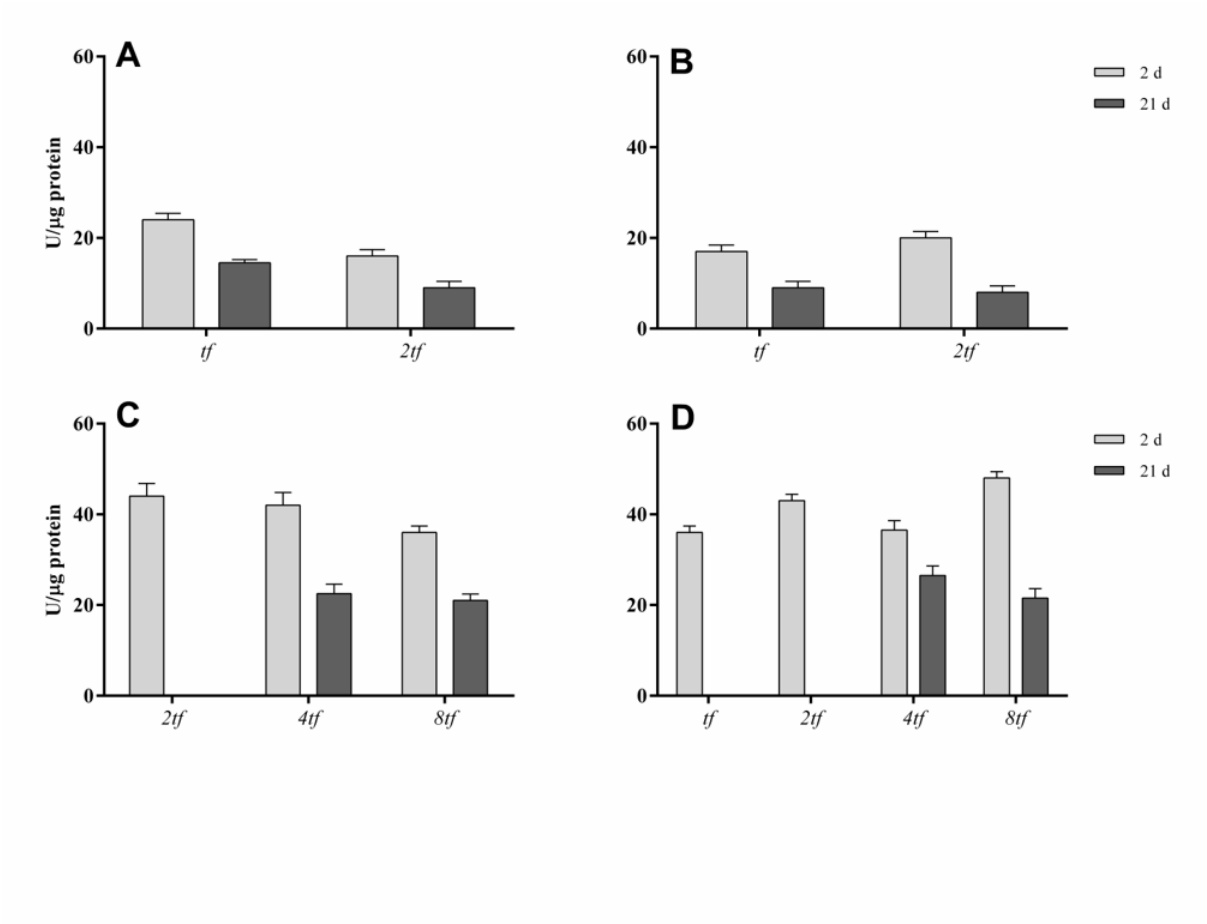


Fig. 8