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Effects of a mixture of glyphosate, 17α-ethynylestradiol and amyl salicylate on cellular and biochemical parameters of the mussel *Mytilus galloprovincialis*

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ABSTRACT

In this study the effects of a mixture of glyphosate (herbicide), 17a-ethinylestradiol (synthetic estrogen) and amyl salicylate (fragrance) to the mussel *Mytilus galloprovincialis* were evaluated. Mussels were exposed for 7 days to two realistic concentrations of the mixture (10 and 100 ng/L) and the effects on total haemocyte counts, haemocyte diameter and volume, haemocyte proliferation, haemolymph lactate dehydrogenase activity and haemocyte lysate lysozyme activity were measured. In addition, superoxide dismutase, catalase, acetylcholinesterase, glutathione-S-transferase and glutathione reductase activities were measured in gills and digestive gland. The survival-in-air test was also performed. Results demonstrated that the mixture affected both cellular and biochemical biomarkers, but not tolerance to aerial exposure of *M. galloprovincialis*. The negative effects recorded in this study suggested that more efforts should be done to assess the ecotoxicological risks posed by contaminant mixture to aquatic invertebrates.

1. Introduction

Various classes of pollutants generally occur in aquatic environments forming complex mixtures, toxicity of which is largely unknown. Consequently, in the last few years need of studying the risk posed by complex mixtures towards aquatic organisms is increased (Heys et al., 2016). In this context, particular attention has been addressed to emerging contaminants. They are natural or synthetic chemicals, the environmental occurrence and effects of which are poorly monitored (Smital, 2008; Geissen et al., 2015). Generally, emerging contaminants show low acute toxicity, but can have chronic effects at low levels (Smital, 2008). Among the large number of emerging contaminants detected in the European aquatic environments (www.norman-network.net) (Geissen et al., 2015), in the present study we focused on glyphosate, 17 α-ethynylestradiol (EE2) and amyl salicylate.

Glyphosate is a broad-spectrum, systemic, non-selective, and post-emergence herbicide used worldwide as a regulator of plant growth. The worldwide annual use of glyphosate has risen till to 600–750 thousand tons, with an expected increase up to 740–920 thousand tons in 2025 (Maggi et al., 2019). This herbicide has a relatively high persistence in both freshwater (7–142 days) (Annett et al., 2014) and seawater (267–315 days in aphotic condition at 25 °C and 31 °C, respectively) (Mercurio et al., 2014). Glyphosate has been detected not only in water bodies near agricultural areas (up to 290 μg/L) (Coupe et al., 2012) and in estuarine habitats (up to 1.69 μg/L) (Skeff et al., 2015), but also in Ocean (hundreds of μg/L in the Western Pacific) (Wang et al., 2016). Glyphosate and its commercial formulations have been shown to affect numerous biological responses in marine invertebrates (Matozzo et al., 2020).

Among endocrine disrupting chemicals (EDCs) occurring in aquatic environments the synthetic hormone EE2 (CAS: 57-63-6) is one of the most detected. EE2 is mainly used in contraceptive pills and derives from the natural hormone 17 β-estradiol (E2) (Barreiros et al., 2016). The estimated annual estrogen excretion amounts to 4.4 kg per millions of inhabitants (Combalbert et al., 2010), with an annual (March 2006–March 2007) use of EE2 of about 88 kg in USA, 51 kg in Germany and 19.9 kg in Italy (Hannah et al., 2009). In addition, annual estrogen excretion by livestock in the year 2000 was 33 and 49 tons in UE and USA, respectively (Lange et al., 2002), with an important presence of EE2 (Aris et al., 2014). EE2 is more persistent than E2 and may exert higher estrogenic effects than natural hormone (Aris et al., 2014). Half-life in rivers is 4–6 days for EE2 and 2–3 days for E2 (Ying et al.,

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2002). In addition, Jurgens et al. (2002) reported a half-life of 17 days for EE2, that raised to 81 days in aquifer media under aerobic conditions (Ying et al., 2003). The environmental concentrations of EE2 are highly variable. Usually, it has been detected either at low levels (few ng/L) or even below the detection limit (Ternes et al., 1999; Heberer, 2002; Ying et al., 2002; Gong et al., 2011). However, higher concentrations can be recorded. For example, in influents of wastewater treatment plants (WWTPs) a mean concentration of 187.9 ng/L has been found in Beijing (China) (Zhou et al., 2010), while in effluents of WWTPs EE2 has been found at concentrations of 42 ng/L and 15 ng/L in Canada and Germany, respectively (Ternes et al., 1999). In USA streams, Kolpin et al. (2002) found a mean concentration of 73 ng/L, with a maximum value of 831 ng/L, whereas a concentration of 35.6 ng/L was observed in a China river (Lei et al., 2009). In Brazilian rivers, Torres et al. (2015) measured EE2 concentrations up to 194 ng/L, while de França et al. (2020) recorded EE2 concentrations ranging from 4.5 to 48.2 μg/L. As for seawaters, EE2 concentrations ranged from 10 to 269 ng/L in China coastal areas (Liu et al., 2010), reaching 101.9 ng/L in Portugal estuarine waters (Ribeiro et al., 2009). In the Baltic Sea, Beck et al. (2005) found an EE2 concentration of 17 ng/L, whereas in the Lagoon of Venice Pojana et al. (2007) measured EE2 concentrations of 34 ng/L and 41 ng/L in seawater and sediments, respectively. In addition, it has been demonstrated that marine musses, such as *Mytilus galloprovincialis*, can bioaccumulate EE2, at values of 38 ng/g dry weight or 163.2 ng/g dry weight (Pojana et al., 2007; Chiu et al., 2018).

Amyl salicylate, also known as pentyl salicylate (CAS: 2050-08-0), belongs to the family of salicylates, which includes many other compounds, such as methyl, butyl, benzyl, and hexil salicylate. Amyl salicylate is a fragrance used not only in the perfumery industry, but also in personal care products (PCPs), such as shampoo, soap, sunscreen, hair spray and house detergent (Gaudin, 2014). A strong increase in the amyl salicylate consumption has been recorded, with an annual worldwide use that reached 6800 metric tons in 2010, more than double the ten years before (Gaudin, 2014). It has been detected in Svalbard snow (3.2–32 ng/L) (Vecchiato et al., 2018) and in Terra Nova Bay near the Mario Zucchelli Italian Antarctic station (4.2–29 ng/L) (Vecchiato et al., 2017). Vecchiato et al. (2016) detected concentrations of amyl salicylate in the range of 10–301 ng/L in the Lagoon of Venice, and of 7–59 ng/L in the open seawaters of the Sicily canal (Vecchiato et al., 2018).

The compounds described above can be present concomitantly in aquatic ecosystems. Consequently, in this study, we assessed for the first time the effects of a mixture of glyphosate (as active ingredient), EE2 and amyl salicylate to the mussel *M. galloprovincialis*. Mussels were exposed for 7 days to two environmentally realistic concentrations of the mixture and the effects on several biomarkers were evaluated. The hypothesis we tested was that the mixture can affect cellular and biochemical parameters in mussels, as well as their survival in air.

2. Materials and methods

2.1. Mussel acclimation and exposure

Adults of *M. galloprovincialis* (about 6 cm shell length) were sampled in a licensed area for bivalve culture in the southern basin of the Lagoon of Venice (Italy) and acclimated in the laboratory for 7 days in large aquaria filled with aerated seawater (salinity of 35 ± 1, temperature of 16 ± 0.5 °C, and suspensions of the microalgae *Isochrysis galbana* as food supply). Only mussels in apparent good conditions (absence of broken shell, secretion of new byssal threads) were used for the experiment.

A single stock solution of the mixture containing 5 mg/L of each compound, namely glyphosate, EE2 and amyl salicylate (Sigma-Aldrich, Milano, Italy) was prepared in distilled water. The concentration of the stock solution was established considering the lowest solubility in water for the three compounds tested, namely that of EE2 (9.2 mg/L in pure water) (Shareef et al., 2006). Mussels (70 in total per each concentration tested) were exposed for 7 days to 0 (control), 10 and 100 ng/L of the mixture in 35 L glass tanks (two tanks per each concentration with 35 mussels per tank) at the same thermohaline conditions of the acclimatisation period. The tested concentrations were chosen based on information concerning the environmental levels of the three compounds in seawater (see Introduction section). Seawater, as well as stock and working solutions of the mixture and microalgae (at an initial concentration of about 5×10^8 cells/L) in exposure tanks were renewed every 24 h. As suggested by Blanco-Rayon et al. (2019), food supply can reduce the interference of fasting during short-term, as well as long-term, toxicological experiments.

2.2. Tissue collection

Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe and stored in Eppendorf tubes at 4 °C. At the end of exposure, 6 pools of haemolymph (from five mussels each) from each experimental condition were prepared. A part of pooled haemolymph was immediately used to measure total haemocyte count (THC), haemocyte diameter and volume, haemocyte proliferation and lactate dehydrogenase (LDH) activity. The remaining part of pooled haemolymph was centrifuged at 780×*g* for 10 min and the supernatant was discharged. Pellets (=haemocytes) were resuspended in distilled water, centrifuged at 780×*g* for 10 min and the supernatants corresponding to haemocyte lysate (HL) were collected, frozen in liquid nitrogen and stored at −80 °C until analyses (lysozyme activity assay). Gills and digestive glands from mussels (the same used for haemolymph collection) were excised, pooled to obtain six different pools of five mussels each, divided in aliquots and frozen in liquid nitrogen and stored at −80 °C until analyses.

2.3. Haemocyte parameters

The total haemocyte count (THC) as well as haemocyte diameter and volume were determined using a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA). A volume of 20 μL of haemolymph was added to 2 mL of Coulter Isoton II diluent. The THC was expressed as the number of haemocytes (10^6)/mL of haemolymph, while haemocyte diameter and volume were expressed in μm and picolitres (pL), respectively.

Haemocyte proliferation was evaluated using a commercial kit (*Cell proliferation* Kit II, Roche). Briefly, 200 μL of the reaction mixture provided with the kit was added to 400 μL of pooled haemolymph and incubated for 4 h in a dark humidified chamber. The absorbance at 450 nm was recorded using a Beckman 730 spectrophotometer. The results were normalised to THC values of each experimental groups and expressed as optical density (OD) at 450 nm.

A commercial kit (*Cytotoxicity Detection* Kit, Roche) was used to measure lactate dehydrogenase activity (LDH) in cell-free haemolymph (CFH). Pooled haemolymph (500 μL) from treated and untreated mussels (controls) was centrifuged at 780×*g* for 10 min, and the supernatant (=CFH) was then collected for the assay following the manufacturer's instructions. The results were expressed as optical density (OD) at 490 nm.

To measure lysozyme activity 50 μL of HL (obtained as described above) was added to 950 μL of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer (pH 6.2), and the decrease in absorbance (ΔA/min) was continuously recorded at 450 nm for 3 min at room temperature. The results were expressed as μg lysozyme/mg of protein. Protein concentrations in the HL were quantified according to Bradford (1976).

2.4. Enzyme activity assays

Gills and digestive gland samples were homogenised at 4 °C with an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA and protease inhibitor cocktail (Sigma-Aldrich), and centrifuged

at 12,000 g for 45 min at 4 °C. Supernatants (SN) were collected for analyses. In all enzyme activity assays, SN protein concentrations were quantified according to Bradford (1976).

Total SOD activity was measured in both gills and digestive glands in triplicate using the xanthine oxidase/cytochrome *c* method proposed by Crapo et al. (1978). Enzyme activity was expressed as U/mg protein, one unit of SOD being defined as the amount of sample causing 50% inhibition under the assay conditions.

CAT activity was measured in gills and digestive gland SN in triplicate following the method of Aebi (1984). The enzyme activity in a volume of 10 μL of tissue SN were measured at 240 nm and expressed as U/mg protein; one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min.

The method of Ellman et al. (1961) was used to measure acetylcholinesterase (AChE) activity in gill SN (200 μL), following the colorimetric reaction between acetylthiocholine and the reagent dithiobisnitrobenzoate. Changes in absorbance were then recorded at 405 nm for 5 min on a microplate reader at room temperature. The results were expressed as nmol/min/mg of protein.

Glutathione S-transferase (GST) activity was measured in digestive gland SN according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. GST activity was expressed as nmol/min/mg protein.

Gill and digestive gland glutathione reductase (GR) activity was indirectly evaluated according to Smith et al. (1988) measuring the (5-thio (2-nitrobenzoic acid)) TNB production at 412 nm. The enzyme activity was expressed as U/mg protein.

2.5. Survival in air

At the end of exposure, 30 mussels from each experimental condition were maintained in closed plastic boxes in humidity-saturated conditions, at a constant temperature of 16 °C. Mussel mortality was recorded daily. Animals were considered dead when their shells gaped and did not shut again after external stimulus.

2.6. Statistical analysis

The normal distribution of data (Shapiro-Wilk's test) and the homogeneity of the variances (Bartlett's test) were assessed. The results obtained were compared using a one-way ANOVA, followed by Tukey's HSD post hoc test. All results are expressed as means ± standard deviation (SD), n = 6.

As for the survival-in-air test, median lethal time (LT_{50}) values were determined according to Kaplan and Meier (1958), and the significance of differences between groups was tested using the Gehan and Wilcoxon test (Gehan, 1965). Lastly, a canonical correlation analysis (CCA) was performed, with exposure concentrations and cellular and biochemical parameters measured as set of variables.

The software packages Statistica 13.4 (TIBCO Software Inc.), R (R Core Team, 2020, Austria) with the CCA package (González and Déjean, 2009) and the r41sqrt10 package (Finos, 2020) were used for the statistical analyses.

3. Results

Pairwise comparisons revealed a significant decrease in THC values in mussels exposed for 7 days to the highest concentration of the mixture ($p < 0.001$) (Fig. 1A), whereas no significant changes in both diameter and volume of haemocytes were observed in mussels exposed to the mixture, with respect to control (Fig. 1B and C).

Haemocyte proliferation increased significantly in mussels exposed for 7 days to 100 ng/L of the mixture ($p < 0.001$), when compared with the control (Fig. 2A). Interestingly, a significant negative correlation between haemocyte proliferation and THC was recorded ($r = -0.88$, $p < 0.001$).

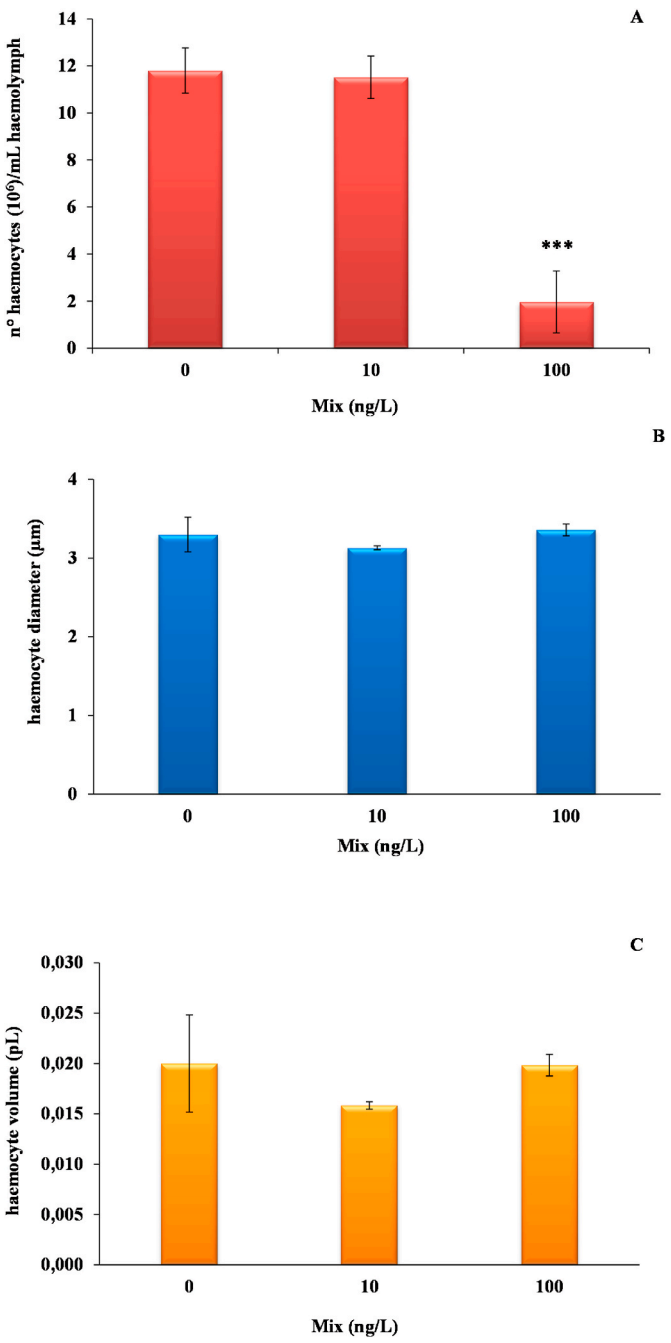

Fig. 1. Total haemocyte count (THC), expressed as the number of haemocytes (10^6)/mL of haemolymph **(A)**, haemocyte diameter, expressed in μm **(B)**, haemocyte volume, expressed in fL **(C)**, in *M. galloprovincialis* exposed to the mixture. The values are mean ± SD (n = 6). The asterisks indicate significant differences in comparison with controls: ***$p < 0.001$.

LDH activity increased significantly ($p < 0.001$) in mussels exposed for 7 days to the highest concentration of the mixture, compared to controls (Fig. 2B).

No significant alterations in lysozyme activity in HL of mussels exposed to the mixture were observed when compared with the controls (Fig. 2C).

As for enzyme activities, statistical analysis demonstrated that the exposure to the highest concentration tested significantly ($p < 0.05$) increased SOD activity in gills (Fig. 3A) and CAT activity in digestive gland (Fig. 3C) of mussels. Conversely, SOD and CAT activity did not vary significantly in digestive gland (Fig. 3B and D).

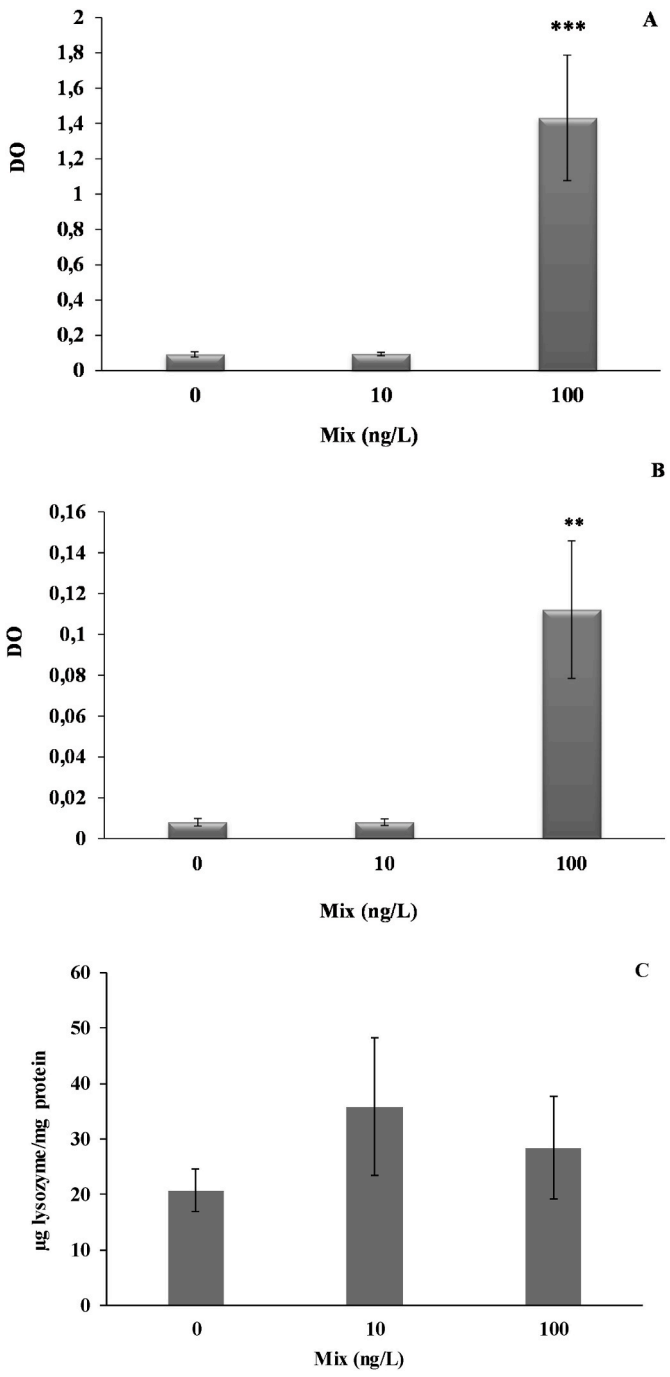

Fig. 2. Haemocyte proliferation, expressed as OD **(A),** cell-free haemolymph lactate dehydrogenase activity, expressed as OD **(B),** and haemocyte lysate lysozyme activity, expressed as µg lysozyme/mg protein **(C)** in *M. galloprovincialis* exposed to the mixture. The values are mean ± SD (n = 6). The asterisks indicate significant differences in comparison with controls: **p < 0.01, ***p < 0.001.

Exposure to the mixture caused a significant ($p < 0.05$) increase in gill AChE activity in mussels treated with 10 ng/L (Fig. 3E), whereas the mixture did not affect digestive gland GST activity (Fig. 3F).

GR activity did not vary significantly in gills (Fig. 3G), whereas it increased significantly ($p < 0.001$) in digestive gland of mussels exposed to 10 ng/L, whit respect to control (Fig. 3H).

As for the survival in air test, the resistance to air exposure of mussels was not affected significantly by the exposure to the mixture, LT_{50} values of the three experimental groups resulting to be similar (about 9–10 days) (Fig. 4).

Lastly, the CCA showed strong correlations between the first two pairs of canonical axes (0.973 and 0.782, respectively). The three samples separated well on the surface. In particular, samples of the concentration 2 (=10 ng/L) were characterized by low volume and diameter and high levels of HL lysozyme and gill AChE activities, while samples of the concentration 3 (=100 ng/L) showed low levels of THC and high levels of LDH and cell proliferation (Fig. 5).

4. Discussion

Except for amyl salicylate, effects of glyphosate (Matozzo et al., 2018; Milan et al., 2018) and EE2 (Canesi et al., 2007; Ciocan et al., 2010) have already been investigated individually in mussels. In this study, we decided to assess the effects of environmentally realistic concentrations of a mixture of the three compounds on a battery of biomarkers measured in target tissues of mussels, such as haemolymph, gills and digestive gland.

As for haemocyte parameters, this study demonstrated that exposure to the highest concentration tested caused a significant reduction in THC of mussels, values falling from 11.8 (10^6) in controls to 1.9 (10^6) cells/mL of haemolymph in molluscs exposed to 100 ng/L. Even though the mechanism underlying this event is not well understood, our results suggested that the mixture can reduce markedly the number of circulating haemocytes in *M. galloprovincialis*. Pipe and Coles (1995) and Parry and Pipe (2004) suggested that THC values can increase because of cell proliferation or mobilisation of haemocytes from peripheral tissues to the haemolymph, whereas THC can decrease due to cell lysis or increased mobilisation of haemocytes from the haemolymph towards tissues. In this study, a statistically significant negative correlation between THC and haemocyte proliferation was recorded (see Results section). Consequently, we hypothesised that the reduction observed at the highest concentration tested was caused by an increased mobilisation of haemocytes from haemolymph to peripheral tissues. The significant increase in cell proliferation recorded in mussels treated with 100 ng/L was probably an attempt of animals to restore - at least partially - the dramatic reduction in circulating cell number. In a previous study, no significant alterations in THC values were recorded in mussels injected with different concentrations of a mixture of estrogens, including E2, EE2, mestranol, nonylphenol, nonylphenol monoethoxylate carboxylate, bisphenol and benzophenone (Canesi et al., 2007). Conversely, a significant reduction in THC values was observed in the green mussel *Perna viridis* following exposure for 7 days to the highest concentration of a mixture of carbamazepine, bisphenol A and the herbicide atrazine (Juhel et al., 2017). In a field study, Höher et al. (2012) observed that mussels (*Mytilus edulis*) collected at a sampling site characterised by pollutant mixtures deriving from larger city, iron-melt industry and freshwater runoff from agriculture had high THC values, together with cell morphological abnormalities (vacuolation) and reduced phagocytic activity. The authors suggested that the relationship between high THC and increased vacuolation in mussels from mixture-contaminated site was a compensation mechanism of reduced cell viability. As single compounds, no information concerning the effects of EE2 and amyl salicylate on THC in molluscs is available in the literature, to our knowledge at least. Conversely, glyphosate was shown to significantly reduce THC values in *M. galloprovincialis* following exposure for 7 and 14 days to glyphosate (Matozzo et al., 2018). A marked decrease in THC values was also observed in shrimps (Hong et al., 2018) and crabs (Hong et al., 2017) exposed to glyphosate.

This study demonstrated that exposure to the mixture did not affect both diameter and volume of haemocytes in *M. galloprovincialis*. It is very difficult for us to compare results of our study with those from the literature, due to the absence of information on the effects of contaminants on these important cell parameters in aquatic animals. Just for comparison, glyphosate was shown to significantly increase cell volume in mussel haemocytes exposed for 7 (at 1000 µg/L) and 21 days (at 10

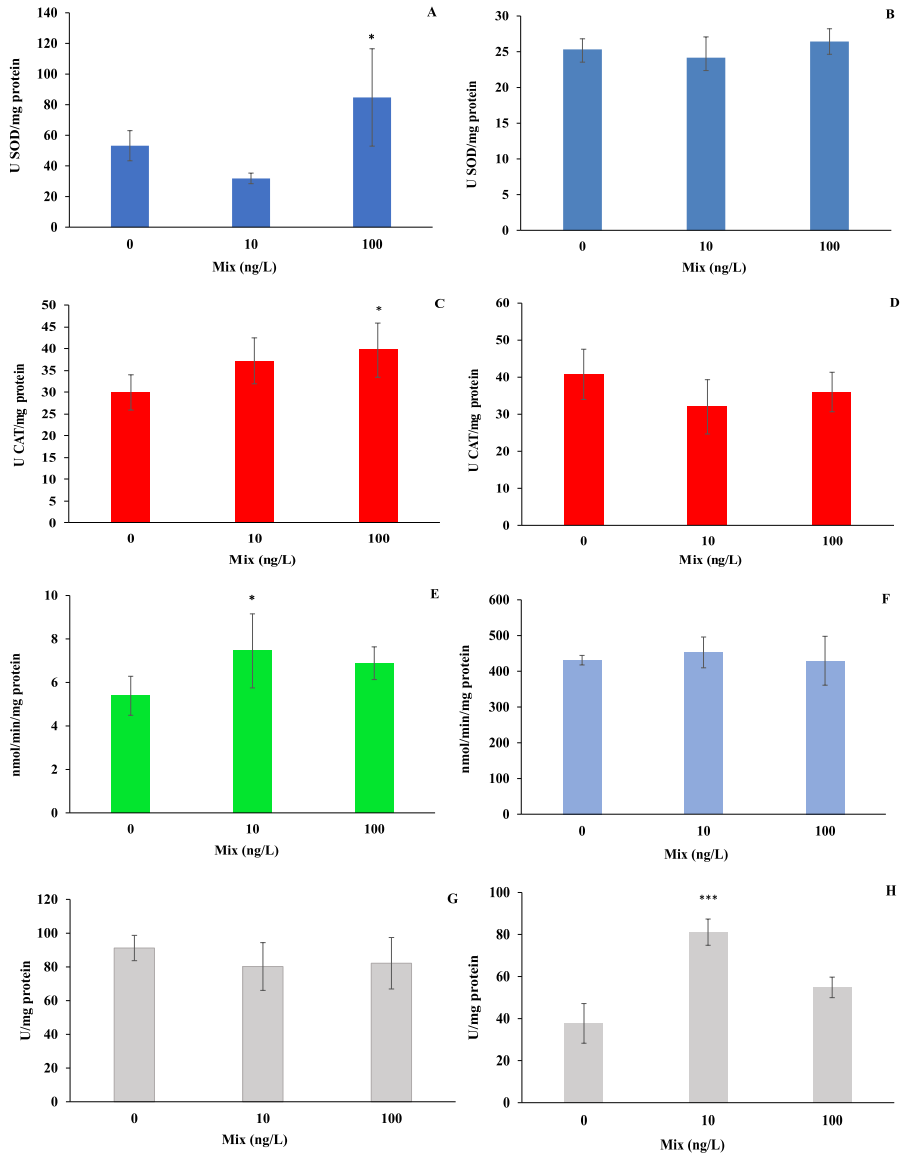

Fig. 3. SOD activity, expressed as U/mg protein, in gills (**A**) and digestive gland (**B**), CAT activity, expressed as U/mg protein, in gills (**C**) and digestive gland (**D**), gill AChE activity, expressed as nmol/min/mg protein (**E**), digestive gland GST activity, expressed as nmol/min/mg protein in (**F**), and GR activity, expressed as U/mg protein, in gills (**G**) and digestive gland (**H**) of *M. galloprovincialis* exposed to the mixture. The values are mean ± SD (n = 6). The asterisks indicate significant differences in comparison with controls: *p < 0.05, ***p < 0.001.

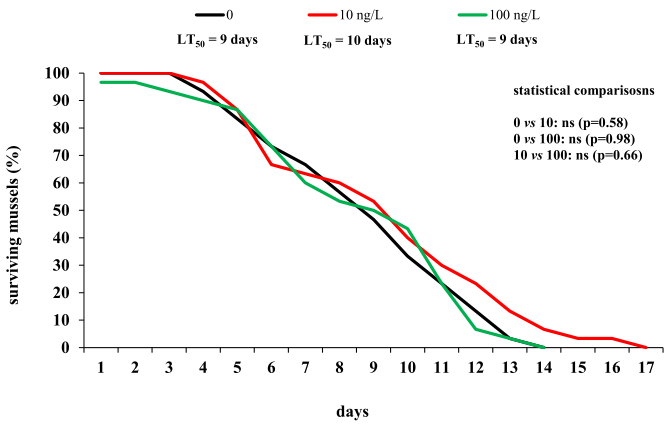

Fig. 4. Survival in air response of *M. galloprovincialis*, expressed as percentage of mussels surviving after 7 days of exposure to the mixture. LT_{50} values (in days) are reported. Inset: significance of comparisons between experimental groups.

µg/L) (Matozzo et al., 2018). Overall, we can state that the mixture did not cause morphological alterations in mussel haemocytes, at least under the experimental conditions tested in this study.

A significant reduction in haemocyte membrane stability was observed in mussels exposed to the highest concentration of the mixture. Indeed, LDH activity (a cytoplasmic enzyme that can be released into the extracellular fluids following to cell membrane destabilisation) increased significantly in haemolymph from mussels exposed to 100 ng/L. Interestingly, Canesi et al. (2007) demonstrated that a mixture of EDCs (its composition is reported above) significantly affected lysosomal membrane stability and lysozyme release in *M. galloprovincialis*, both *in vitro* and *in vivo* (=injection). In mixture-injected mussels (at nominal concentrations of 0.0796, 0.796 and 7.96 pmol/mL haemolymph/mussel), a dose-dependent lysosomal destabilisation (up to 71% with respect to controls) was recorded, as well as a marked release of lysozyme (+32% and + 61% at 0.796 and 7.96 pmol/mL) (Canesi et al., 2007). As for individual compounds, *in vitro* experiments demonstrated that EE2 can induce lysosomal membrane destabilisation in mussel haemocytes, but not lysozyme release into the haemolymph (Canesi et al., 2007). In a recent study, we observed a significant increase in LDH activity in cell-free haemolymph of mussels exposed for 7 (at 10 and 1000 µg/L) and 14 days (at 10 µg/L) to glyphosate (Matozzo et al.,

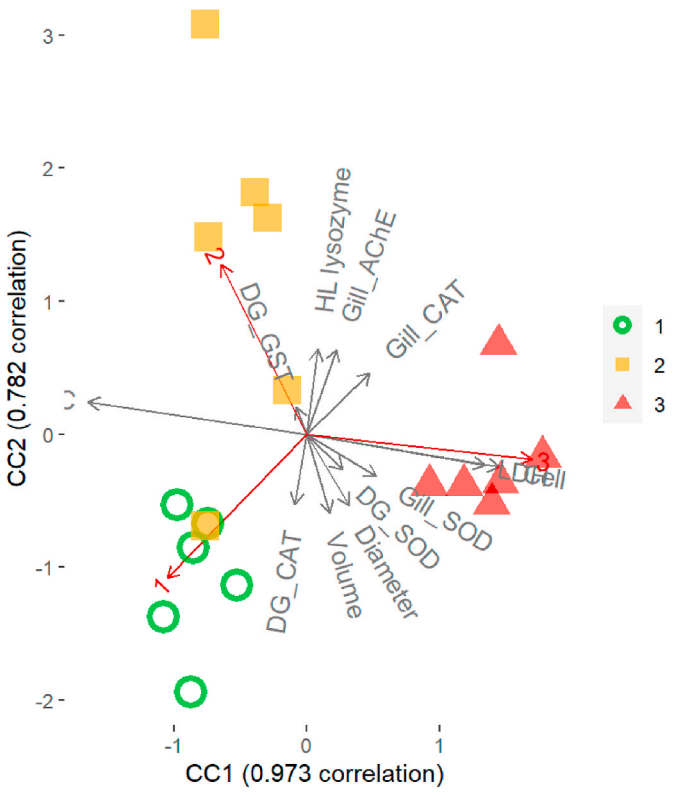

Fig. 5. CCA analysis. Cellular and biochemical parameters measured in mussels and experimental concentrations (1 = 0 ng/L, 2 = 10 ng/L, 3 = 100 ng/L) were used for CCA analysis.

2018). Conversely, glyphosate did not induce significant alterations in LDH activity of clams (*Ruditapes philippinarum*) (Matozzo et al., 2019a). Considering that alterations in cell/lysosomal membrane stability have extensively been reported for molluscs exposed to differing environmental contaminants, we can state that haemocyte membrane stability is an important target for the mixture of glyphosate, EE2 and amyl salicylate.

In this study, we tested the hypothesis that the mixture can act intracellularly affecting hydrolytic enzyme activity. Exposure to the mixture did not cause significant alterations in HL lysozyme activity in mussels, suggesting that such enzyme is not a target for the mixture, at least under the experimental conditions tested. Conversely, Juhel et al. (2017) demonstrated that exposure to both medium and high concentrations of a mixture of carbamazepine, bisphenol A and atrazine induced a significant decrease in lysozyme activity in cell-free haemolymph of *P. viridis*, suggesting that extracellular lysozyme is a sensitive biomarker of exposure to the mixture, as well as to individual compounds. Considering the single compounds, we observed that exposure for 7 days to 1000 μg/L and for 21 days to 100 and 1000 μg/L of glyphosate reduced acid phosphatase activity in HL of *M. galloprovincialis*, while enzyme activity increased after exposure for 14 days to 1000 μg/L (Matozzo et al., 2018).

As for gill and digestive gland enzyme activities, this study demonstrated that exposure to the mixture induced a significant increase in gill SOD and CAT activities, as well as in digestive gland GR activity, suggesting an activation of defence systems against oxidative stress mediated by reactive oxygen species (ROS). As a first defence line against ROS-mediated stress, SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide and oxygen, while CAT catalyses the conversion of H_2O_2 to water and molecular oxygen. Consequently, a relationship between the two enzymes can be hypothesised in gills from *M. galloprovincialis* exposed to the mixture. Indeed, the increase in CAT activity could be a consequence of increased SOD activity, which promoted hydrogen peroxide formation, which in turn led to the activation of CAT. As for digestive gland, in this study no significant variations in SOD, CAT and GST activities were observed, whereas GR activity increased significantly. It is well known that GR catalyses the reduction of glutathione disulfide (GSSG) to glutathione (GSH), which plays a key role in detoxifying ROS. Based on the results obtained in this study, we can hypothesise that protection against mixture-mediated oxidative stress was driven by SOD and CAT in gills, and by GR in digestive gland. In a previous study, exposure for two days of the clam *Venerupis decussata* to a mixture of permethrin and anthracene was shown to significantly increase SOD, CAT and GST in both gills and digestive gland (Sellami et al., 2015). In that study, the authors suggested a relationship between SOD and CAT activities in both tissues and hypothesised that the increase in enzyme activities was a signal of the activation of defence pathways against ROS. Canesi et al. (2008) observed a significant decrease in CAT activity in digestive gland of *M. galloprovincialis* at 24 h post-injection with the lower concentrations of a mixture of seven EDCs, followed by a significant increase at the highest concentration tested. In the same study, a significant increase in GST activity was also recorded at 24 h post-injection, whereas enzyme activity decreased at 72 h post-injection with the highest concentration of the mixture (Canesi et al., 2008).

With regards to AChE activity, this study demonstrated that exposure to the mixture significantly increased enzyme activity in gill mussels. A significant increase in AChE activity was recorded in gills from *M. galloprovincialis* exposed for four days to the lowest concentration of a mixture of Cu (5 μg/L) and chlorpyrifos (0.05 μg/L), whereas a significant decrease in enzyme activity was recorded following exposure to the highest concentration of the mixture (15 μg/L Cu + 5 μg/L chlorpyrifos) (Peric and Buric, 2019). In *P. viridis,* a significant decrease in AChE activity was found in cell-free haemolymph of mussels exposed to low, medium and high concentrations of a mixture containing carbamazepine, bisphenol A and atrazine (Juhel et al., 2017). Exposure for 7, 14 and 21 days to a mixture of glyphosate and its main breakdown product aminomethylphosphonic acid (AMPA (100 + 100 μg/L) induced a non-linear response of mussels, AChE activity decreasing after 7 days of exposure, increasing after 14 days, and decreasing again after 21 days (Matozzo et al., 2019b). In that study, the authors hypothesised that the increase in AChE activity recorded at 14 days of exposure was an attempt of mussels to face the initial decrease at 7 days, whereas after 21 days of exposure mussels were unable to counteract mixture effects. According to Perić and Burić (2019), stimulation of AChE activity observed in this study at lower levels of the mixture can be considered as a response in terms of activation of important defence pathways in molluscs.

The survival-in-air test is one of the simplest, most feasible, sensitive, reproducible and cost-effective methods for evaluating contaminant-induced stress in bivalves (Eertman et al., 1993; de Zwaan and Eertman, 1996). In this study, tolerance to aerial exposure did not change significantly in mussels exposed for 7 days to the mixture, LT_{50} values being similar among the experimental groups. This suggested that the mixture did not affect physiological performance of bivalves. It is difficult to compare results of our study with those from the literature, as to our knowledge there is no information about the effects of contaminant mixture on tolerance to aerial exposure in bivalves, but of single compounds only.

5. Conclusions

Summarising, results of the present study indicated that exposure to a mixture of environmentally realistic concentrations of glyphosate, EE2 and amil salicylate can affect haemocyte and tissue biochemical parameters, but not tolerance to aerial exposure of *M. galloprovincialis*. At the cellular level, this study demonstrated that the mixture affects markedly the number of circulating haemocytes and cell membrane stability. At the tissue level, results obtained suggested that gills were

more affected by the exposure to the mixture than digestive gland. Although further studies are needed to clarify better the mode of action of the contaminants tested (alone or in combination), results reported here suggest a potential ecotoxicological risk for bivalve molluscs.

Credit author statement

Jacopo Fabrello: Writing – original draft, Writing – review & editing. Investigation, Methodology, Conceptualization, Luciano Masiero: Writing – review & editing. Investigation, Methodology. Livio Finos: Writing – review & editing. Investigation, Formal analysis, Maria Gabriella Marin: Writing – review & editing. Investigation, Methodology, Valerio Matozzo: Funding acquisition, Project administration, Writing – review & editing. Writing – original draft, Investigation, Formal analysis, Conceptualization, Methodology.

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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