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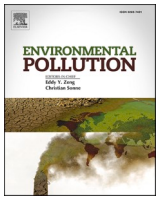

Fitness assessment of *Mytilus galloprovincialis* Lamarck, 1819 after exposure to herbicide metabolite propachlor ESA☆

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ABSTRACT

The lack of data on the chronic effects of chloroacetanilide herbicide metabolites on non-target aquatic organisms creates a gap in knowledge about the comprehensive impacts of excessive and repeated pesticide use. Therefore, this study evaluates the long-term effects of propachlor ethanolic sulfonic acid (PROP-ESA) after 10 (T1) and 20 (T2) days at the environmental level of 3.5 $\mu g.L^{-1}$ (E1) and its 10x fold multiply 35 $\mu g.L^{-1}$ (E2) on a model organism *Mytilus galloprovincialis*. To this end, the effects of PROP-ESA usually showed a time- and dose-dependent trend, especially in its amount in soft mussel tissue. The bioconcentration factor increased from T1 to T2 in both exposure groups – from 2.12 to 5.30 in E1 and 2.32 to 5.48 in E2. Biochemical haemolymph profile and haemocyte viability were not affected by PROP-ESA exposure. In addition, the viability of digestive gland (DG) cells decreased only in E2 compared to control and E1 after T1. Moreover, malondialdehyde levels increased in E2 after T1 in gills, and DG, superoxidase dismutase activity and oxidatively modified proteins were not affected by PROP-ESA. Histopathological observation showed several damages to gills (e.g., increased vacuolation, over-production of mucus, loss of cilia) and DG (e.g., growing haemocyte trend infiltrations, alterations of tubules). This study revealed a potential risk of chloroacetanilide herbicide, propachlor, via its primary metabolite in the Bivalve bioindicator species *M. galloprovincialis*. Furthermore, considering the possibility of the biomagnification effect, the most prominent threat poses the ability of PROP-ESA to be accumulated in edible mussel tissues. Therefore, future research about the toxicity of pesticide metabolites alone or their mixtures is needed to gain comprehensive results about their impacts on living non-target organisms.

1. Introduction

Over the last several decades, the widespread use of various chemical compounds in the agricultural sphere of human society has resulted in global concern about environmental health. In agriculture, the intensive and repeated use of pesticides and fertilisers has been the most effective way to maintain agricultural production for a still-growing human population (Kannan et al., 2023; Wang et al., 2020; Bilal et al., 2019).

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One of the widely used groups of pesticides, chloroacetanilide, is applied in the fields to control annual grass and broadleaf weeds (Ma et al., 2021; Antić et al., 2015). Moreover, nowadays, around 30–50% of the total annual pollution of aquatic ecosystems is caused by humans because of commercial pesticide usage for gardening (Antić et al., 2015; Kolpin et al., 2006; Blanchoud et al., 2004).

These herbicides and their residues are commonly detected in aquatic ecosystems worldwide (e.g., Kiefer et al., 2021; Ma et al., 2021; Tan et al., 2020; Xu et al., 2019; Hvězdová et al., 2018; Kolpin et al., 2000, 1996), where threaten living organisms in different trophic levels, thanks to food web links (Olowoyo and Mugivhisa, 2019). Water contamination caused by pesticides is influenced by several factors, mainly the absorption and mobility of chemicals in soil, in which the content of soil organic matter has a critical role (Zhang et al., 2012). However, the leaching of pesticides into the environment is a complex system that also depends on the decomposition or oxidation mechanism of pesticide, climate conditions, and agricultural treatment of crops and soils (Dao et al., 2023). Since during last few decades, our knowledge of pesticides and their metabolites occur in the environment has been studied well (e.g., Velisek et al., 2020; Hvězdová et al., 2018; Al-Mamun, 2017; Zhang et al., 2012). Nevertheless, there are still gaps in the scientific literature about their impact on non-target organisms. The long persistence of these agrochemicals must also be considered because they might affect living organisms and their habitats decades after their use or after their banning use (e.g., Dao et al., 2023; Tresnakova et al., 2022).

After alachlor and metolachlor, the most used pre-emergence herbicide in Europe and China is propachlor [(2-chloro-N-(1-methylethyl)-N-phenyl-acetanilide)] (Liu et al., 2012; Lambropoulou et al., 2002; Konstantinou et al., 2000). It has been used on the field mainly with corn, sorghum, pumpkins, flax, and flowers since 1965 (WHO and IPCS, 1993). Its properties like log K_{ow} 2.41 and water solubility 700 $mg.L^{-1}$ make propachlor quickly transfer throughout the soil into water (Zhang et al., 2012; Lambropoulou et al., 2002; Clegg et al., 1991). Its half-life is 4 days, according to Beestman and Deming (1974), and less than 3 days, according to the U.S. Environmental Protection Agency (1998). The crucial role in its biodegradation in soil and water has microorganisms (Elsayed, 2015; Hatvani et al., 2013; Zhang et al., 2012; WHO and IPCS, 1993), especially the sulphate-reducing bacteria (Stamper et al., 1997) or *Bacillus cereus* and *B. thuringiensis* (Wang et al., 2008). Furthermore, propachlor can also be removed to a particular amount in biological systems due to glutathione-acetanilide conjugates (Stamper and Tuovinen, 1998).

Propachlor ESA [2-Oxo-2-(N-propan-2-ylanilino)ethanesulfonic acid] (PROP-ESA), with a molecular weight of 257.31 g $mol^{-1,}$ is an organosulfonic acid and one of the predominant biodegradation products of herbicide propachlor (National Center for Biotechnology Information, 2023; Zheng et al., 2004; WHO and IPCS, 1993). U.S. EPA (2023) shows propachlor biodegradation time is 4.91 days, fish biotransformation half-life (km) is 0.222 days, soil adsorption coefficient is 123 L kg^{-1}, and the bioconcentration factor is 8.5.

There are no scientific data about the adverse effects of PROP-ESA on non-target aquatic organisms. Regarding chloroacetanilide herbicides and their metabolites, Junghans et al. (2003) evaluated the same mode of action of eight herbicides, including propachlor, in algae. Their toxicity was observed to be higher than their mixture. On the other hand, biodegradable products might evince even more toxicity than their parental or commercial chemicals (Anagnostopoulou et al., 2022). It will probably depend on each component of the testing mixture because, during the laboratory experiment, it is known that the exact mixture content in comparison with natural waters is a mixture of hundreds of compounds, so the identification is often intricated (Dao et al., 2023; Anagnostopoulou et al., 2022; Tresnakova et al., 2022, 2021; Velisek et al., 2020). Therefore, we hypothesised that PROP-ESA might have similar effects on non-target organisms as its parental compound. For example, the EC50 value is reported for algae *Scenedesmus vacuolatus* at level of 35.2 $\mu g.L^{-1}$ and NOEC 19.9 $\mu g.L^{-1}$ (Junghans et al., 2003); for zooplankton species *Daphnia magna* are reported values of LC50 at level 7.8 and 6.9 $mg{\cdot}L^{-1}$ and NOEC less than 5.6 $mg{\cdot}L^{-1}$; 96hLC50 value for rainbow trout is 0.17 $mg{\cdot}L^{-1}$ and NOEC of 21-day exposure is 0.019 $mg{\cdot}L^{-1}$ (WHO and IPCS, 1993); and slightly toxic to birds (U.S. EPA, 1998). It was also found that propachlor is cytotoxic for rats and human cells (Dierickx, 1999). Nevertheless, it is still thought that propachlor and its metabolite toxicities are low because of non-persistence and fast environmental reduction (U.S. EPA, 1998). Since information about PROP-ESA toxicity is missing, we studied the long-term toxicity of metabolite PROP-ESA on the bioindicator bivalve species Mediterranean mussel *Mytilus galloprovincialis*.

The Mediterranean mussel has several advantages in its usage for ecotoxicological and immunological research, mainly due to its long-life cycle, filter-feeding, wide range of tolerance to environmental stressors, and a critical link in the food web (Tresnakova et al., 2023; Curpan et al., 2022; Stara et al., 2021; Třešňáková et al., 2020; Waykar and Deshmukh, 2012). Therefore, this study investigated the 10- and 20-day effects of PROP-ESA on model species *M. galloprovincialis* at the environmental level (3.5 $\mu g{\cdot}L^{-1}$) and its ten multiply (35 $\mu g{\cdot}L^{-1}$). To sum up, bioaccumulation of PROP-ESA and its effect on digestive gland cells and oxidative stress was determined.

2. Materials and methods

2.1. Experimental design

A marine bivalve, *Mytilus galloprovincialis* Lamarck, 1819, was selected as a model organism to investigate the toxicity of herbicide metabolite propachlor ESA. The test mussels were purchased from a commercial shellfish farm, FARU SRL Frutti di Mare, on Faro Lake in Messina (Italy). It is a small marine lake with a mean depth of 3 m and a maximum depth of 30 m. The 300 mussels with a mean length of 6 ± 0.54 cm were immediately transported to the University of Messina and randomly divided into six tanks with continuous aeration and 20 L of filtered lake water for acclimatisation to laboratory conditions. The water for one week of acclimatisation and main experiments was renewed three times per week (T: 22.20 ± 0.13 °C; pH: 7.20 ± 0.05; salinity 3.2 ± 0.01‰) with filtered fresh lake water, and the bivalve was kept at natural a light condition (16 h light: 8 h dark). Mussels were not fed due to nutrients contained in natural lake water.

Two PROP-ESA concentrations, E1 (3.5 $\mu g.L^{-1}$), E2 (35 $\mu g.L^{-1}$), and the control group (no exposure treatment) were carried out in duplicates in the experimental design (N = 46 per tank). Tested environmental concentration (E1) and its multiply (E2) were chosen according to US Environmental Protection Agency (U.S. EPA, 1998). Stock dosing solutions of PROP-ESA (Sigma – Aldrich, USA: purity ≥98,0%; CAS: 947601-88-9) were prepared in distilled water. The same concentrations were redosing after each renewal of water volume (three times per week - Mon, Wed, Fri). Every thirty mussels were analysed after 10 and 20 days of exposure to PROP-ESA. At the sampling time, mussels were randomly selected and anaesthetised on an ice flake before dissection.

2.2. Bioaccumulation

2.2.1. Chemicals

HPLC-grade methanol, ACS-grade ammonium acetate and Ultra-pure HPLC-grade water were obtained from Sigma-Aldrich (Steinheim, Germany). The ENVI-CARB cartridges were purchased from Supelco (Bellefonte, PA). The calibration standard was prepared in deionised water containing 5 mM ammonium acetate. A solution of 10 mM ammonium acetate in methanol was prepared by adding 0,77 g ammonium acetate to 1 L of methanol; 5 mM ammonium acetate in methanol was prepared by adding 0.385 g ammonium acetate to 1 L of deionised water.

2.2.2. Water analyses

Water samples from each tank were collected before and after each water exchange from each tank and stored at - 20 °C until their processing (Andreu-Sánchez et al., 2012). The extraction of PROP-ESA from water samples was made according to a method by Shoemaker (2002). In brief, water samples were extracted with ENVI-CARB carbon cartridges (6 mL, 0.5 g). Samples were dechlorinated with ammonium chloride, 100 mg.L^{-1}. The cartridges were previously conditioned with 20 mL of 10 mM ammonium acetate in methanol and 30 mL of deionised water. The water samples were passed through the cartridges at a flow rate of 10 mL min^{-1}. Then, the cartridges were washed with 5 mL of deionised water and dried for 3 min. The target analyte was eluted with 15 mL of 10 mM ammonium acetate in methanol from the cartridges. Extracts were evaporated to dryness and reconstituted to 1 mL with 5 mM ammonium acetate prepared in deionised water. A blank was made using the same reagents and method in the absence of the contaminant.

2.2.3. Soft tissue

The extraction of PRL from the tissues of 20 mussels taken from each experimental group was performed by taking the entire soft tissue of the mussel and weighing the individual samples belonging to the three experimental groups to obtain three corresponding mussel tanks. The extraction procedure was performed by considering the previous methods of Shoemaker (2002) and Stara et al. (2021). After the homogenisation of each pooled sample, an aliquot of homogenate (20 g) was mixed with 25 mL of 10 mM ammonium acetate in methanol and shaken for 30 min at 35 °C. The obtained mixture was centrifuged for 3 min at 4200 rpm. Hence, the upper phase was decanted, evaporated to 0.5 mL with a rotavap (Büchi Heating Bath B-490, Büchi Labortechnik AG, Switzerland) and dissolved in 30 mL of ultrapure water. The extracts were purified with ENVI-CARB carbon cartridges (6 mL, 0.5 g). The cartridges were previously conditioned with 20 mL of 10 mM ammonium acetate in methanol and 30 mL of deionised water. The water samples were passed through the cartridges at a flow rate of 10 mL min^{-1}. Then the cartridges were washed with 5 mL of deionised water and dried for 3 min. The target analyte was eluted with 15 mL of 10 mM ammonium acetate in methanol from the cartridges. All extracts were evaporated to dryness and, before the analysis, reconstituted to 1 mL with 5 mM ammonium acetate prepared in deionised water.

2.2.4. HPLC-ESI-MS/MS analysis

The HPLC-ESI-MS/MS analysis was performed on an LC apparatus (Prominence UFLC XR system, Shimadzu, Kyoto, Japan) consisting of a controller (CBM-20 A), binary pumps (LC-20AD-XR), degasser (DGU20A3 R), column oven (CTO-20AC), and autosampler (SIL-20 A XR). A column Agilent Hypersil (2.1 × 100 mm, 5 μm) C18) was used to separate the target analyte at a flow rate of 0.4 mL min^{-1} and column temperature of 35 °C. The injection volume used was 100 μL. The binary mobile phase gradient composition was (A) 10 mM ammonium acetate and (B) methanol. The gradient program was: 0–8 min, 10–20% of B, 8–12 min, 20–25% B, 12–17 min 25% B, including final column washing and re-equilibration steps. An electrospray ionisation (ESI) source interfaced the LC system with a triple quadrupole mass spectrometer (MS) (LCMS-8040, Shimadzu, Kyoto, Japan) and was employed in positive ionisation mode. The precursor ion $[M-H]^+$ was first fully scanned over the m/z range 100–500. Then, selected reaction monitoring (SRM) MS/MS experiments were performed by selecting the precursor ion $[M-H]^+$ (m.z^{-1} 258) with the first quadrupole mass analyser (Q1) and setting the third quadrupole mass analyser (Q3) to sequentially scan product ions m/z 216, 217 and 258 (Table 1). Data were acquired by LabSolution software (v 5.53 SP2, Shimadzu, Kyoto, Japan). For each mussel pool and water sample, triplicate measurements were conducted.

2.2.5. Bioconcentration factor

The bioaccumulation of pollutants in the aquatic organism is generally given via the bioconcentration factor (BCF), which identifies the ratio between the pollutant concentrations in the aquatic organism and water (Arnot and Gobas, 2006; Costanza et al., 2012). BCF is measured under controlled laboratory conditions and is expressed as a ratio between the concentration of chemical pollutants in the aquatic organism and the water under steady-state conditions (Davies and Dobbs, 1984). BCF is given by the following formula:

$$BCF = \frac{C_{tissue}}{C_{water-total}}$$

where BCF is the bioconcentration factor, C_{tissue} is the concentration of PRL in soft mussel tissue, and $C_{water\text{-}total}$ is the concentration measured in water.

Table 1
Retention time (Rt), molecular weight, precursor ion and products ions of the target analyte herbicide metabolite propachlor ESA (PROP-ESA).

Analyte	Retention time (min)	Molecular weight (g.mol^{-1})	Precursor (m.z^{-1})	Product ions (m.z^{-1})
PROP-ESA	6.3	257.31	258	216; 217; 258

2.3. Biochemical profile of haemolymph and haemocyte viability

The haemolymph was taken with a 5 cm syringe from the adductor muscle of fifteen mussels, according to Bolognesi and Fenech (2012). Three haemolymph samples were pooled to ensure a sufficient multiplicity of haemolymph samples for both biochemical analyses and viability assays.

The haemolymph samples were centrifuged at 1000 rpm for 10 min at 4 °C and stored at −80 C° until the future analyses of electrolytes (calcium (Ca^{2+}), sodium (Na^+), potassium (K^+), chloride (Cl^-), phosphorus (PHOS), magnesium (Mn^{2-})) and the biochemical parameters (lactate dehydrogenase (LDH), glucose (GLU), aspartate transaminase (AST), alanine transaminase (ALT), creatine kinase (CK)). These parameters were evaluated following the methodology presented by Stara et al. (2020a; 2020b).

The Haemocyte viability test was conducted using two different colourimetric assays: the Neutral Red retention assay performed by Moore et al. (2009) and the Trypan Blue exclusion method. In brief, the lysosomal membrane stability was evaluated with both methods under a light microscope (Carl Zeiss Axioskop 20, Wetzlar, Germany) with a Burker chamber at a magnification of 40×. The percentage of viability was established according to the following formula:

$$\text{cells viability } (\%) = \frac{\text{number of viable cells}}{\text{total number of cells}} \text{ x } 100$$

2.4. Digestive gland cells analyses

Digestive glands (DG) from four mussels were used to evaluate the effects of PROP-ESA on cell viability and regulation of volume decrease (RVD). Dissected DG was placed in a calcium and magnesium-free solution (CMSF) and processed following the protocol described in Pagano et al. (2017) and Faggio et al. (2011); here, the following is briefly specified.

2.4.1. Cell viability

DG cell viability was assessed by the same procedure (Neutral Red and Trypan Blue assays) as the haemocyte viability, which is described above in subchapter 2.3.

2.4.2. Regulation of volume decrease

The RVD is evaluated based on microscopical observation of samples under a light microscope (Carl Zeiss Axioskop 20, Wetzlar, Germany) and a magnification of 100× connected to the digital camera (Canon

550D) following the protocol of Pagano et al. (2017) and Tresnakova et al. (2023). Briefly, the cells were spread out and left to adhere for 2 min on the bottom of a thermostated Plexiglas chamber (18 °C) covered with poly-L-lysine (0.01%). A gravity flow of solution from reservoirs was kept at 18 °C by a water jacket constantly perfusing the chamber. After 3 min cell incubation in an isotonic solution, the cells underwent an osmotic shock with a hypotonic solution. As a result, a total the 17 images were taken in 40 min and analysed with the computer software ImageJ (NIH, Bethesda, MD, USA), where cells' area from exposed mussels (Aexp) was compared with the control group (Ai).

2.5. Oxidative stress

Tissues (gills and DG; N = 6 for each group) were homogenised in cold 100 mM Tris/HCl buffer (pH 7.2) containing a mixture of protease inhibitors. An aliquot of homogenate was used to test lipid peroxidation, protein oxidative damage and superoxide dismutase activity (Filice et al., 2023), as the following specified.

2.5.1. Lipid peroxidation

Lipid peroxidation (LPO) was determined by measuring the concentration of 2-thiobarbituric acid-reacting substances (TBARS), as reported by Filice et al. (2021). Briefly, a reaction mixture containing sample homogenate (0.2 mL, 10% w/v) in Tris/HCl buffer (100 mM, pH 7.2), 2-thiobarbituric acid (TBA; 0.8%, 0.2 mL), and trichloroacetic acid (TCA; 20%, 0.2 mL) was kept in a water bath at 100 °C for 10 min as follow the centrifugation at 7000 rpm for 10 min. TBARS levels were determined in the supernatant by assessing malondialdehyde (MDA) concentration at 540 nm; TBARS values were reported as nmol MDA per mg of protein (MDA extinction coefficient:156000 M^{-1} cm^{-1}).

2.5.2. Assay of carbonyl groups of oxidatively modified proteins (OMP) level

The 2,4-dinitrophenylhydrazine (DNPH) method, described by Levine et al. (2000), was used to measure carbonyl group content, indicative of oxidative protein damage. In brief, homogenates were centrifuged at 5000 g for 5 min at 4 °C, and an aliquot of the supernatant was incubated for 1 h at room temperature with 10 mM DNPH in 2 M HCl. Proteins were precipitated with 2 vol of TCA and centrifuged at 7000 rpm for 20 min. The pellet was washed thrice with ethanol-ethyl acetate (1:1; v/v) to remove DNPH excess and dissolved in 6 M guanidine in 2 N HCl. The concentration of carbonyl groups was measured spectrophotometrically at 370 nm (aldehydic derivates) and 430 nm (ketonic derivates) using the extinction coefficient of 22000 $M^{-}1.cm^{-1}$. Results were expressed as nmol per mg protein.

2.5.3. Superoxide dismutase (SOD) activity

The SOD activity was determined by the pyrogallol method described by Maklund and Maklund (1974) and modified according to Tresnakova et al. (2023). The inhibitory effect of SOD on the auto-oxidation of pyrogallol at pH 8.20 was assayed spectrophotometrically at 420 nm and 25 °C. The reaction was run in 50 mM Tris-HC1, 1 mM EDTA, 0.2 mM pyrogallol and monitored every 30 s for 5 min. One unit of SOD activity was defined as the amount of the enzyme that inhibits 50% of pyrogallol auto-oxidation. Results were expressed in U. mg^{-1} protein.

2.6. Histological procedures

Histological analysis was performed after 10–20 days (T1-T2) of exposure to a xenobiotic, collecting two specimens of *M. galloprovincialis* from each experimental tank (C, E1 and E2). Following the protocol proposed by Tresnakova et al. (2023), gills and digestive glands (DGs) were removed and immediately fixed in 10% buffered formalin solution. Tissue samples thus collected were subsequently washed with distilled water and dehydrated through an increasing range of alcohol solutions. Before being embedded in paraffin, the histological samples were infiltrated with xylene. A microtome (EG11504 Leica Biosystems) was used to obtain serial sections of 3 μm of thickness from paraffin blocks, whereas haematoxylin and eosin (H&E) and xylene and Eukitt (Bio--Optica) were used to stain, clear, and mount the sections mentioned above, respectively. All the histopathological evaluations were performed under a light microscope (Leica DM6 B, Leica Microsystems GmbH Ernst- Leitz-Strasse, Wetzlar, Germany) with a built-in digital camera (Leica DFC 7000 T), using several objectives 10X, 20X, 40X, 63X, 100X. The Leica Application Suite X (LAS X) software was finally used to process the images acquired during gill and digestive gland tissue observations.

2.7. Histopathological condition indices

Semi-quantitative histopathological indices (*Ih*) were estimated for each specimen of *M. galloprovincialis* studied to assess the effects of Propachlor administration, according to Bernet et al. (1999), with appropriate modifications for bivalves (Pagano et al., 2022a, b; Costa et al., 2013). This approach involves assigning a weight to each histological alteration, based on biological relevance, with a ranging value between 1 and 3 (maximum severity). In addition, a score is attributed similarly, with values between 0 (no sign of alteration) and 6 (widespread alteration). The following formula was used to estimate the histopathological condition indices.

Where I_h is the histopathological condition index for the individual h; w_j the weight of the j_{th} histopathological alteration; a_{jh} is the score attributed to the h_{th} individual for the j_{th} alteration and M_j is the maximum attributable value for the j_{th} alteration (i.e., weight x maximum score). In the given equation, the denominator normalises I_h to a value between 0 and 1, allowing a comparison between distinct organs. The indices related to gills and digestive glands were thus estimated and subdivided by "reaction patterns" (cellular and morphological epithelial alterations). In the observations performed under the light microscope, a weight was established for each histopathological alteration, supported by data previously reported concerning invertebrates' (Costa et al., 2013) and vertebrates' histopathology (Costa et al., 2013, 2011; Bernet et al., 1999). According to the literature, haemocyte infiltration had the lowest weight ($w = 1$) along with intracellular melanin/lipofuscin-like aggregates, followed by epithelial cell hyperplasia ($w = 2$) and necrosis ($w = 3$) for both gills and digestive glands.

2.8. Statistical analyses

The viability cells, the RVD test and bioaccumulation were assessed using a one-way ANOVA comparing the control group with the experimental groups. Parameters of biochemical haemolymph profile, oxidative stress analysis and the histopathological indices (I_h) were analysed through a two-way ANOVA. The tests were followed by Tukey's post hoc test for multiple comparative analyses with a significance level of $p < 0.05$ was accepted as significant. The statistical analyses were evaluated using Graphpad Prism software, version 5 (Graphpad Software Ldt., USA, 2003). The results are reported as the mean ± standard deviation (S.D.).

3. Results

3.1. Bioaccumulation

3.1.1. Analytical verification of propachlor ESA concentrations

The method's sensitivity was assessed by determining the limit of detection (LOD) and limit of quantification (LOQ) for PROP-ESA. The mean LOD for water and soft tissue samples was 0.04 ng g^{-1}, and the LOQ was 0.13 ng g^{-1}. PROP-ESA concentrations in water were >80% of the nominal concentration throughout the exposure period, averaging 84%. The lake water from the control tank was PROP-ESA-free, and

detailed concentrations are listed in Table 2.

3.1.2. Bioconcentration factor

The BCF was calculated from analytical verified concentrations in water and soft mussel's tissue (Table 2) after the first 10 days (T1) of exposure – E1: 2.12, E2: 2.30; and at the end of the experiment (T2) – E1: 5.30, E2: 5.48.

3.2. Biochemical profile of haemolymph and haemocyte viability

Between indices of biochemical haemolymph's profile, were no detected significant differences ($p > 0.05$) for all experimental groups (PROP-ESA-free, E1, E2) in both exposure times (T1, T2). Detailed values of individual electrolytes and biochemical parameters obtained in this study are shown in Table 3.

The statistical analyses ($p > 0.05$) confirmed no effect of PROP-ESA on haemocyte viability calculated based on the TB and NR assays for all experimental groups (PROP-ESA-free, E1, E2) during both exposure times (T1, T2) (Table 4).

3.3. Digestive gland cells analyses

3.3.1. Cell viability

After T1, DG cell viability assessed with TB assay significantly decreased *($p < 0.05$)* in E2 (94.08 ± 4.86%) compared to control and E1 groups (99.15 ± 0.4%, and 99.10 ± 0.32%, respectively). No significant difference ($p > 0.05$) was measured between the control and E1 groups. The same trend was observed even for DG cell viability assessed with an NR assay at the same exposure time (PROP-ESA-free: 98.77 ± 1.12%, E1: 99.25 ± 0.46%, E2: 95.45 ± 5.11).

After twenty days of exposure, the cells did not show significant ($p > 0.05$) results for both methods – PROP-ESA-free: 99.06 ± 0.46%, E1: 98.99 ± 0.61%, E2: 98.49 ± 0.63% for TB assay, and PROP-ESA-free: 98.77 ± 1.12%, E1: 98.63 ± 0.79%, E2: 98.22 ± 1.30% for NR assay.

3.3.2. Regulation of volume decrease

The analyses conducted on the DG cells for evaluating the capacity of RVD after an explosion to a hypotonic solution showed that after T1, the maximum swell reached by the cells ware of 86% in E1 and 83% in E2 (Fig. 1 A). In both cases, the cells were not returned to their original volume. However, compared to the cells from the control group, the One-Way ANOVA analyses highlighted a significance only for the cells from E2 group *($p < 0.05$)*. After T2, the cells reached faster the maximum swelling compared to the T1 with a percentage of swell respectively of 83% (E1) and 89% (E2) (Fig. 1 B). Also, in this case, the cells were not returned to the initial volume. Nevertheless, the data was not statistically significant ($p > 0.05$) compared to the control in both exposure groups.

Table 2
The concentration of Propachlor ESA in lake water during the experiment and soft tissue of *Mytilus galloprovincialis* exposed to 3.5 μg.L^{-1} (E1) and 35 μg.L^{-1} (E2) after 10 (T1) and 20 (T2) days of exposure.

Sample	Exposure time (days)	Experimental groups		
		Control (PROP-ESA free)	E1 (3.5 μg. L^{-1})	E2 (35 μg. L^{-1})
Water (μg. L^{-1})	T1 (10)	< LOD	2.87 ± 0.15	29.31 ± 0.20
	T2 (20)	< LOD	2.92 ± 0.18	30.97 ± 0.23
Soft tissue (ng.g^{-1})	T1 (10)	< LOD	7.427 ± 0.027	185.52 ± 0.134
	T2 (20)	< LOD	8.147 ± 0.018**	191.91 ± 0.745**

LOD: 0.04 ng g^{-1} and LOQ: 0.132 ng g^{-1}. Data are presented as mean ± S.D. Water samples (n = 12) from each experimental group were collected before and after each water exchange. The pool of soft tissues of mussels (n = 6) was taken after 10 and 20 days of exposure. ** indicate a significance of $p < 0.01$, in relation to the same concentration at the shorter time, respect to the control group.

Table 3
Biochemical characteristics of haemolymph of *Mytilus galloprovincialis* treated with propachlor ESA at levels 3.5 μg.L^{-1} (E1) and 35 μg.L^{-1} (E2) after duration of 10 (T1) and 20 days (T2).

Indices	Exposure time (days)	Experimental groups		
		Control (PROP-ESA free)	E1 (3.5 μg. L^{-1})	E2 (35 μg. L^{-1})
AST (U.L^{-1})	T1 (10)	1.50 ± 2.12	–	2.00 ± 1.00
	T2 (20)	7.50 ± 7.78	7 ± 4.24	15.50 ± 13.44
ALT (U.L^{-1})	T1 (10)	–	–	–
	T2 (20)	1.50 ± 2.12	1.00 ± 1.41	4.00 ± 4.24
CK (U.L^{-1})	T1 (10)	2.00 ± 0	2.50 ± 2.12	1.00 ± 1.00
	T2 (20)	–	–	–
GLU (mg. dL^{-1})	T1 (10)	7.05 ± 1.34	1.60 ± 0	5.10 ± 0.10
	T2 (20)	6.85 ± 1.20	6.50 ± 0.28	7.85 ± 0.07
ISE Cl^{-} (mmol.L^{-1})	T1 (10)	464.90 ± 4.20	467.55 ± 10.11	458.30 ± 4.60
	T2 (20)	415.70 ± 46.95	465.00 ± 0.07	467.70 ± 5.94
ISE K^{+} (mmol.L^{-1})	T1 (10)	14.70 ± 0	13.55 ± 1.06	14.40 ± 0
	T2 (20)	12.20 ± 2.12	13.95 ± 0.21	14.30 ± 0.42
ISE Na^{+} (mmol.L^{-1})	T1 (10)	495.50 ± 2.12	484.00 ± 26.87	522.50 ± 2.50
	T2 (20)	453.00 ± 52.33	489.00 ± 4.24	499.00 ± 0
Ca^{2+} (mmol. L^{-1})	T1 (10)	10.44 ± 0.01	11.10 ± 0.05	10.77 ± 0.03
	T2 (20)	9.95 ± 1.07	10.96 ± 0.11	10.88 ± 0.07
PHOS (mg. dL^{-1})	T1 (10)	1.10 ± 0	0.85 ± 0.35	0.80 ± 0
	T2 (20)	0.85 ± 0.21	0.70 ± 0	0.75 ± 0.07
Mn^{2-} (mg. dL^{-1})	T1 (10)	11.55 ± 0.21	11.75 ± 0.07	11.70 ± 0
	T2 (20)	11.65 ± 0.07	11.50 ± 0.42	11.75 ± 0.07

The values are the mean ± S.D. AST: aspartate transaminase; ALT: alanine transaminase; CK: creatine kinase; GLU: glucose; ISE; ions selective electrodes; PHOS: phosphorus.

Table 4
Viability test on the haemocytes cells of *Mytilus galloprovincialis* using Trypan Blue exclusion method (TB) and Neutral Red retention assays (NR).

Method	Exposure time (days)	Experimental groups		
		Control (PROP-ESA free)	E1 (3.5 μg. L^{-1})	E2 (35 μg. L^{-1})
TB	T1 (10)	97.47 ± 1.82	91.03 ± 4.33	95.07 ± 3.46
	T2 (20)	98.80 ± 1.53	96.26 ± 2.23	95.68 ± 1.77
NR	T1 (10)	96.42 ± 0.84	96.31 ± 2.34	95.74 ± 1.39
	T2 (20)	97.67 ± 3.27	96.41 ± 2.92	94.32 ± 6.23

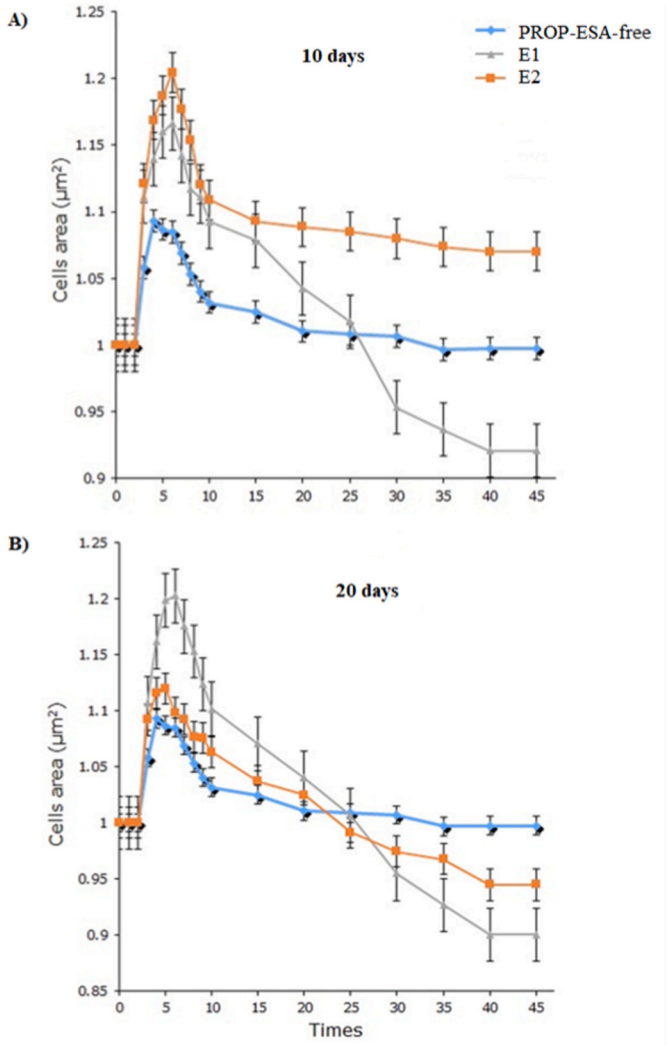

Fig. 1. Regulation volume decrease (RVD) of digestive gland cells of *Mytilus galloprovincialis* after 10 days (A) and 20 days (B) of exposition to herbicide metabolite propachlor ESA (PROP-ESA). Rhombuses represent control (PROP-ESA-free); triangle represent E1 (3.5 $\mu g \cdot L^{-1}$); squares represent E2 (35 $\mu g \cdot L^{-1}$). Two-way ANOVA was applied to compare the tested group with the control. The value represents the mean ± SD significance for $p < 0.05$.

3.4. Oxidative stress

In gills, SOD enzyme activity was unaffected ($p > 0.05$) in both E1 and E2 treatment after T1 (Fig. 2). However, concerning the control group, after T2, a significant increase ($p < 0.05$) in enzyme activity was measured in the gills of mussels exposed to both E1 and E2 concentrations (Fig. 2). No significant differences ($p > 0.05$) were observed in the DG at both concentrations after T1 nor T2 (Fig. 3).

The TBARS levels in both gills and DG of mussels increased after T1 in the E2 group. An increase in TBARS levels was also observed after twenty days of exposure to the lower concentration (E1), although with significant differences ($p < 0.05$) only in the DG tissue (Figs. 2 and 3).

In the gills, high levels of aldehydic derivatives of OMP were detected after T1 in the E2 group, while ketonic derivates significantly increased under all conditions tested (Fig. 2). No changes ($p > 0.05$) in aldehydic and/or ketonic derivates were observed in the DG tissue (Fig. 3).

3.5. Histological responses

The histopathological assessment revealed significant lesions of tissues analysed, which emerged from comparison with samples collected from the control tanks. More specifically, acute responses and several damages were noted after T2, mainly in the gills of *M. galloprovincialis* from E1 and E2, with moderate differences between the two experimental groups (Fig. 4). Among the reactions documented in gill tissues, lamellar deformations and epithelial alterations were the most frequent, together with haemocytes infiltration as a sign of possible diapedesis caused by inflammation. A significant increase ($p < 0.05$) of vacuolation was observed in mussels from E2 compared to those collected from E1 after T2. Other histological responses documented were the presence of granulocytes and lipofuscin aggregates, over-production of mucus and loss of cilia, characterised by a growing trend for control specimens.

Regarding the DG tissues analysed, the reaction patterns were less severe, except for haemocytes infiltration, which showed an increasing trend like that observed in the gills (Fig. 5). The deposit of lipofuscin was documented even in this case, together with inter-tubular tissue changes and other alterations of tubules. No signs of necrosis or presence of melanin aggregates were noted.

According to histopathological condition index results (Table 5), the general trend of tissue responses to inflammation appeared time and dose-dependent. All these inferences were supported by statistical analysis conducted on the abovementioned results. The analysis of variance was preliminarily performed on the Ih of control groups to ensure the accuracy of statistical results obtained in the subsequent analyses, showing no significance ($p > 0.05$) among specimens tested. The comparison between experimental groups and controls revealed a close correlation among reaction patterns, time of exposure and concentration of PROP-ESA in both organs analysed. As shown in Fig. 6, a significant growing trend of Ih was observed in gill tissues ($p < 0.001$), strictly related to increased concentration of the PROP-ESA (E1 and E2) and to the exposure times (T1 and T2). Similarly, the increasing trend of Ih noted in DG was time-dependent, as emerged from the comparison between specimens of the same experimental group. The digestive tubule alterations (TA) and the intertubular tissue changes (ITC) were compared, showing relevant differences among individuals exposed to both concentrations of PROP-ESA with a significant increase of I_h values observed between T1 and T2 (Fig. 6). Moreover, other considerable differences related to both PRL concentrations were noted between the I_h values of ITC observed after T1 ($p < 0.01$).

4. Discussion

Industrial and agricultural operations, waste management, and urbanisation have posed severe risks to the aquatic ecosystem in recent years. Pollution related to these human activities directly or indirectly affects wildlife habitats and organisms (Porretti et al., 2022; Zicarelli et al., 2022; Rashid et al., 2010). Therefore, pollutant impacts assessment on aquatic species is crucial to identify the main polluting activities, their origins, and their destiny in the aquatic environment via testing model organisms (Ravi et al., 2023; Amoatey and Baawain, 2019). Herbicide propachlor belongs to these pollutants, making non-target organisms like molluscs vulnerable (Tulcan et al., 2021). However, not only do parental compounds threaten biological systems, but their biodegradable products might be more toxic than the compound itself.

A good example might be this present study, which focuses on the toxicity of PROP-ESA – one of the primary metabolites of the parental compound propachlor (chloroacetanilide). Only a few studies about the toxicity of propachlor on non-target organisms have already been conducted (e.g., Dierickx, 1999; U.S. EPA, 1998; WHO and IPCS, 1993), but information about its metabolite PROP-ESA is missing. Since it is supposed that chloroacetanilide herbicides and their metabolites have similar modes of action, the toxic effects, including immune and

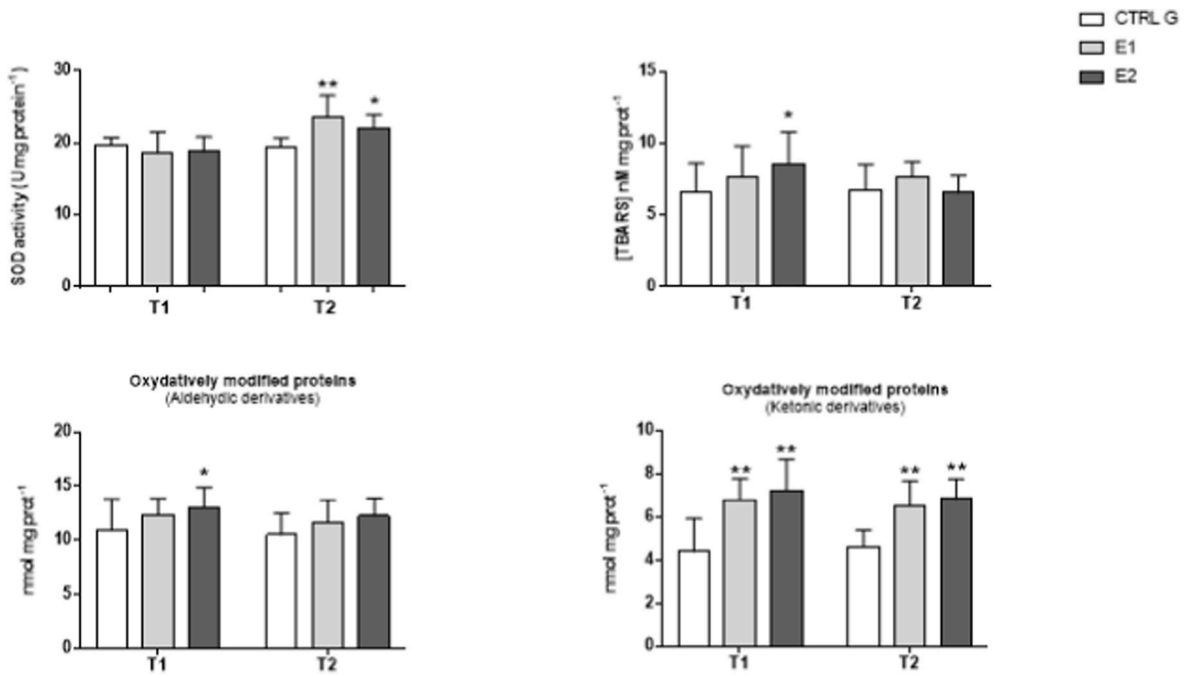

Fig. 2. Effects of propachlor ESA - treatment on oxidative stress biomarkers. Levels of SOD activity, TBARS, and OMP in the gills of control and propachlor ESA-treated mussels at different exposure times (T1: 10 days; T2: 20 days). Data are expressed as mean ± s.e.m. of absolute values from individual experiments (n. 6 each test). The statistic was assessed by two-way ANOVA followed by 'Tukey's multiple comparison test (*: CTRL *vs* E1 or E2; *$p < 0.05$; **$p < 0.01$).

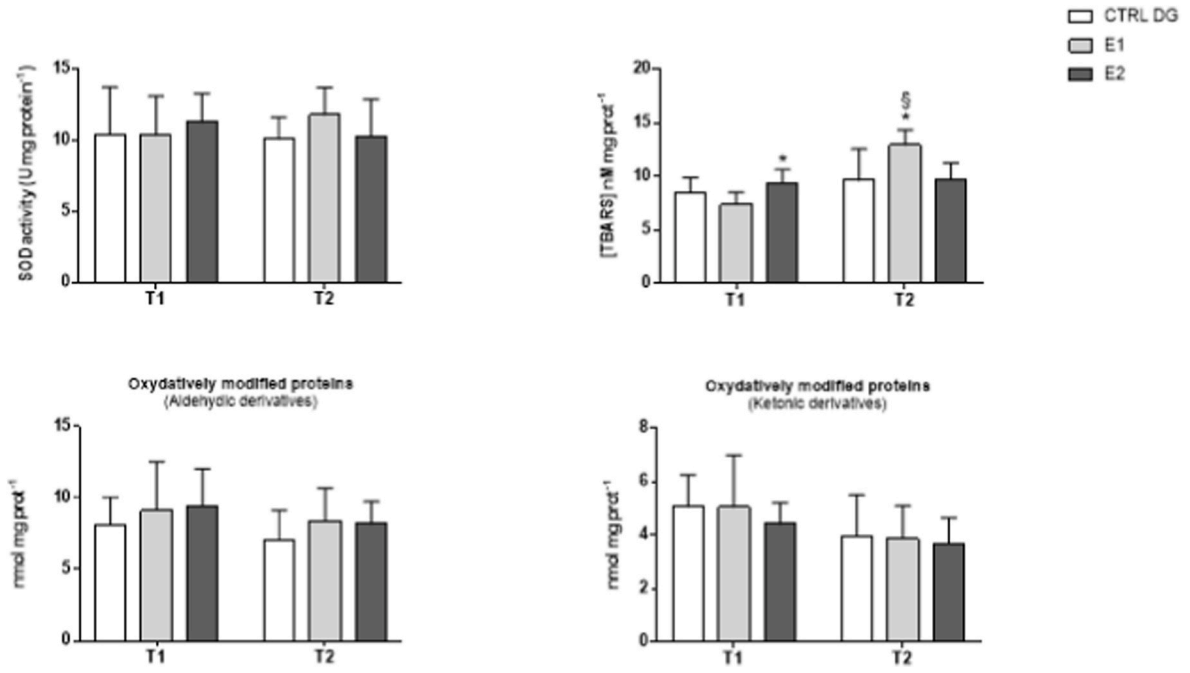

Fig. 3. Effects of propachlor ESA - treatment on oxidative stress biomarkers. Levels of SOD activity, TBARS, and OMP in the digestive gland of control and propachlor ESA-treated mussels at different exposure times (T1: 10 days; T2: 20 days). Data are expressed as mean ± s.e.m. of absolute values from individual experiments (n. 6 each test). The statistic was assessed by two-way ANOVA followed by 'Tukey's multiple comparison test (*: CTRL *vs* E1 or E2; *$p < 0.05$; **$p < 0.01$; §: E1 *vs* E2; §$p < 0.05$).

developmental toxicity, endocrine disruption, DNA damage, and enzyme activity (Ramesh et al., 2023; Kumar et al., 2022; Mai et al., 2014; Tu et al., 2013), might be expected as results from future research. For these reasons, the results gained are valuable and could be considered a step forward in protecting management against residual concentrations in aquatic ecosystems. Mainly due to the analytical verification of PROP-ESA concentrations in the soft tissue of *M. galloprovincialis*, which is one of the essential organisms between food chain links, because the knowledge about PROP-ESA bioaccumulation (kinetics and metabolism) in this animal is not well understood.

Regarding the results obtained from the analytical assessment of PROP-ESA concentrations in mussel soft tissue and water, an increase was observed in the E2 (T1: 2.12; T2: 5.40) group compared to E1 (T1: 2.12; T2: 5.30) group after both exposure times. Our results show lower

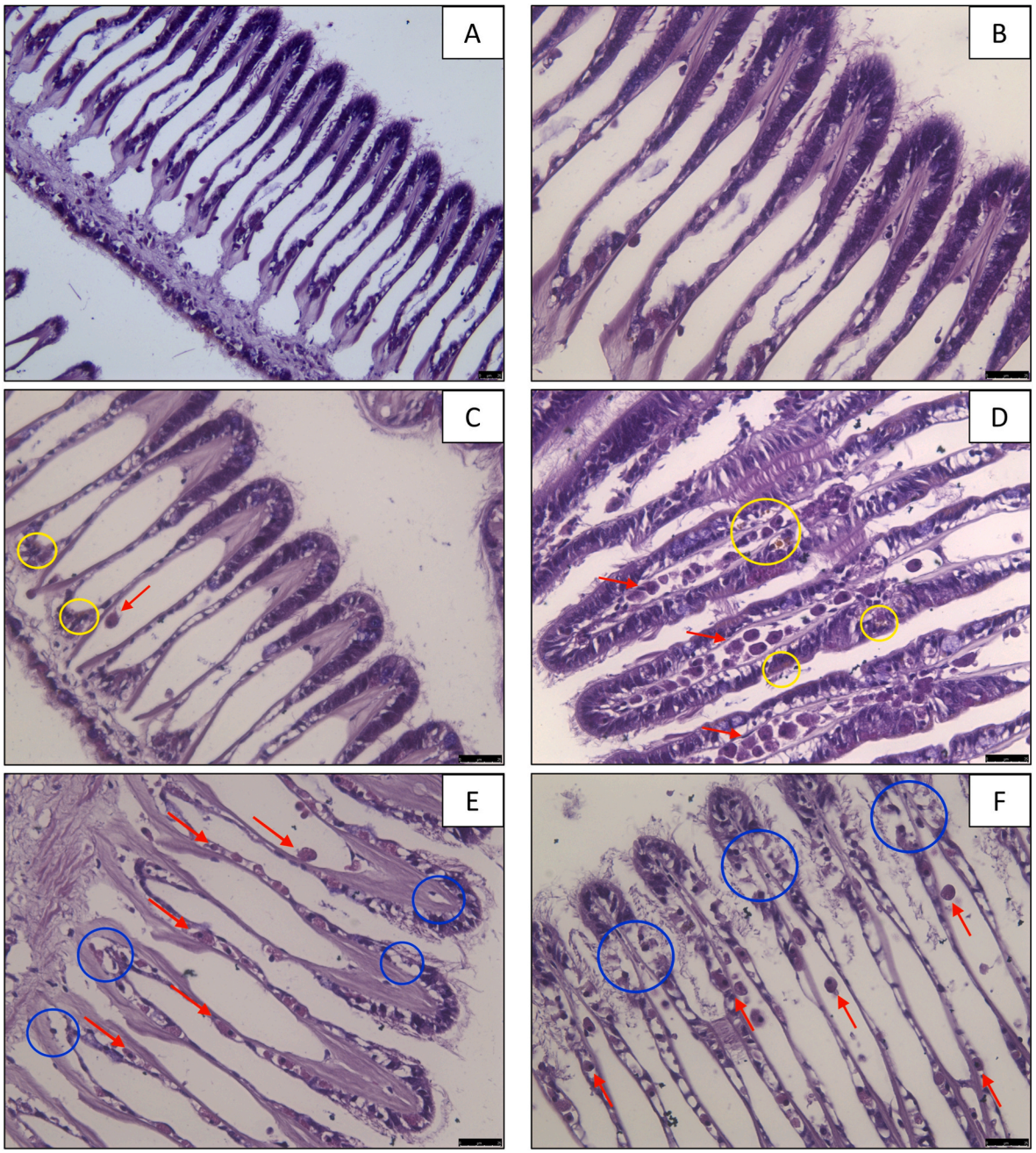

Fig. 4. Gill tissues of *Mytilus galloprovincialis* stained with haematoxylin and eosin, observed under a light microscope: mussels from control tanks 20 × (**A**) and 40 × (**B**) – Scale bars: 25 μm; specimens exposed to 3.5 μg/L (**C**) and to 35 μg/L (**D**) of propachlor ESA after 10 days of exposure (40 ×) – Scale bars: 25 μm; specimens exposed to 3.5 μg/L (**E**) and to 35 μg/L (**F**) of propachlor ESA after 20 days of exposure (100 ×) – Scale bars: 25 μm. Red arrows indicate haemocyte infiltration, yellow circles indicate lipofuscin aggregates and blue circles indicate epithelial alterations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

BCF (even in E2 group, where we tested ten multiply of environmental concentration) compared to its parental compound, which its BCF is given at level 8.5 by U.S. EPA (1998). In control group, the concentration of PROP-ESA was detected in mussel tissue as well as in lake water below LOD. Therefore we might supposed, that the shellfish farm run on the lake is not contaminated with this metabolite. The same results were obtained from Camps Bay (Cape Town, South Africa) study (Ojemaye et al., 2020), where all chloroacetanilide herbicides in limpets (*Cymubula granatina*), *M. galloprovincialis*, and sea urchin (*Parachinus angulosus*) were below the LOD. Moreover, according to the EU classification, a chemical's bioaccumulation potential in an organism is BCF >100. However, PROP-ESA can be classified as "dangerous to the environment" since it could impair the health of organisms, leading to the transfer and accumulation of the substance along the food chain (Costanza et al., 2012; Chapman et al., 1996).

Mussels, and fish, in general, are organisms that can filter xenobiotic substances with which they come into contact (Jebara et al., 2021; Di Bella et al., 2018a, b,c, d, 2017, 2010). The accumulation of these often-toxic substances in the organism of these molluscs is, therefore, easy. Previous studies confirm this ability, showing how *M. galloprovincialis* expose to different concentrations from 0 to 40 μg. L^{-1} of Yttrium, a rare metal, is significantly accumulated in the mussel tissue. Even if BCF levels decrease as the concentration increases, it could assume a form of safeguarding the animal, preserving itself from

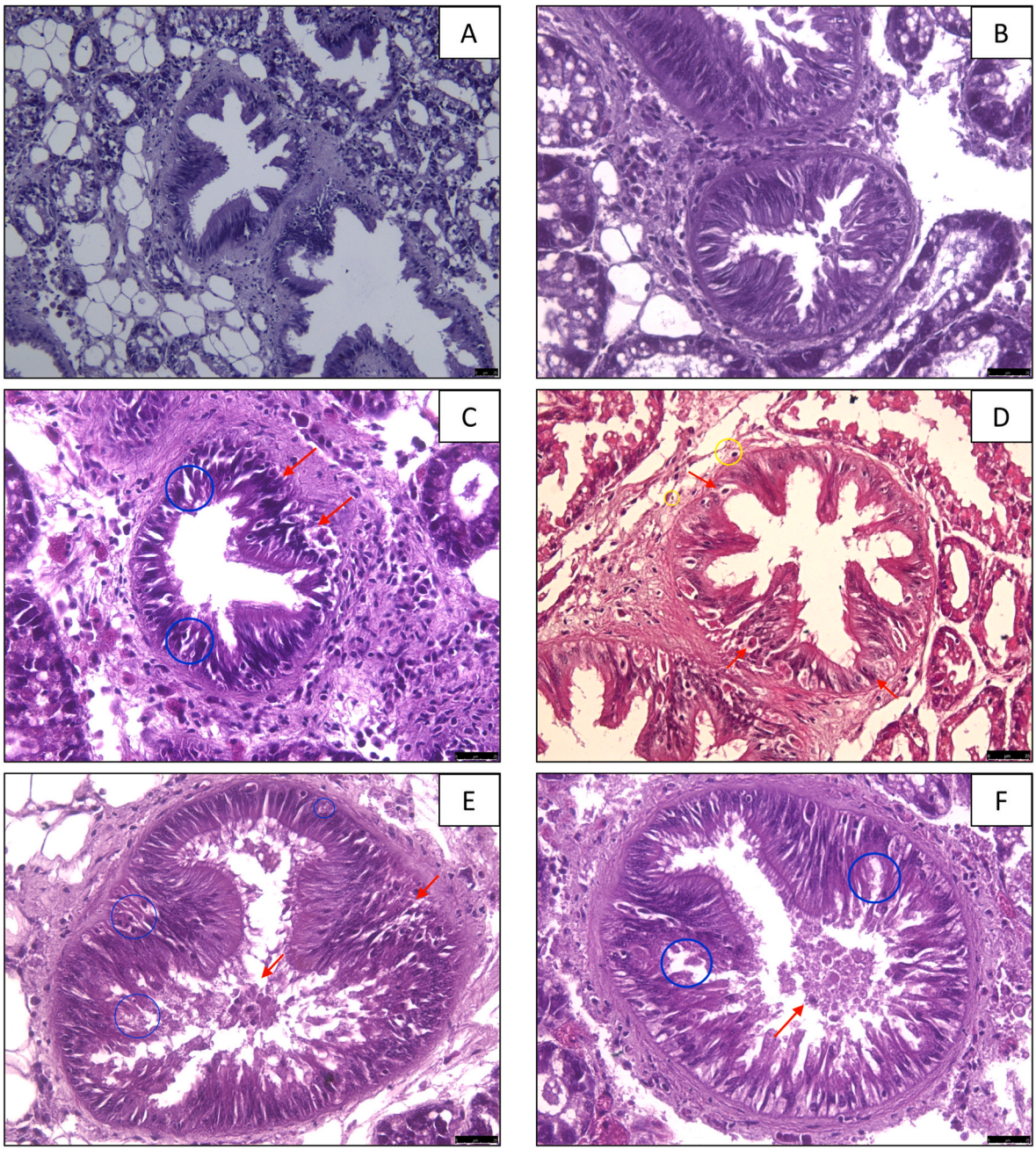

Fig. 5. Digestive gland tissues of *Mytilus galloprovincialis* stained with haematoxylin and eosin, observed under a light microscope: mussels from control tanks 20 × (**A**) and 40 × (**B**) – Scale bars: 25 μm; specimens exposed to 3.5 μg/L (**C**) and to 35 μg/L (**D**) of propachlor ESA after 10 days of exposure (40 ×) – Scale bars: 25 μm; specimens exposed to 3.5 μg/L (**E**) and to 35 μg/L (**F**) of propachlor ESA after 20 days of exposure (100 ×) – Scale bars: 25 μm. Red arrows indicate haemocyte infiltration, yellow circles indicate lipofuscin aggregates and blue circles indicate epithelial alterations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the toxic effects of this metal (Andrade et al., 2023). Our study showed PROP-ESA levels in the tissue increased with exposure concentration and time. In fact, at T1, the E1 and E2 concentration levels were 7.43 ng g^{-1} and 185.52 ng g^{-1}; at T2, 8.15 ng g^{-1} and 191.91 ng g^{-1}, respectively. From T1 to T2, both concentrations (E1, E2) show there is a significant difference ($p < 0.01$) compared to the control group (Table 2). A similar trend is seen for exposure to the neonicotinoid herbicide thiacloprid, which also shows a gradual increase in concentration with increasing exposure duration, from the lowest exposure concentration (4.5 μg.L^{-1}) to the highest concentration (450 μg.L^{-1}) (Stara et al., 2021).

It is known that herbicide propachlor is a stable compound with a half-life in an aqueous solution of up to 890 days (Liu et al., 2011) and a low K_{ow} (coefficient octanol-water) logP = 1.6 (PPDB, 2023). However, no information is available for PROP-ESA. Generally, the K_{ow} is useful for estimating the distribution of chemicals inside an organism. A low K_{ow}, like our case, is correlated with hydrophilic compounds, which are mainly found in aqueous regions rather than lipophilic comportment, such as lipids. Moreover, the low K_{ow} of its precursor and the levels of bioaccumulation and BCF that are determined, it can be presumed that PROP-ESA is poorly absorbed by aquatic organisms such as *M. galloprovincialis*. Although the amount of accumulated PROP-ESA in the mussel body is not enormous, this does not detract from the fact that the quantities detected in our study can mediate toxic effects in animals.

Table 5
Histopathological condition index I_h of *Mytilus galloprovincialis* exposed to 3.5 μg/L (E1) and 35 μg/L (E2) of PROP-ESA after 10 (T1) and 20 (T2) days.

Organs	Reaction pattern	Exposure time (days)	Experimental groups		
			Control (PROP-ESA free)	E1 (3.5 μg. L^{-1})	E2 (35 μg. L^{-1})
Gills	Cellular and morphological changes	T1 (10)	0.108 ± 0.012	0.158 ± 0.002	0.198 ± 0.002
		T2 (20)	0.118 ± 0.012	0.255 ± 0.007	0.297 ± 0.004
Digestive gland	Tubule alterations	T1 (10)	0.105 ± 0.007	0.156 ± 0.005	0.197 ± 0.003
		T2 (20)	0.117 ± 0.010	0.238 ± 0.002	0.266 ± 0.004
	Intertubular tissue changes	T1 (10)	0.095 ± 0.007	0.136 ± 0.004	0.186 ± 0.004
		T2 (20)	0.108 ± 0.011	0.218 ± 0.002	0.255 ± 0.007

The values are presented as the means ± S.D (n = 4).

The fact that there are no significant changes in the composition of the biochemical profile of the haemolymph is in correlation with insignificant cell viability results. Haemolymph, a fluid with a similar function to blood, is a crucial information carrier, allowing us to better understand the processes underlying cells in every day and stressful situations caused by pollutants. Therefore, cell viability assays are also useful and comprehensive tools for a better understanding of the health status of *M. galloprovincialis* (Faggio et al., 2016). In this case, exposure to PRO-ESA at two concentrations recorded no significant changes in overall viability after both T1 and T2. It might be referred to as some form of adaptation or resistance to the presence of this pollutant.

The composition of the haemolymph varies according to several parameters, including the water quality like pH, temperature, salinity, ion concentration and the presence or absence of xenobiotics (Capillo et al., 2018). The Ca^{2+}, Mg^{2+}, K^{+}, Cl^{-}, Na^{+} ions, and PHOS are essential components of haemolymph as they are involved in various functions, including metabolism, enzymatic functions, shell formation, osmoregulation, anabolic and catabolic processes and generally the maintenance of the animal's homeostasis. As an osmoregulatory organism, the mussel is able to maintain its internal osmolarity similar to that of the water in which it lives (Pagano et al., 2022a, b). The ability of this species to adapt or resist the presence of pollutants such as the herbicide metabolite PROP-ESA is confirmed by the non-significant changes in biochemical parameters.

The DG of *M. galloprovincialis* carries out the digestion of nutrients necessary for the body's survival. Furthermore, DG has an essential detoxification function. On the other hand, this organ has a solid predisposition for pollutant accumulation (Tresnakova et al., 2023; Curpan et al., 2022; Pagano et al., 2022a, b). Due to these reasons, cell viability tests are valuable for understanding the animal's health status (Impellitteri et al., 2022). Based on RVD methods, DG cell viability decreased significantly in E2, with a viability of 94% in TB and 95% in RN method after T1 compared with control and E1, with 99% for both groups with both colourings. This decrease in cell viability may indicate damage to the cell membrane. These results are comparable to others in the literature (e.g., Tresnakova et al., 2023; Pagano et al., 2022a, b; Stara et al. (2020a; 2020b); Messina et al., 2014), and they may highlight affection in physiological mechanisms essential for the organism's survival by chronic exposure to residual concentration in their natural environment. However, after T2, DG cell viability was not significantly affected. These results could indicate an adaptation of the cells and the whole organism to the chemical stress condition imposed. Indeed, mussels are extremely resilient organisms and can adapt to the adverse environmental conditions they live in (Lassoued et al., 2019; Nardi et al., 2017).

The detrimental interaction of PROP-ESA with DG cells is demonstrated not only by lysosomal accumulation and subsequent lysosomal instability, verified by the neutral red test but also by the inability of the cells to recover their cell volume upon exposure of the cells to a hypotonic solution. It shows that both the cytoskeleton and channel protein damage, as demonstrated by the RVD experiment, and damage to the cell membrane, as conducted by the TB exclusion test, occurred. Cell volume regulation for DG cells is necessary to maintain organ function (Barmo et al., 2013; Dimitriadis et al., 2004). In fact, under normal physiological conditions, in which the animal is not exposed to the pollutant, cells can respond to osmolar changes, restoring their cell volume. Therefore, the herbicides and their metabolites probably inhibit the cellular structures that regulate osmotic changes, creating damage. In fact, at both exposure times T1 and T2 (Fig. 1A and B) and both E1 and E2, the pollutant affected the DG cells' ability to regulate their cell volume. It shows a significant trend for DG cells from E2 group after T1 ($p < 0.05$) compared to the control. On the contrary, after T2, the DG cells did not show a significant change at both concentrations in relation to the control group. While the cells retained the ability to swell in the

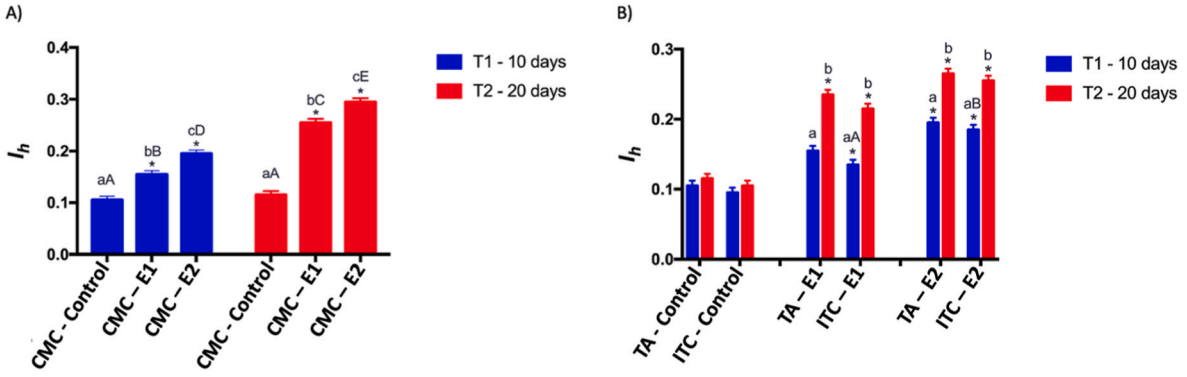

Fig. 6. Plotting the histopathological condition index values (I_h) of tissues of *Mytilus galloprovincialis* exposed to different concentrations of Propachlor ESA (**E1** and **E2**) for 10 and 20 days (**T1** and **T2**): gills (**A**) and digestive glands (**B**). All values are shown as mean ± S.D. (n = 4); asterisks represent significant differences between experimental groups and controls after the same time of exposure. Significant statistical differences are represented by letters ($p < 0.05$ – two-way ANOVA test/ 'Tukey's multiple comparisons test). Different small letters indicate significant differences between the two xenobiotic concentrations tested at the same time point (T1 or T2). Different capital letters represent significant differences between the same PROP-ESA concentration after different exposure time. **E1** = 3.5 μg/L; **E2** = 35 μg/L; **CMC** = cellular and morphological changes; **TA** = tubular alteration; **ITC** = intertubular tissue changes.

presence of the administered hypotonic solution, they could not restore their initial condition. These modifications imply that the damage involves cytoskeletal elements and K^+ and Cl^- ion transport systems regularly implicated in cell volume-regulating activities (Torre et al., 2013; Silva and Wright, 1994). This inability to return to initial conditions, considered optimal by the organism, is mainly evident only after T1. By prolonging the exposure period, the data obtained support the thesis that mussels can adapt somehow to the mediated poisonous effects.

Data about the influence of PROP-ESA on the oxidative status of aquatic animals are lacking. A modulation of pro-oxidant and antioxidant markers has been reported in response to other members of chloroacetanilides, although only in very few species of cyprinid fish. For example, exposure to butachlor-induced LPO and antioxidant enzyme activity in liver, kidneys, and gills of *Cyprinus carpio* (Tramboo et al., 2010), while alachlor affected the hepatic antioxidant system of *Carassius auratus* (Yi et al., 2007). Our study is the first to show a direct effect of PROP-ESA on the oxidative status of the aquatic model *M. galloprovincialis*. Specifically, we observed that, after T1, the higher concentration of PROP-ESA tested increased LPO in both gills and DG; after T2, and only at the lower concentration used, in the DG. Also, protein carbonylation results were affected by PROP-ESA, but only in gills; high levels of ketonic derivatives were observed in each condition tested, while aldehydic derivatives increased only after T1 in E2.

The introduction of carbonyl groups (i.e., reactive aldehydes and ketones) into proteins may occur by oxidation of amino acid side chains or through oxidative cleavage of proteins by α-amidation pathway or by oxidation of glutamyl side chains, thus leading to the formation of ketonic derivatives. Also, the conjugation with aldehydes, produced during lipid peroxidation, and with reactive carbonyl derivatives, generated by reaction with reducing carbohydrates, can cause protein carbonylation (Møller et al., 2011). The results of our study show that PROP-ESA may activate, in a tissue-specific manner, different intracellular oxidative pathways of protein carbonylation. The linear relation between aldehydic derivatives and lipid peroxidation observed in gills but not in DG may sustain this hypothesis.

SOD enzyme, by converting the superoxide anion ($O_2^{\bullet -}$) to H_2O_2, represents a first line of defence against oxygen radicals. Exposure to pesticides has been associated with oxidative stress induction and a different modulation of cellular antioxidant systems, the last sometimes resulting inhibited (Sule et al., 2022). After T2, we observed a significant increase in SOD activity only in the gills of animals treated with PROP-ESA at both concentrations. A different tissue activation of the SOD enzyme in response to pollutants is not uncommon in Mytilus. In particular, in *M. galloprovincialis*, exposure to the herbicide glyphosate affected SOD activity in a tissue-specific manner, with a reduction in the DG and no effect on gills (Matozzo et al., 2018). The result from our study suggests that gills are susceptible to oxidative damage; likely, the increase in SOD activity we observed may represent a mechanism of defence against protracted ROS production.

Relying on the histopathological evaluations performed, the most frequently documented reaction patterns, such as haemocyte infiltrations, epithelial alterations, and lipofuscin aggregates, suggest an infiltrative inflammation as a response to PROP-ESA exposure (De Vico and Carella, 2012). The target organs for histological analysis were chosen based on their role in performing vital functions for bivalves, like feeding and breathing in the case of gills (Stara et al. (2020a; 2020b; Azevedo et al., 2015; Canesi et al., 2012) or metabolic regulation and xenobiotics detoxification, mainly operated by the digestive gland (Pagano et al., 2016; Gomes et al., 2012; Koehler et al., 2008). From a functional point of view, gills represent the first interface with the aquatic environment, while the DG could be compared to the liver of vertebrates (Tresnakova et al., 2023). For all these reasons, gills and DG are considered valuables sentinel tissues to determine the response to an inflammation using the histopathological condition index (Costa et al., 2013). Considering the histological responses observed in mussel tissues analysed, the presence of lipofuscin deposits confirmed the course of an inflammatory process, unequivocal signs of the immune activity of the "pro-phenoloxidase (PO) activating chain systems" mainly involved in phenomena like foreign matter recognition and encapsulation (Stara et al., 2021; De Vico and Carella, 2012). Even the widely documented haemocyte infiltration was related to the inflammation, resulting from a growing haemolymph flow necessary for defensive cells to reach the site of inflammation and then penetrate the epithelium through diapedesis (Pagano et al., 2016). As mentioned in the results section, these two reaction patterns, mainly noted in the gill tissues, showed an increasing trend related to the concentration of the xenobiotic and the time of exposure. Other frequent gill aberrations ascribable to exposure to propachlor were lamellar alterations, vacuolation and epithelial alterations that seriously threaten the survival of bivalves, compromising the breathing and feeding function (Pagano et al., 2016; Carballeira et al., 2011; Auffret, 1988). Regarding the DG tissues analysed, the less severe extent of lesions documented was probably due to the detoxification as mentioned above function of this organ, as previously reported in the literature (Tresnakova et al., 2023).

5. Conclusion

This study is the first to highlight the long-term effects of PROP-ESA on a model organism *M. galloprovincialis*, mostly related to time- and dose-dependent trends. This study revealed links between the organism's biochemical and histological response and chemical stress caused by PROP-ESA-contaminated water. Mainly considerable awareness among the results is the ability of PROP-ESA bioaccumulation in edible mussel tissue. These findings are important for the shellfish farms run on natural waters, especially considering Bivalve species as an essential food chain link. Furthermore, the excessive and repeated application of chloroacetanilide pesticides in agriculture, hence their metabolites, is necessary to reach higher crop production for increasing population growth; the importance of screening their potential risks on non-target aquatic organisms is still challenging. Therefore, these data are valuable for regulatory agencies, providing a toxicity database of environmental pollutants and highlighting future research needs.

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Author contribution

NT: Conceptualization, Methodology, Investigation, Data Curation, Writing-Original draft preparation; FI: Conceptualization, Methodology; SF: Formal analysis, Investigation, Writing-Original draft preparation; MP: Methodology, Investigation, Data Curation, Writing-Original draft preparation; MC: Investigation, Formal analysis, Writing-Review & Editing; AC: Investigation, Formal analysis, Writing-Review & Editing; SS and CD: Investigation, Writing-Review & Editing; SI: Resources, Writing-Review & Editing; AA: Investigation, Writing-Review & Editing; IV: Investigation; AS: Conceptualization, Writing-Review & Editing; GDB: Resources, Writing-Review & Editing; JV: Conceptualization, Resources, Project administration, Writing-Review & Editing; CC: Resources, Supervision, Project administration, Writing-Review & Editing. All authors read and approved the final manuscript.

Ethical approval

This study did not require ethical approval for human ethics. Any permission for this experiment is not needed. However, all the methods for sampling and protocols used in the present study followed relevant guidelines and regulations: EU Directive 2010/63/EU for animal experiments and the recommendations in the ARRIVE guidelines.

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.

Data availability

Data will be made available on request.

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