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Original scientific paper

Oxidative stress response as biomarker of exposure of a freshwater invertebrate model organism (*Unio mancus* Lamarck, 1819) to antifouling copper pyrithione

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SUMMARY

Sublethal effects of copper pyrithione (CuPT) on an invertebrate model organism, freshwater mussel (*Unio mancus*), were assessed using oxidative stress parameters and histopathology. The environmentally relevant concentration of 5 µg/l copper pyrithione was applied as a 96 h semi-static exposure. MDA levels in digestive gland (DG) significantly increased, compared to a control group ($p < 0.001$). Gill MDA levels also increased, but not significantly ($p > 0.05$). GSH level in DG increased significantly ($p < 0.05$), and gill tissue levels also increased but not significantly ($p > 0.05$). Levels of SOD, GPx activities, and AOPP levels did not change significantly ($p > 0.05$). The most prominent histopathological alterations in the gills were haemocyte infiltration, lipofuscin aggregates and lamellar deformations with tubule degeneration and accumulated lipofuscin aggregates in DG tubules. CuPT impacts antioxidant defense systems even during sublethal exposure of *Unio mancus* as the freshwater model organism.

Keywords: biocides, antifouling copper pyrithione, biomarkers, oxidative stress, freshwater mussel

INTRODUCTION

Freshwater ecosystems are under pressure of continuous water quality and biodiversity decline, impacting global human and animal populations with health risks, augmented by agriculture, forestry and modern technical developments. Comprehensive biomonitoring programs using biomarkers for changes in biological responses enable early warning of human, animal and ecosystem health risks. They extend to risk assessment and ecotoxicology (Guidi et al., 2017).

Aquatic organisms accumulate on submerged external surfaces of marine structures, such as boat hulls, and cause biofouling. Extra cleaning and maintenance expenditure, higher fuel consumption, reduced manoeuvrability and increased frictional drag on boats are other detriments enhancing the impact. Furthermore, the affected surface may pose food safety risks (Muller-Karanassos et al., 2019; Nogueira et al., 2018). Marine biofouling not only damages boat surfaces, but also increases carbon dioxide emission while reducing boat performance. Antifouling paints (coatings) containing biocides are used to prevent this significant marine concern. (Martins et al., 2018). Tributyltin (TBT) and its derivatives were effective antifouling paints during the 1970s, with annual global production of around 4000 tons, providing protection for up to five years. TBTs have been considered advantageous due to their long-term protection effect (Arai et al., 2009). However, their long half-life caused accumulation in sediments and aquatic organisms, including non-target aquatic organisms even at low concentrations. Furthermore, biomagnification in the food web at increasing levels raise concern. Representative marine products at different trophic levels in Danish coastal waters were shown to accumulate in the food web. Levels were determined in flounder (60-259 ng g^{-1}, w/w), eider duck (12-202 ng g^{-1}, w/w), and harbour porpoise (134-2283 ng g^{-1}, w/w) (Strand & Jacobsen, 2005). Shell malformations were reported in larvae of *Crassostrea gigas* found in the Gulf of Arcachon, France (Arai et al., 2009). In 1990, tin-free antifouling paints were developed as alternatives. The IMO (International Maritime Organization) recommended that countries ban the use of TBT-based antifouling paints for vessels shorter than 25 m due to serious environmental impact. Some organisms exposed to TBT showed imposed imposex and the USA, Canada, Sweden and The Netherlands have imposed restrictions on TBT release from ship surface (Okay, 2004; Gittens et al., 2013).

Alternative antifouling compounds, such as diuron, irgarol, zinc pyrithione (ZnPT, presently used also in antidandruff shampoos as antimicrobial) and copper pyrithione (CuPT) are proposed chemicals developed for use as "booster" biocides in Cu-based antifouling paints. Pyrithione salts, ZnPT and CuPT, were commercialised together during the 1990s. Environmental pollution by direct leaching from hull and paint particle discharges during maintenance and cleaning has raised concern since both biocides are usually present as mixtures in the marine environment. Some had the potential to cause harmful effects on many non-target organisms, algal growth and photosynthesis (Dupraz et al., 2018), energy production, biological response to genomic activities, such as stress responses, genotoxic damage, immune-supressed protein expression, oxidation and nerve conduction (Qian et al., 2013). Their apolar properties and high degradability have made them choice biocides as replacements for TBT (Martins et al., 2018). Copper pyrithione, registered for antifouling use, was approved by the European Union in 2015 as an active ingredient in anti-degradation products, as part of its program for evaluation of active substances in biocidal products.

Copper pyrithione, a Cu(I) used as the main biocide in antifouling paints, is a highly effective broad-spectrum biocide, but it also has adverse effects on several aquatic organisms. CuPT degrades rapidly by photolysis in seawater/sediment systems. Significant metabolites (pyrithione sulfonic acid [OMSA] and 2-pyridine sulfonic acid [PSA]) are produced via aerobic degradation (Mochida et al., 2008). CuPT is very toxic to aquatic invertebrates at the µg/l level. CuPT 96 h EC_{50} was 11 µg/l and 96 h NOEC growth was 6.9 µg/l to the marine eastern oyster (*C. virginica*). Tresnakova et al. (2020) reported sublethal toxicities of CuPT and ZnPT alone (10 µg/l) and in combination (5 µg/l) to the freshwater mussel *Unio crassus*. CuPT toxicity to the copepod *Tigriopus japonicus* (24 h LC_{50} = 41 µg/l) was higher than ZnPT (24 h LC_{50} > 500 µg/l) (Shipbuilding Research Association of Japan, 2002, cited in Yamada, 2006). Another early life stage embryotoxicity and embryo mortality report was made for sea urchins (Wang et al., 2011; Xu et al., 2011). An EC_{50} value > 100 mg/l was determined for *Paracentrotus lividus* embryotoxicity, where this exposure concentration was not very toxic to early embryos (2-cell stage) but inhibited the pluteus stage of larval development (EC_{50} = 0.011 mg/l) (Gutner-Hoch et al., 2019).

Mochida et al. (2006) reported that CuPT was more toxic than ZnPT to the fish *Pagrus major* and toy shrimp *Heptacarpus futilirostris* (LC_{50} values of CuPT and ZnPT: 9.3 and 98.2 µg/l for *P. major*, and 2.5 and 2.5 µg/l for *H. futilirostris*, 96 h, respectively). Skeletal muscle atrophy

and peripheral nerve damage are the result of repeated exposures to high concentrations (Arch Chemicals, 2008). In addition, potential endocrine disrupting effects have raised concerns over mutagenicity and impairment of endocrine signalling pathways (Cui et al. 2014).

Globally the aquaculture industry is a good example of sustainable ecosystem services, providing a healthy protein source in seafood, where bivalves are of economic importance. Bivalve molluscs, both marine (*Mytilus*) and freshwater (*Unio*, *Anodonta*), respond to rapid changes in their environment, such as pollutant load, habitat salinity, temperature (e.g. climate change stressors), pH or pollutant mixtures, and therefore they serve as bioindicator species for biomonitoring (Kholodkevich et al., 2019).

Today bivalves also have another unprecedented role as invertebrate model organisms for research in chronobiology, neuroendocrinology, bacterial endosymbiosis, innate immunity, biomineralization, aging and various biotechnological applications, as well as for environmental health monitoring (Robledo et al., 2019). Mussels are abundant in seas; they accumulate metals in high concentrations and keep them in their bodies for a long time and are one of the biological indicators that reflect pollution in water. *Unio mancus* is an indicator species physiologically suitable for ecotoxicological studies. It is the first species reported to disappear after exposure to pollution stress. The *Unio* genus has the advantage of being sensitive to even low levels of environmental pollutants, have a long life, are widely distributed, and large enough for providing tissues for analyses (Van Hassel & Farris, 2007).

Biochemical, molecular and physiological biomarkers are reliable integral responses of model/indicator organisms both for whole organism level changes in the environment and state of the habitat (ecological status) of aquatic organisms and lower organisational levels under the impact of environmental stressors (Newton & Cope, 2006). Metabolic and physiological monitoring provides invaluable information about prolonged thermal stress depleting energy sources (Ganser et al., 2015) and adjustments in filtration rates (Ferreira-Rodriguez, 2019).

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are enzymatic defense line antioxidants. When oxidative stress persists, enzyme inactivation, lipid peroxidation, DNA damage and even cell death are unavoidable. Reactive oxygen species (ROS) production is also caused by the loss of mitochondrial integrity, damage to critical cellular macromolecules and activation of programmed cell death (Wang et al., 2018). Aerobic life forms are under a threat of ROS targeting lipids where cellular level membrane lipid peroxidation damage is measured by malondialdehyde (MDA, a secondary product) (De Las Heras et al. 2018). Parameters used as biomarkers of ROS and defense systems are widely employed in life sciences and ecotoxicological research.

There is limited toxicity data on antifoulants, and more specifically on CuPT effects on non-target freshwater organisms. Pollution biomonitoring is mostly limited to crustaceans, which are the most affected benthic organisms. Marine coastal ecosystems depend on benthic organisms for energy transfer between pelagic and benthic ecosystems. Biocidal metals, Cu and Zn, were shown to accumulate in experiments with non-target organisms after exposure to antifouling biocides (Muller-Karanassos et al., 2019). No studies have been conducted to investigate ecotoxicological effects of the antifouling compound copper pyrithione on freshwater mussels. This study aimed to test the hypothesis that CuPT toxicity to the non-target model organism *Unio mancus* acts through multiple mechanisms with diverse biomarkers involved, such as those of oxidative stress (MDA, GSH, SOD, GPx, AOPP) in gill and digestive gland (DG) tissues. In addition, total hemocyte counts and histopathological effects were assessed.

MATERIALS AND METHODS

Freshwater mussels and exposure experiments

Freshwater mussels (Mollusca: Bivalvia: Unionidae), *Unio mancus*, Lamarck 1819 (Bourguignat, 1860) were collected manually from shallow locations of Karasu Irmağı (Karasu River), Sinop, NE Turkey. Mussels were brought to the Ecotoxicology Laboratory of the Biology Education Department, Gazi Faculty of Education, in containers providing cool and humid environment where direct contact with daylight was avoided. The specimens were acclimatized for at least two weeks in aerated glass aquaria. In this process, the mussels were fed on freshwater algae. Fifteen mussels were distributed to each 20 l glass aerated aquarium with 15 l of water. The mean total weight and length of the control (24.83 ± 7.50 g; 59.37 ± 5.37 cm) and experimental groups (25.90 ± 8.91 g; 59.74 ± 0.06 cm) of freshwater mussels were measured. Water quality parameters were: temperature 19.2 ± 0.14°C, conductivity 249.1 µS/cm, pH 8.12 ± 0.2, dissolved oxygen (DO) 6.30 mg/l.

In preliminary dose-range finding experiments (1, 2, 4, 8, 10, 12 and 50 µg/l, 100% mortality was recorded for the highest concentration) copper pyrithione (CuPT) (Arch Chemicals, UK, purity: 95-100%; CAS: 14915-37-8;

molecular weight: 315.86 g/mol) was weighed and a stock solution was prepared by dissolving the amount in a specified volume of DMSO, and dosing solutions were diluted from this stock using DMSO. Exposure concentrations were based on environmentally relevant concentrations in available literature (Almond & Trombetta, 2016). All chemicals, solutions and aquaria were covered with aluminium foil to avoid photodegradation. In a sublethal experiment, 15 specimens were exposed to 5 µg/l CuPT (in DMSO) in 20 l volume aquaria. Two control 20 l aquaria (DMSO and negative control) contained 15 specimens each. Sublethal exposure duration was 96 h. The bioassay system was constructed according to the OECD (1993) and national legislation (Turkish Official Gazette, 1991).

Total haemocyte counting

Hemolymph was obtained from each mussel from the anterior adductor muscle using disposable syringe with 1:1 dilution with 4% formaldehyde. Haemolymph total hemocyte counts (THCs/ml) were estimated by a modified hemocytometer method of Sepici-Dinçel et al. (2013).

Tissue analysis

After hemolymph collection, mussels were dissected and tissues (gill and DG) of 10 specimens were reserved for biochemistry. The tissues were wrapped in aluminium foil before immediate freezing in liquid nitrogen and storage at -80°C.

Oxidative stress biomarker analyses (MDA, GSH, AOPP, SOD, GPX)

Dissected, frozen tissues were thawed on ice and about 100 mg was weighed and homogenized using 900 µl trichloroacetic acid (TCA, ice-cold 10%, 1:10, w:v). Homogenate supernatants were kept on ice or stored at -20°C until analysis.

Lipid peroxidation levels (MDA analysis) were measured as TBARS equivalents by slightly modified methods of Casini et al. (1986), Kurtel et al. (1992) and Yildirim et al. (2011). To 250 µl supernatant, 10 µl 1% BHT (butylated hydroxytoluene) was added, and 250 µl 0.67% TBA (thiobarbituric acid). The mixture was boiled at 100°C for 15 min, and absorbance measured at 535 nm. Molar extinction coefficient of $\varepsilon_{535} = 1.56 \times 10^5\ M^{-1}cm^{-1}$ was used to calculate TBARS equivalents (nmole/g tissue).

Reduced glutathione levels (GSH). A slightly modified method of Parihar et al. (1997)

Kurtel et al. (1992), Yildirim et al. (2011) and Flohe & Günzler (1984) was used. Supernatant in the amount of 100 µl was mixed into 400 µl 0.3 M Na_2HPO_4 (disodium hydrogen phosphate) and 50 µl DTNB (Ellman's reagent [1959]; 5,5′-dithiobis[2-nitrobenzoic acid]), incubated at room temperature for 10 min and absorbance was measured at 412 nm. Reduced GSH values were calculated as µmole/g tissue, assuming a molar extinction coefficient of $\varepsilon_{412} = 14.150 \times 10^5\ M^{-1}cm^{-1}$.

AOPP levels: Ice-cold Tris-HCl buffer, 20 mM, pH=7.4, was used as dilution medium (100 mg gill and DG tissue sample +400 µl buffer, 1:5, w:v) for tissue homogenization and the homogenate centrifuged at 5.000*xg* for 10 min. Supernatant was analyzed at 340 nm for AOPP levels using the spectrophotometric method of Witko-Sarsat et al. (1996) (Biochrom Libra S22® UV-VIS spectrophotometer) in the presence of potassium iodide. When necessary the supernatant was diluted 1:3 with PBS and 200 µl of sample was analyzed, calibrated with chloramine-T solution. AOPP levels were expressed in micromoles chloramine-T equivalents per liter. The values are expressed as nmole/mg protein.

SOD enzyme assay: The principle of the assay is based on tissue superoxide degradation by SOD enzyme and the production of O_2^- with xanthine oxidase. After the reaction with nitroblue tetrazolium (NBT), color change due to the end product was determined spectrophotometrically (Sun et al., 1988). Tissue samples, 50 mg of DG and gill each, were weighed and diluted 1:5 (w:v) with 20 mM HEPES (pH=7.2, containing 1 mM EDTA, 210 mM mannitol, 70 mM sucrose) before homogenization. Homogenates were centrifuged at 1.500*xg* for 5 min at +4°C. Tissue SOD activity (U/mg protein) was calculated using the Cayman SOD assay kit at 440-460 nm following manufacturer's instructions.

GPx enzyme assay: Cumene hydroperoxide is formed by GPx activity after oxidation of GSH; glutathione reductase (GR) and NADPH reduce the oxidized glutathione to reduced GSH. $NADP^+$ is the oxidized product of the reaction (Paglia & Valentine, 1967). Tissue samples, 50 mg of DG and gill each, were weighed and diluted 1:5 (w:v) with 50 mM Tris-HCl (pH=7.5, containing 5 mM EDTA and 1 mM DTT) before homogenization. Homogenates were centrifuged at 10.000*xg* for 15 min at +4°C. Tissue GPx activities (nmole/min/mg protein) were calculated using the Cayman GPx assay kit at 340 nm following manufacturer's instructions. Bradford's micromethod version was used for tissue protein level measurements

using bovine serum albumin (BSA) as the standard (Bradford, 1976). Absorbance measurements were made using the Molecular Devices VERSAmax tunable microplate reader®.

Histopathological analysis

Following ice anesthesia, a complete necropsy of the Unionid mussels was performed and tissues (digestive gland, mantle, intestine, nephridium, gill, gonads) transferred to prelabelled histology cassettes and stabilized into invertebrate Davidson's fixative (composed of 115 ml of acetic acid (glacial), 220 ml of formalin, 330 ml of 95% ethyl alcohol and 335 ml distilled water) for 48 hours. Afterwards, histological cassettes were embedded in 70% ethanol. Routine histological protocols were processed (dehydration in ethanol series, clearing with xylene, embedded in paraffin, sectioned with microtome, stained via H & E). Preparations were visualized under light microscope.

Statistical analysis

The exposure group mean values of MDA, GSH, AOPP, SOD and GPx were tested for homogeneity of variances and normal distribution. If these conditions were met, then parametric t-test and one-way ANOVA were used for comparison between groups. Otherwise, the non-parametric counterparts Mann-Whitney U and Kruskal Wallis-H tests were used for statistical analyses. The significance level was taken as 0.05.

RESULTS

Total hemocyte counts (THCs)

The mean total hemocyte counts of freshwater mussel *Unio mancus* exposed for 96 h to sublethal CuPT were 179667 ± 20552 THCs/ml and 139667 ± 10165 in the controls. The slightly higher cell numbers in the experimental group were not significantly different from the control group ($p > 0.05$).

Lipid peroxidation levels (malondialdehyde levels, MDA, as TBARS equivalents) of DG and gill tissues

Lipid peroxidation and oxidative stress biomarker malondialdehyde (MDA) levels were measured in freshwater mussel DG and gill tissues exposed to sublethal copper pyrithione for 96 h. The mean experimental group DG level increased almost five-fold that of the control group, and the increase was strongly significant ($p < 0.001$). Gill MDA levels did not differ significantly between groups ($p < 0.05$) (Figure 1).

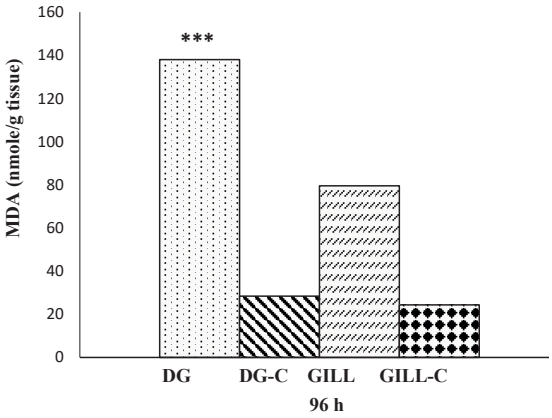

Figure 1. MDA activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 µg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: GILL tissue, GILL-C: Gill-Control tissue, 96 h. *** $p < 0.001$

Tissue reduced glutathione levels (GSH)

Mean reduced glutathione (GSH) levels showed a similar trend with MDA levels in gill and DG tissues of mussels after 96 h exposure to sublethal CuPT. Experimental groups mean GSH levels were almost 2.5-fold that of the control group, and the increase was significant ($p < 0.05$). In the gill tissue experimental group, GSH level increased slightly but not significant ($p > 0.05$) (Figure 2).

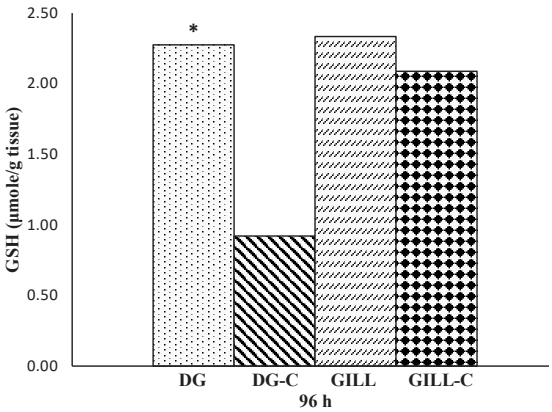

Figure 2. GSH activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 µg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: Gill tissue, GILL-C: Gill-Control tissue, 96 h. * $p < 0.05$

Tissue AOPP levels

Mean AOPP levels were determined in DG and gill tissues of organisms exposed to sublethal CuPT. AOPP levels decreased in both tissues compared to their respective controls, however this effect was not significant ($p > 0.05$) (Figure 3).

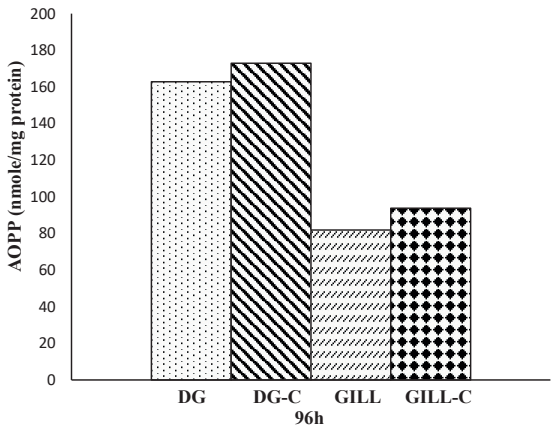

Figure 3. AOPP activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 μg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: Gill tissue, GILL-C: Gill-Control tissue, 96 h.

Tissue superoxide dismutase (SOD) activity

Mean SOD activities in both tissues increased in the experimental groups but the increase was not significant ($p > 0.05$) (Figure 4).

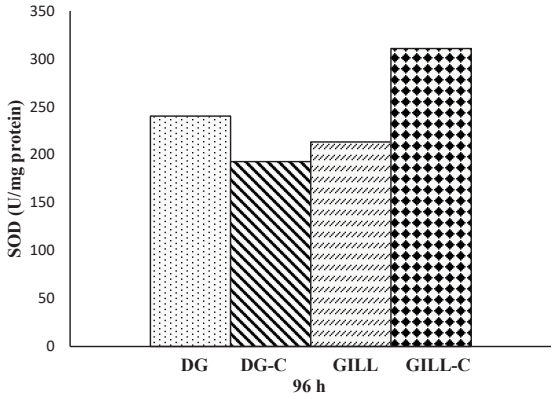

Figure 4. SOD activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 μg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: Gill tissue, GILL-C: Gill-Control tissue, 96 h.

Tissue glutathione peroxidase (GPx) activity

Mean GPx activity in DG decreased in the experimental group, whereas gill level increased when compared with the control group ($p > 0.05$) (Figure 5).

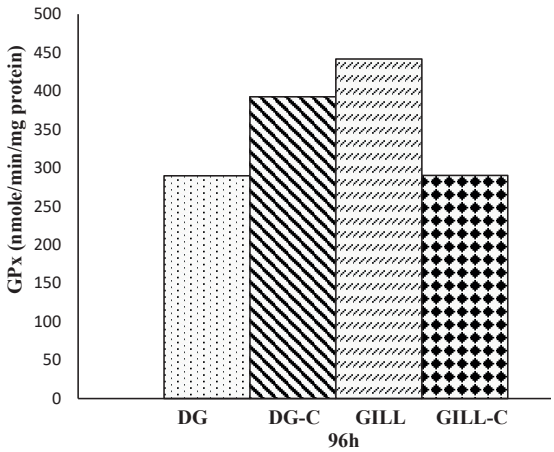

Figure 5. GPx activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 μg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: Gill tissue, GILL-C: Gill-Control tissue, 96 h.

Histological findings

Unionid mussel tissues of the mantle, intestine, nephridium and gonads did not reveal significant adverse effects when exposed to sublethal antifouling copper pyrithione for 96 h. Histopathology is generally the gold standard for evaluating toxic effects at tissue level in toxicology, as well as in ecotoxicology. The gill filaments of control mussels exhibited no pathological changes (Figure 6a). In the control group, gills showed well-preserved lamellae composed of a single layer of epithelial cells and tight hemolymphatic sinus. Compared to control, exposure to copper pyrithione led to mild histopathological alterations in the gills, such as haemocyte infiltration, lipofuscin aggregates and lamellar deformations (shorthening) (Figure 6b). The DG of control Unionids showed a normal structure with digestive tubules composed of epithelial cells (Figure 7a-b). Sublethal copper pyrithione exposed mussels exhibited mild histological change in the structure of DG and accumulated lipofuscin aggregates (Figure 7c-d).

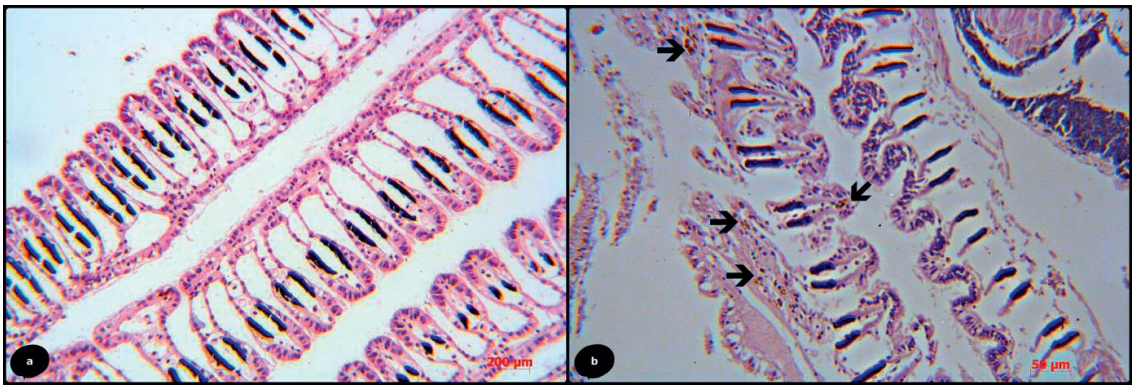

Figure 6. Histologic illustration of the gills of *Unio mancus* exposed to copper pyrithione (H&E). (a) Control mussel gills with normal structure; (b) Lamellar deformation of gill epithelium (shorthening) and lipofuscin aggregates (arrows).

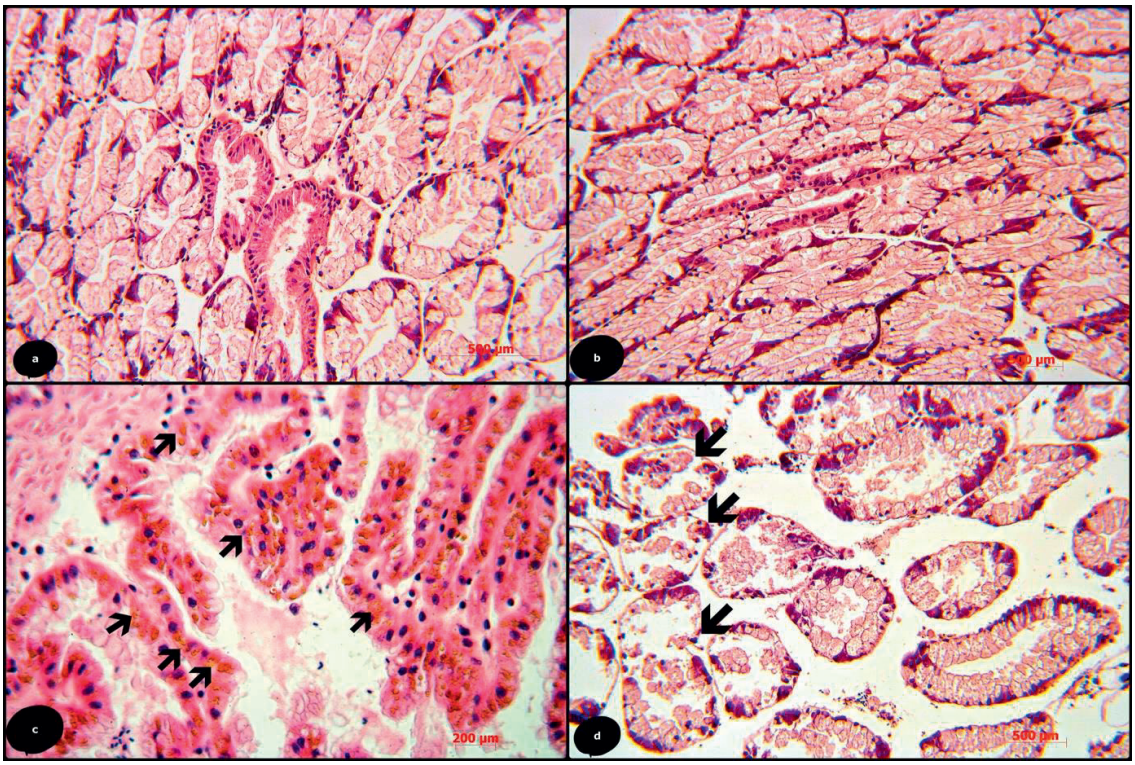

Figure 7. Histologic illustration of the DG of *Unio mancus* exposed to copper pyrithione (H&E). (a-b) Control mussel digestive gland with typical structure; (c) Lipofuscin aggregates; (d) Digestive tubule degeneration (arrows).

DISCUSSION

Contamination of freshwater and marine ecosystems with heavy metals, organic pollutants, pollutants of emerging concern, persistent pollutants and biocidal substances is a major concern worldwide. Macroinvertebrates are widely accepted indicator species in ecotoxicology studies, representing impacts due to biotic and abiotic stressors using a series of parameters for biochemical, physiological, survival, feeding and reproduction activities. Over 95% of all animal species are invertebrates and they have invaluable ecological roles in the lower levels of the food web. Bioindicator mussels have been used for pollution biomonitoring since the early 1950s and their use has increased since then (Van Hassel & Farris, 2007).

Unio mancus mussels were exposed to sublethal CuPT for 96 hours and morphometric, hematological and biochemical parameters were studied. CuPT did not affect weight, length, height or thickness of mussels ($p > 0.05$). Similarly, total hemocyte counts increased after exposure but the change was not significant ($p > 0.05$). Tresnakova et al. (2020) reported significant increase in THCs after 48 h exposure of *Unio crassus* to CuPT and its combination with ZnPT but a significant decrease followed after 7 days.

The micronutrient Cu is essential both for aquatic plants and animals, but may pose toxicity risk at high concentrations, caused by heavy boat traffic, maritime activity and limited water exchange rate regions. Risk is mainly due to intensive use of Cu-based (Cu alone or Cu in combination with booster biocide) antifouling paints (Schiff et al., 2007). Cu as a cofactor and redox component of cellular metabolism may be toxic above optimum levels to aquatic life, including microalgae. In photosynthesis, the mechanism of toxic action (MoA) of Cu is thought to inhibit electron transport of the PSII (Dupraz et al., 2018), decreasing its efficiency. ZnPT and CuPT may both impact cell membrane integrity and membrane transport, and inhibit ATP synthesis. A further impact of CuPT could be through mitochondrial oxidative metabolism and even mediating the apoptotic process through ROS formation and oxidative stress (Gutner-Hoch et al. 2019).

The bioavailable form of copper is the dissolved ionic form Cu^{2+}. Elevated concentrations of Cu in sediments and surface waters of California, USA (Schiff et al. 2007) and Mexico (Jonathan et al. 2011) were attributed to Cu-containing antifouling paints. Cu exhibits higher toxicity in mixtures, compared with pure solutions, due to synergistic interactions.

Antifouling paint particles (APPs) of between 500 mm and > 2 mm in diameter have been identified in silty, intertidal estuarine sediments, in guts of sediment-dwelling ragworm, *Hediste diversicolor*. The ragworm tissues showed high Cu residues (Muller-Karanassos et al. 2019). These sites have high APPs loads due to abandoned boats or boating activities. Epibenthic harpaticoid) *Nitokra* sp. was also studied for APPs residues in sediments and decreased fecundity was reported as potential risk (Soroldoni et al., 2017).

Another study examined the effects of TBT and benzo[a]pyrene (BaP) on the gastropod *Haliotis diversicolor* (abalone) immune system, and long-term chronic exposure to sublethal BaP concentration was observed to modulate immunocompetance as a change in the activity of immune-related parameters. The immunotoxicity biomarkers THC, phagocytosis, membrane stability and lysosome activity were significantly reduced after 21 d of sublethal TBT exposure. After 21 d, 14 d BaP-exposed gastropod parameters recovered. Fourteen days of improvement in TBT-exposed organisms showed no variation. In this case, TBT is thought to be more harmful than BaP (Gopalakrishnan et al., 2011). CuPT is an alternative to the banned TBT, but it also shows non-target species toxicity. In our study, an increase in hemocyte count was observed as a result of exposure to copper pyrithione. This difference can be explained by the different functioning of the immune system. Hemocyanin is the blood pigment in mussels, and the hemoglobin of higher organisms in the hierarchy of species' has iron. In our study, iron binding as the element can be assumed as effective in increasing hemocyte counts. In addition, the use of different model organisms in experiments with CuPT, which is considered as toxic as TBT, suggests that the duration of exposure is significant and that some chemicals may have different effects on each animal species. In fact, the improvement of immune parameters in BaP treatments supports this view.

Ecotoxicity of CuPT and HPT (2-mercaptopyridine N oxide, degradation product) to the brine shrimp, *Artemia salina* was studied together with Cu^{2+} using natural seawater and organic matter-free artificial seawater as exposure media (Lavtizar et al. 2018). Artificial seawater media, with the highest salinity and no organic matter content, caused the highest CuPT toxicity (48 h EC_{50} = 250 µg/l). The corresponding natural seawater EC_{50} value was 556 µg/l, showing less toxicity; organic matter content and salinity decrased toxicity. The marine crustacean *Tigriopus japonicus* was more sensitive than *Artemia salina* to CuPT exposure, and the 24 h EC_{50} = 23 µg/l showed CuPT to be highly toxic (Onduka et al. 2010). Koutsaftis and Aoyama (2007) reported 24 h EC_{50} of 830 µg/l for *Artemia salina* exposed to CuPT in standard artificial seawater, which is highly toxic. Also in agreement were the results reported by Mochida et al. (2006), 96 h LC_{50} = 2.5 µg/l for *Heptocarpus futilirostris* (toy shrimp). CuPT 24 h LC_{50} values for *Tigriopus japonicus* (copepod) (and for the rotifer *Brachionus koreanus*) were in µg/l order of magnitude and highly temperature-dependent (Li et al., 2014). Although nauplii are tolerant to marine toxins to a small extent, 24 h LC_{50} for *Balanus amphitrite* barnacle nauplii exposed to CuPT were in the 4.0-6.1 µg/l range and imposex was recorded in sites heavily polluted by PAH and other endocrine disruptors (Romano et al. 2010). Our results showing high toxicity of CuPT to non-target freshwater

mussels are in agreement with other studies and confirm them (Koutsaftis & Ayoma, 2007; Koutsaftis & Ayoma, 2008; Onduka et al., 2010; Romano et al., 2010; Bao et al., 2011; Li et al., 2014; Lavtizar et al., 2018; Tresnakova et al., 2020).

Biomarker-based results showed prooxidant activity, where catalase and cholinesterase activities increased significantly. Overload of ROS together with weakened antioxidant capacity predispose aquatic species to oxidative stress. Malondialdehyde (MDA) levels of DG increased dramatically with respect to controls, showing lipid peroxidation and tissue unable to cope with oxidative stress caused by CuPT after 96 h exposure, leading us to consider that oxidative stress may contribute to CuPT toxicity in *Unio*. In agreement with results of other researchers, our MDA levels increased in DG and gills of *Mytilus edulis* (mussel) and *Crassostrea gigas* (oyster) when exposed to heavy metals, silver, cadmium, copper and zinc for 21 days (Geret et al. 2002). However, no differences were reported in MDA levels of *Unio crassus* neither with CuPT alone nor in combination with ZnPT.

Flakes of CuPT and ZnPT, which are directly released and reach bottom sediments in a way similar to TBT accumulation in the sediment, have a relatively short half-life of just several months in water bodies but can last for years bound in anaerobic sediment at ppm levels (Warford et al. 2022). Risk due to long-term effects of TBTs is higher and raises concern specifically in estuarine areas, necessitating comprehensive ecotoxicity assessment of sediment organisms such as Polychaete species for pyrithiones that are marketed as alternatives to TBTs. ZnPT has been reported to be toxic to the polychaete *Hediste diversicolor* (Nunes & Costa, 2019. Parameters of oxidative stress (catalase, glutathione S-transferases (GSTs), and lipid peroxidation (GPx), total proteins and neurotoxicity (acetylcholinesterase) were studied after 96 h acute exposure to a 10-160 µg/l dose range. TBARS levels did not change, while AChE decreased and catalase increased, leading the authors to conclude that no clear toxicity mechanisms have been elucidated for ZnPT so far. Evidently, toxicological data are sparse, and more than one MoA may be effective (Nunes & Costa, 2019). As a cofactor for a number of enzymes, Cu^{2+} participates in oxidative stress and mitochondrial morphology with a narrow optimal range between essential and toxic concentrations. Numerous studies in a variety of species have shown that Cu^{2+} exposure resulted in apoptotic and autophagic cell death because of elevated ROS levels (Wang et al. 2018).

Glutathione (GSH), as a major cellular antioxidant defense agent, decreases in the process of oxidative stress due to utilization as antioxidant. GSH levels in exposed mussel DG increased 2.5-fold compared to controls ($p < 0.05$), but increase in gills was not significant ($p > 0.05$) after 96 h exposure. *Unio tumidus* mussels were introduced to four different contaminated areas and exposed for 15 days. In the digestive gland, GSH levels decreased by 70%. It shows that the defense mechanism against oxidative stress was working and reduced glutathione depots (Cossu et al., 2000). The GSH levels in *Unio crassus* tissues were significantly raised after 48 h exposure to 10 µg/l CuPT, while they declined significantly after 7 days exposure. In another experimental study, the effects of 1-4 h high temperature exposure to SOD, the activities of GSH-Px and GSH were investigated in *Heteropneural fossilis*. After exposure at elevated temperatures ranging from 25 (control) to 37°C, SOD activity increased, while GSH-Px activity and GSH content decreased significantly ($p < 0.05$) at 32 and 37°C after 14 h in comparison to control. Nevertheless a transient increase in GSH-Px activity was observed after 1 and 2 h at 32°C. The results at 27°C temperature were non-significant ($p < 0.05$) in comparison to control. During the extended hours (1 to 4 h) of each elevated temperature, a general trend of increase in SOD activity was observed at 32 and 37°C. However, GSH-Px activity and GSH content did not change significantly for most of the extended period of elevated temperature.

Oxidative stress and histopathological parameters were determined in a study examining the effect of heavy metals on *Leuciscus cephalus* species. Fish samples were collected from two sites in the Tur River, NW Romania, upstream and downstream of a pollution source. Histopathological changes were associated with metal bioaccumulation, being more severe in kidneys than in liver. Malondialdehyde (MDA) and advanced oxidation protein products (AOPP) increased significantly in the liver and kidney of fish at the downstream site, compared to the upstream one, whereas GSH decreased. The activities of SOD, catalase (CAT) and glutathione-S-transferase (GST) increased significantly in livers, whereas SOD increased in kidneys (Hermenean et al. 2015). ROS formed during xenobiotic metabolism causes direct inactivation of GST isoenzymes. Furthermore, the use of engineered micro/nanomaterials (EMNMs) as carriers of antifouling booster biocides to control their release and reduce harmful effects on living biota are considered as a potential mitigation strategy, and they are under investigation (Gutner-Hoch et al. 2019).

Although mussels are important bioindicator model organisms in ecotoxicology of aquatic systems, the published research has been carried out mostly on marine bivalves. Histopathological investigations of Unionid mussels related to ecotoxicology are scarce. As sessile filter-feeder organisms, they can easily accumulate water-borne toxicants from the environment and reflect changes in water quality. The studies indicated that DG and gill tissues lead as the most sensitive tissues exposed to chemical toxicants. Since gills have a large surface in direct contact with water-borne toxicants, they are considered a sensitive organ affected by water quality (Aarab et al., 2011; Pinto et al. 2019). Exposure of 96 h to sublethal copper pyrithione revealed haemocyte infiltration, lipofuscin aggregates and lamellar deformations. Similar to the findings in this study, Tresnakova et al. (2020) noticed hemocytic infiltration and lipofuscin-like structure in gill tissues of *Unio crassus* following exposure to copper and zinc pyrithione and their combination for 48 h and 7 days. Lopez-Galindo et al. (2010) observed lipofuscin aggregates in gill tissues with haemocytic infiltrations on gill filaments following exposure to the antifoulants NaClO and Mexels432. Previous research with *Ruditapes phillippiranum,* exposed to contaminated sediments, reported results similar to our study (Martin Diaz et al. 2008). Haemocyte infiltration was noticed in gill tissues of *Mytilus edulis* exposed to treated and untreated sewage (Akaishi et al., 2007). *Mytilus galloprovincialis* and *Ruditapes phillipinarum* field samples were reported to show lipofuscin in cells due to environmental stressors of heavy metals and anoxic conditions (Sarasquete et al. 1992).

Overall mussel health is mainly evaluated looking at DG tissue and the impact of xenobiotics is compared with other tissues (Faggio et al. 2018). Digestive tubule degeneration has heretofore been associated with toxicity of xenobiotic exposure (Bignell et al. 2008). Exposure to antifouling copper pyrithione showed accumulated lipofuscin aggregates and caused mild degeneration of digestive tubules. In agreement with our present research, Tresnakova et al. (2020) observed deformations and loss of digestive tubules of *U. crassus* following exposure to copper-zinc pyrithione combination for 48 h and 7 days. Changes in the structure of DG epithelium in response to toxicants have been investigated for some marine bivalves (Sarasquete et al. 1992). Previous studies have demonstrated degeneration of DG following exposure to different toxicants and an increase in the frequency of DG degeneration may lead to digestive gland dysfunctions. (Lowe & Clarke, 1989). Results of the present study also support that oxidative stress in bivalves can be the source of lipofuscin accumulation in association with xenobiotic exposures.

In conclusion**,** there have been limited data so far on the environmental occurrence, fate, toxicity and persistence of these booster biocides, as well as their potential risks for aquatic ecosystems due to their increased use (Bao et al., 2011). The use of these antifouling biocides is expected to further increase after the complete ban on TBT, yet the results of this study (and others) suggest that some of them could be even as toxic as TBT to aquatic systems. Further studies should be carried out to understand how they affect aquatic life.

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Oksidativni stres kao biomarker kod izlaganja slatkovodne školjke (*Unio mancus* Lamarck 1819) kao model organizma sredstvu protiv obrastanja bakar piritionu

REZIME

Ocenjivani su efekti bakar piritiona (CuPT) na jednu vrstu beskičmenjaka, slatkovodnu školjku (*Unio mancus*) kao model organizma, korišćenjem parametara oksidativnog stresa i histopatoloških nalaza. Koncentracija bakar piritiona od 5 µg/l, koja je značajna za životnu sredinu, primenjena je u polustatičnom izlaganju u trajanju od 96 h. Nivoi MDA u digestivnoj žlezdi (DG) bili su značajno povećani, u poređenju sa kontrolnom grupom ($p < 0.001$). MDA u škrgama su takođe bili povećani, ali ne značajno ($p > 0.05$). Nivo GSH u DG se značajno povećala ($p < 0.05$), dok je u tkivu škrga povećanje bilo bez značaja ($p > 0.05$). Nivoi SOD, GP i AOPP nisu se značajno izmenili ($p > 0.05$). Najznačajnije histopatološke promene u škrgama predstavljali su infiltracija hemocita, akumulacija lipofuscina i lamelarna deformacija sa degeneracijom tubule i akumulacijom lipofuscina u tubulama DG. CuPT utiče na antioksidativni odbrambeni sistem čak i tokom subletalnog izlaganja *Unio mancus* kao slatkovodnog model organizma.

Ključne reči: biocidi, sredstvo protiv obrastanja bakar pirition, biomarkeri, oksidativni stres, slatkovodna školjka