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Histopathology of chironomids exposed to fly ash and microplastics as a new biomarker of ecotoxicological assessment

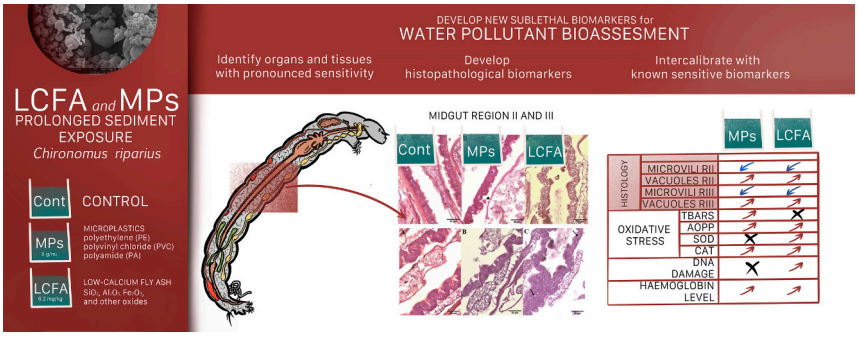
Jelena Stojanović [a,*], Dimitrija Savić-Zdravković [a], Boris Jovanović [b], Jelena Vitorović [a], Jelena Bašić [c], Ivana Stojanović [c], Andrea Žabar Popović [a], Hatice Duran [d,e], Margareta Kračun Kolarević [f], Đurađ Milošević [a]

[a] Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, Niš, Serbia
[b] Department of Natural Resource Ecology and Management, Iowa State University, Ames, IA, USA
[c] Department of Biochemistry, Faculty of Medicine, University of Nis, Bulevar dr Zorana Đinđića 81, 18000 Nis, Serbia
[d] Department of Materials Science & Nanotechnology Engineering, TOBB University of Economics and Technology, Söğütözü Cad. 43, 06560 Ankara, Türkiye
[e] UNAM — National Nanotechnology Research Center and Institute of Materials Science and Nanotechnology, Bilkent University, Ankara, Türkiye
[f] Department of Hydroecology and water protection, Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia

HIGHLIGHTS

- Midgut brush border length is a promising histopathological biomarker.
- Microplastics affected midgut brush border length and oxidative stress parameters.
- Lignite coal fly ash (LCFA) significantly affected midgut brush border length.
- LCFA caused vacuolization of the midgut digestive cells.

GRAPHICAL ABSTRACT



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ABSTRACT

In the last few decades, industrial pollution has gained extensive attention in terms of its effect on the aquatic environment. This imposes the need to develop sensitive biomarkers for early detection of pollutant toxicity in ecotoxicological assessment. The advantages of histopathological biomarkers are many, including quick reaction to the presence of contaminants, and the small number of individuals needed for efficient analysis. The present study analyzed the negative effect of lignite coal fly ash (LCFA) and microplastic particles (MPs) on *Chironomus riparius*, a suggested model organism by the Organization for Economic Cooperation and Development (OECD). This study aimed to perform histological analyses of larval tissues and target potential changes in treated groups that could serve as promising histopathological biomarkers of the contaminant's negative effects. Following that, other known sensitive sub-organismal biomarkers were analyzed and paired with the histopathological ones. Histological analysis of larvae showed a significantly decreased length of microvilli in midgut regions II and III in

* Corresponding author.
E-mail address: jelena.conic@pmf.edu.rs (J. Stojanović).

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both treatments. Treatments with MPs affected oxidative stress parameters: thiobarbituric acid reactive substances (TBARS), advanced oxidation protein products (AOPP), superoxide dismutase (SOD), and hemoglobin levels, while LCFA significantly affected all tested sub-organismal biomarkers (DNA damage, levels of AOPP, SOD, and hemoglobin), except catalase (CAT) and TBARS. When observing histological slides, a significant shortage of brush border length in the posterior parts of the midgut was detected in all treatments. In the case of LCFA, the appearance of intensive vacuolization of digestive cells with inclusions resembling apoptotic bodies, in mentioned regions was also detected. This study demonstrated high sensitivity of brush border length to the MPs and LCFA exposure, complementary to other tested sub-organismal biomarkers. Revealing the great potential of this histopathological biomarker in ecotoxicological studies contributes to the international standard ecotoxicology assessment of emerging pollutants.

1. Introduction

The modern industrial era has ushered in a diverse range of synthetic materials that have become integral to our daily lives. However, this progress has also resulted in vast amounts of industrial waste and byproducts (Harja et al., 2022). As we become increasingly aware of the importance of environmental protection, more attention is being directed towards emerging pollutants, a term coined by the United States Environmental Protection Agency (EPA) to refer to chemicals or materials that pose actual or potential threats to both human health and the environment (US EPA, 2013). The presence of these emerging contaminants in freshwater has raised concerns about the health of vulnerable aquatic ecosystems (Bashir et al., 2020), which serve as the very foundation of all life.

Among the emerging pollutants that have garnered global attention, microplastic particles (MPs) stand out due to their widespread distribution across various ecosystems and their potential impact on environmental health (Maddela et al., 2022). The prevalence of these tiny plastic particles raises concerns about their long-term effects on our natural surroundings and living organisms. MPs vary in size, ranging from 1 μm to 5 mm, which makes them easily ingested by living organisms (Horton et al., 2017). Among these organisms, many freshwater invertebrate species have been found to ingest MPs (Scherer et al., 2017). This has raised concerns about potential disruptions to nutrient cycling, as there is a worry that benthic invertebrates might consume MPs, instead of organic particles (Silva et al., 2021).

Exposure of invertebrates to MPs affects their growth and survival, as was shown in studies with *Chironomus tepperi* (Ziajahromi et al., 2018), with this negative impact being linked to energy deficiency resulting from the interference of MPs with food uptake (Trestrail et al., 2020). Notably, *Chironomus riparius* larvae, due to their nonselective feeding behavior, have been confirmed to ingest MPs, with the quantity of particles in the gut depending on various factors such as size, concentration, and type of MPs, as well as organism behavior (Silva et al., 2021). Moreover, Silva et al. (2021) demonstrated that MPs could obstruct the gut passage of *C. riparius* larvae due to their irregular shape and potential to aggregate, leading to a false sense of satiation that affects the organism's physiological status.

Research focusing on low-density polyethylene (LDPE) MPs has revealed various alterations at molecular and cellular levels in *C. riparius* larvae. Environmental concentrations of LDPE MPs were found to decrease the carbohydrates and increase lipids, modifying the energy reserve composition. Additionally, these MPs altered the expression of genes associated with the endocrine system, immune system, and DNA repair in the larvae (Muñiz-González et al., 2021). Moreover, several studies have linked polyethylene MPs to oxidative stress and changes of antioxidant enzymes levels in the 4th instar larvae of *C. riparius* (Muñiz-González et al., 2021; Silva et al., 2021). Furthermore, it has been observed that environmental concentrations of MPs can affect the developmental time of *C. riparius* (Stanković et al., 2020a). Altogether, these findings emphasize the potential risks that microplastics pose to aquatic ecosystems and the importance of understanding their effects on various organisms, especially invertebrates like *C. riparius*.

Another pollutant that is drawing significant public interest is fly ash, a byproduct generated from coal combustion in power plants. Fly ash is primarily composed of silica, alumina, iron oxide, calcium, oxygen, and trace amounts of other heavy metals (Upadhyay and Edrisi, 2021), while its properties, such as mineral density, type, and particle size, exhibit variations influenced by factors like coal type (lignite or stone coal), combustion temperature, and sampling location (Kozbek et al., 2022). Lignite fly ash (LCFA) typically has higher silica, alumina, and iron oxide content with a smoother surface and spherical particles, while stone coal ash may have additional minerals, such as calcium salts. The toxicity of fly ash arises from various sources, including organic molecules such as polychlorinated biphenyls (PCB), polyaromatic hydrocarbons (PAH) as well as trace elements such as zinc (Zn), lead (Pb), cadmium (Cd), nickel (Ni), arsenic (As), chromium (Cr), and copper (Cu) (Upadhyay and Edrisi, 2021). Chironomids, as inhabitants of freshwater ecosystems, can be influenced in varying ways by fly ash. While certain species demonstrate remarkable resistance to elevated fly ash concentrations, extended exposure may have adverse effects on their survival and growth (Cherry et al., 1979; Bae et al., 2014; Sherrard et al., 2015). Chironomid ability to withstand toxins is even reflected in their potential as a bio-sorbent material for removing Cd from wastewaters (Cherry et al., 1979; Bae et al., 2014). However, long-term exposure of benthic organisms to fly ash, as observed in post-dredging studies for the Tennessee Valley Authority (TVA) Kingston ash spill, revealed a moderate risk to the analyzed benthic community when the fly ash concentration exceeded 40 %, with the survival and growth of *Chironomus dilutus* being affected in both analyzed rivers where the ash was spilled (Sherrard et al., 2015). Fly ash, with its diverse composition and potential environmental impact, has become a subject of great concern, underscoring the need for further research, and understanding its effects on freshwater ecosystems and the resilient inhabitants like chironomids (Sherrard et al., 2015).

In the quest to understand and address the potential toxic effects of emerging pollutants and safeguard ecosystems, comprehensive investigations are essential. Ecotoxicological tests, which encompass sub-organismal (biomarkers and in vitro bioassays), whole-organismal, population, and community responses, play a crucial role in assessing the impact of chemicals on various biological and ecological levels (Schuijt et al., 2021). Sub-organismal biomarkers, including genetic, biochemical, cellular, physiological, and histological changes, exhibit high sensitivity to the presence of toxins, enabling a rapid stress response and serving as early indicators of pollutant effects (Smit et al., 2009). Identifying these biomarkers promptly allows for appropriate actions to be taken to prevent irreversible damage to the ecosystem. While current aquatic ecotoxicology testing primarily relies on conventional whole-organismal biomarkers like survival, emergence rate, development rate, sex ratio, weight, and length of individuals, their lower sensitivity compared to sub-organismal biomarkers has led to the introduction of novel approaches to enhance the efficiency of detecting toxic effects caused by emerging contaminants (Schuijt et al., 2021).

In the pursuit of determining most effective and reliable tools for ecotoxicity assessment, it becomes crucial to utilize suitable model organisms and identify precise biomarkers. In ecotoxicological studies, aquatic macroinvertebrates, acting as sensitive bioindicators, play a vital role in assessing water quality (Uherek and Pinto Gouveia, 2014).

One of the model organisms used in ecotoxicological test protocols by the Organization for Economic Cooperation and Development (OECD, 2004a) is *C. riparius*. Chironomids are widely distributed aquatic organisms, capable of adapting to diverse water ecosystems. As sediment serves as the primary sink for many pollutants, chironomids, particularly their larvae, which are predominantly benthic and consistently in contact with water and sediment, become crucial test subjects for studying the impact of water pollutants. To effectively test the toxicity of these unregulated chemicals, the development of biomarkers capable of detecting their presence in both sediment and water becomes imperative (Poynton and Vulpe, 2009).

The present study proposes the potential histopathological biomarker of *C. riparius* sensitive to the MPs and LCFA exposure, where we analyze microvilli length and correlate the changes with other sensitive sub-organismal biomarkers. We hypothesized that MPs and LCFA affect the microvilli length in midgut of *C. riparius* larvae, changes of which can be correlated with the levels of DNA damage, oxidative stress parameters and hemoglobin (Hb) concentrations. To achieve this, we set the following specific objectives: i) to identify organs and tissues of *C. riparius* larvae that exhibit pronounced sensitivity to the presence of contaminants; ii) to establish quantitative biomarkers based on target tissues of chironomid larvae; iii) to correlate histopathological changes with other sensitive sub-organismal biomarkers, including DNA damage, oxidative stress, and Hb concertation.

2. Materials and methods

2.1. Test organisms

In this study, 1st instar larvae of *C. riparius* Meigen, 1804 were used. Larvae were obtained from the stock population housed at the laboratory of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis. The population was established and maintained following OECD guidelines (OECD, 2004[a]), housed in glass tanks, on cellulose sediment, filled with a mixture of tap and deionized water at the temperature 23 °C ± 2 °C. Tanks were constantly aerated and a 16 h photoperiod was used. The larvae were fed with TetraMin® finely ground food.

2.2. Test substances

The present study examined two test substances, namely microplastic particles (MPs) and lignite coal fly ash (LCFA). A mixture of ultra-high molecular weight polyethylene-PE (Sigma-Aldrich cat no. 434272), high molecular weight polyvinyl-chloride-PVC (Sigma-Aldrich cat no. 81387), and polyamide for column chromatography-PA (Carl Roth cat. no. 9620.1) was used as an MPs, in ratio 50:25:25, respectively. This mixture was used in the previous study with MPs (Stanković et al., 2022). Lignite coal fly ash was obtained from a Turkish thermal power plant that does not wish to disclose its name to the public.

Scanning Electron Microscopy (SEM) images and Energy Dispersive X-Ray Analysis (EDAX) spectroscopy results of LCFA were obtained using a Zeiss Evo 50 scanning electron microscope equipped with an Oxford EDAX detector. Morphology, particle size, and size distributions of LCFA were analyzed by SEM (FEI Quanta 200 FEG SEM with 4 nm resolution). A Brunauer–Emmett–Teller (BET) surface area analysis of adsorbents was performed with an Autosorb-iQ Station 1 (Quantachrome Instruments) in an N_2 atmosphere. The BET surface areas of LCFA particles were obtained from N_2 adsorption and desorption tests at 77.350 K. The samples were degassed at 120 °C for 24 h before measurements were taken. EDAX (Energy Dispersive X-Ray Spectroscopy) and X-Ray (Rigaku model ZSX Primus II XRF) analyses were performed to determine the elemental composition of the components in LCFA. X-ray photoelectron spectroscopy (XPS) for elemental compositions and bonding information was performed using Thermo Scientific K-Alpha. The Mg Kα (1486.6 eV) X-ray source was operated at 300 W. The survey scans were carried out with a pass energy of 117.40 eV. The spectra were recorded using a 60° take-off angle relative to the surface normal. The UV/vis absorption spectra of the dyes were analyzed using a Hitachi U-5100 spectrophotometer.

2.3. Bioassay design

The purpose of the bioassay design was to assess the following parameters: brush border length (BB) and cell area (CA) ratio (μm), microvilli length (μm), tail intensity (TI%), concentration of thiobarbituric acid reactive substances (TBARS), concentration of advanced oxidation protein products (AOPP), catalase activity (CAT), superoxide dismutase activity (SOD) and hemoglobin concentration (Hb).

The OECD tests No. 218 and No. 219 were used as guidelines to modify chronic exposure of *C. riparius* larvae to the aforementioned test substances (OECD, 2004a, 2004b). Following these guidelines, the test is considered valid if no >15 % of the larvae showed any signs of immobilization in the control group. Since we needed a total of 30 larvae from each jar for further analysis, we have added 15 % more larvae to the jars. The test was modified and terminated earlier for the purpose of further analyses that require 4th instar larvae. A total of 420 1st instar larvae were divided into 12 replicates, i.e. glass jars (35 larvae in each jar) with a volume of 700 mL each. The jars were filled with 105 cm^3 (1/5 of jar volume) of coarse quartz sediment (with test substances in treated groups and without them in the control group) and 4/5 mixture of tap and deionized water (1:1). The constant temperature (23 °C ± 2 °C), aeration and 16/8 h photoperiod were provided. The experimental setup was formed 48 h before adding larvae to stabilize the environment. The experiment was terminated after the larvae reached the 4th instar.

The experimental design consisted of three distinct treatment groups, with each group comprising four replicates. The control group consisted of sediment and water only, while, in treatment groups pollutants were mixed with coarse quartz sand. Treatment with MPs contained polyethylene (PE), polyvinyl chloride (PVC), and polyamide (PA) in a ratio of 50:25:25 (%), respectively (Stanković et al., 2022). The concentration used was 8 g per m^2 of the sediment, as it was previously considered an environmentally relevant concentration by (Stanković et al., 2020a). Microplastic contamination from equipment was prevented by using glassware instead of plasticware. Treatment with LCFA contained 6.9 mg of LCFA per 1 kg of sediment. This concentration was empirically determined as a 48 h LC20 (Lethal Concentration for 20 % of exposed chironomid larvae) prior to the start of the experiment. To determine the 48 h LC20 a series of concentrations was used as previously explained (Stanković et al., 2022) in an acute OECD 219 spiked water test (OECD, 2004[b]), in the absence of sediment. LC20 was determined using the Risk Assessment Tool Analysis Software - RA V1.0. and converted to mg/kg to spike sediment in future exposure. In order to achieve randomization, the jars were arranged in the experimental setup without any specific order. The blinding was achieved during the analysis of sub-organismal biomarkers phase, described below. The larvae were sent to the laboratories for further analyses without marking the specific treatment that they belonged to. Physical-chemical parameters were measured at the start and the end of the experiment, as suggested by the OECD 233, of which results are presented as Supplementary material S1 (OECD, 2018). The ethical statement is uploaded as a Supplementary material S2.

2.4. Histology analysis

For detecting histological changes in the tissues, 5 larvae were selected from each sample and transferred to Bouin's solution (25 % formalin solution, 70 % picric acid, and 5 % acetic acid). After fixation, larvae were washed in 70 % ethanol for 6 h and then dehydrated with a series of increasing concentrations of ethanol (70 %, 80 %, 90 %, and 96 %). The samples were immersed into toluene for 10 min and placed in tissue-embedding paraffin overnight. The day after, samples were

embedded and cooled down to be prepared for sectioning. Longitudinal sections, 5 μm thick, were made on a Leica® RM 2125RT microtome and then stained using a combination of hematoxylin and eosin (H&E). Slides were observed and photographed under a light photomicroscope (Leica® DM 2500), to detect any changes in normal tissue architecture. Brush border length (BB) and cell area (CA) of digestive cells were measured using the ImageJ® program (Schneider et al., 2012) and BB/CA ratio was calculated.

2.5. Comet assay

The genotoxicity of tested substances was evaluated using the single-cell gel electrophoresis assay (SCGE), also known as a comet assay. The method was performed following the protocol by Bernabò et al. (2017) without isolating individual cell types. 10 larvae from each sample, as well as positive (larvae treated with 20 mM H_2O_2 for 1 h) and negative control (untreated larvae), were gently homogenized using mortar and pestle and washed through a filter with 1.5 mL of suspension buffer in 1.5 mL Eppendorf tubes. The samples were centrifuged for 15 min at 1500 rpm at 4 °C. Resulted pellet was resuspended in 300 μL of suspension buffer. Using the Neubauer chamber, number of cells in the suspension was calculated and the cell suspension was diluted to 1000 cells/μL. Following that, 30 μL of cell suspension was mixed with 70 μL of 1 % low melting point agarose (Sigma-Aldrich), out of which 70 μL was transferred to the comet assay slide and the gel was solidified for 5 min at 4 °C. Slides were transferred to the lysis buffer (Tris 10 mM, EDTA 100 mM, NaCl 2.5 M, DMSO 10 %, Triton X-100 10 %, pH = 10) overnight. The day after, slides were immersed in the alkaline buffer for 45 min at 4 °C and then transferred to an electrophoresis chamber. Cells were exposed to an electric field of 1 V/cm, 300 mA for 25 min in an alkaline buffer, washed with ddH_2O for 30 min, and left to dry overnight. Staining was performed using 20 μL of GelGreen (Biotium) and slides were examined under a fluorescence microscope (Leica DM4 B, Austria, under magnification 400×, excitation filter 450–490 nm, barrier filter 510). On each slide, 50 nuclei were scored (150 per treatment group) using TriTek CometScore Pro 2.0.0.38 Software (TriTek Corp., Sumerduck, USA). Brightness and length of the comet tail relative to the head, called tail intensity (TI %), was used as a measure of DNA damage.

2.6. Oxidative stress analyses

Five larvae from each sample were homogenized in 1.25 mL Tris-EDTA (TE, pH 7.8) and then centrifuged for 15 min on 500 G (RCF). Supernatant was centrifuged again on 12.000 G (RCF) for 30 min. The supernatant was used to analyze oxidative stress enzymes: thiobarbituric acid reactive substances (TBARS), advanced oxidation protein products (AOPP), catalase activity (CAT), and superoxide dismutase activity (SOD). Total protein concentration was analyzed following the method described by Lowry et al. (1951), with bovine serum albumin as a standard.

Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS) according to the method of Andreeva et al. (1988). The pink chromogen produced by the reaction of TBA with MDA was measured spectrophotometrically at 532 nm on Multiscan Ascent 96/384 spectrophotometer (Thermo Labsystems). Concentration of TBARS was determined using an extinction coefficient of 1.56 × 105 M^{-1} cm^{-1} and the results were expressed in μmol/L and then converted to mg of proteins.

Concentration of AOPP was determined by the spectrophotometric method of Witko-Sarsat et al. (1996). Calibration was carried out using chloramine-T solutions, which absorb the light at λ = 340 nm in the presence of potassium iodide. The data were calculated and expressed in μmol/L of chloramine T equivalents and finally related to total protein levels (μmol/mg of proteins).

Activity of CAT was determined by Góth's (1991) spectrophotometric method, based on the ability of CAT to decompose the substrate (H_2O_2), whereby the enzymatic reaction was stopped by the addition of ammonium molybdate, and the resulting yellow complex of H_2O_2 and molybdate was measured at 405 nm against the reagent blank. The enzyme activity was expressed in U/L and then converted to mg of proteins.

Activity of SOD was measured by the method of Minami and Yoshikawa (1979), based on formazan-colored product formation. In the reaction with NBT (nitroblue tetrazolium), superoxide anion, produced by pyrogallol autooxidation, forms a colored product. SOD, as a superoxide anion scavenger, inhibits this reaction. The enzyme activity was expressed in U/L and then converted to mg of proteins. One unit of SOD activity was defined as the amount of enzyme causing 50 % inhibition of the NBT photoreduction rate.

2.7. Hemoglobin concentration

The concentration of hemoglobin was detected using a method described by Stanković et al. (2020b). Immediately after termination of the experiment, 5 fresh 4th instar larvae were decapitated, and hemolymph was sampled using the capillary tube. Samples were analyzed by photometric method on a hematology analyzer (Medonic M16M/M20M, Sweden). Results were expressed in g/L of hemolymph.

2.8. Data analysis

To analyze the difference between control, MPs and LCFA treatments for microvilli length, BB/CA ratio and TI% One-Way ANOVA was applied, while for post hoc comparison between control and each treatment the Dunnett test was applied. The difference between control group and treatment samples for oxidative stress parameters and hemoglobin was tested using Man-Whitney test. All statistical tests were performed in SPSS statistical software package.

3. Results

3.1. Chemical analysis and particles characterization of lignite coal fly ash

The chemical composition of the LCFA was analyzed with XRF before and after washing is given in Table 1. The substance was washed with distilled water at 50 °C and the solution was stirred at 350–400 rpm for 4 h with the aid of a magnetic stirrer. Then, the solution was filtrated through a Whatmann filter paper. This process was repeated 3 times until the supernatant was clear. Since LCFA contains water-soluble minerals and Cl salts, washing steps are applied for the removal of these salts. Pristine LCFA contains approximately 53.19 % SiO_2, 27.86 % Al_2O_3, 7.02 % Fe_2O_3, and 11.93 % other oxides. The silica and alumina content slightly increased after the washing procedure while the percentage of iron oxide decreased. However, the most significant change was observed in particle morphologies after the washing. As shown in Fig. 1, the water-washing process ensures that the ash particles have smooth surfaces and that the impurities in the large particle were removed from the fly ash. There were minerals deposited on the pristine

Table 1
Chemical composition of LCFA before and after distilled water washing measured by XRF.

Oxide wt% (excluding oxide compounds below 1 %)	Pristine LCFA	After washing (distilled water at 50 °C)
SiO_2	53.193	55.021
Al_2O_3	27.860	28.289
Fe_2O_3	7.018	6.2488
K_2O	4.500	5.1300
MgO	2.068	1.9201
CaO	1.818	1.6317
TiO_2	1.232	1.2182

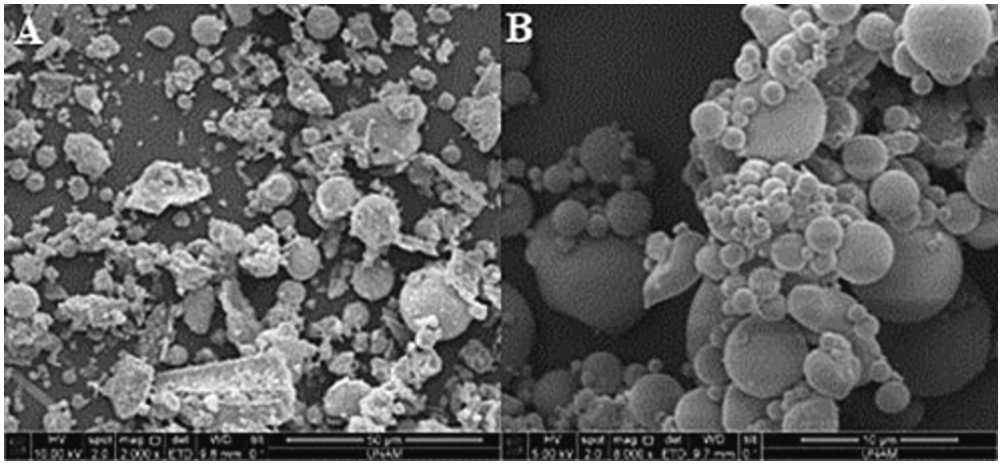

Fig. 1. SEM images of (A) pristine LCFA, (B) after washing with distilled water at 50 °C.

LCFA surfaces and the particle surfaces are rougher. According to the EDX results, the weight percentages of elements such as K, Ca, Mg, and Na were higher on pristine LCFA compared to water-treated fly ash. Dimensional analysis of LCFA was performed by SEM analysis in a 20 μm^2 area showed that the fly ash particle size varies between 0.5 μm and 120 μm. The specific surface area of the LCFA measured with BET analysis was 173.18 $m^2\ g^{-1}$ and the predominant pore diameters were at around 1.9 nm and 2 nm.

3.2. Histology analyses

Histological analyses showed changes in the alimentary canal in both treatments (MPs and LCFA). The normal tissue architecture of the midgut was disturbed by the appearance of the vacuoles and potential apoptotic bodies as well as brush border alterations. Intensive vacuolization appeared in an LCFA treatment. Out of 20 analyzed larvae, 11 had vacuoles in midgut region II (Fig. 2) and 6 in midgut region III (Fig. 3), while region I showed no appearance of vacuoles.

Microvilli length measurement revealed a tendency to shorten when exposed to the MPs and LCFA and compared to the control (Figs. 4 and 5). Region II of the midgut showed the most significant microvilli shortening in both treatments (One-way ANOVA $F(2.257) = 189.179$, $p < 0.001$; Dunnett test, $p<0.001$) (Fig. 4). Midgut region III had also significant length alterations in both treatments when compared to the control (One-way ANOVA $F(2.597) = 434.788$, $p < 0.001$; Dunnett test, $p<0.001$) (Fig. 4). BB/CA ratio in the midgut region II was significantly higher in LCFA treatment when compared to the control (One-way ANOVA $F(2,587) = 28.678$, $p<0.001$; Dunnett test, $p<0.001$), while MPs treatment values were not significantly different from the control. In the midgut region III, only the MPs treatment had a significantly lower BB/CA ratio (One-way ANOVA $F(2.587) = 5.535$, $p = 0.004$; Dunnett test, $p = 0.001$), which was not observed in LCFA treatment (Fig. 5).

3.3. DNA damage

One-way ANOVA and Dunnett post hoc test showed a significantly higher level of DNA damage in LCFA treatment (One-way ANOVA $F(2.397) = 119.154$, $p<0.001$; Dunnett test, $p<0.001$) while MPs

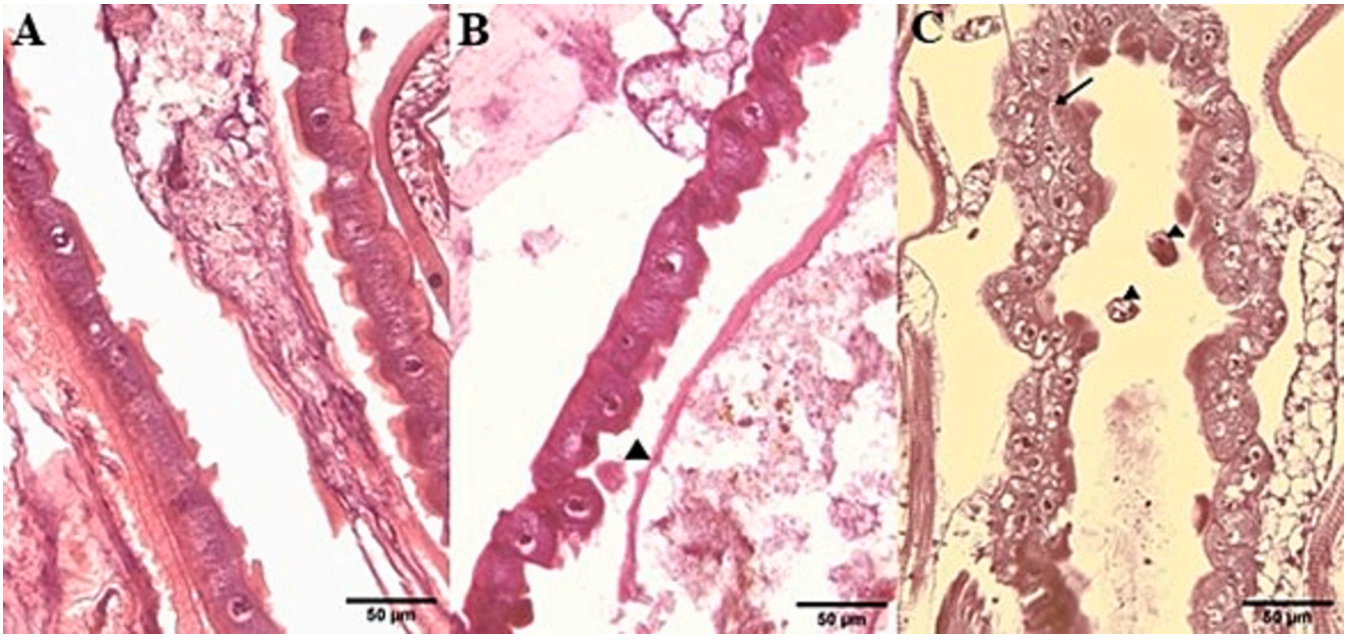

Fig. 2. Photomicrograph of midgut region II digestive cells of *C. riparius* from (A) control group; (B) MPs treatment; (C) LCFA treatment showing numerous vacuoles with potential apoptotic bodies (arrow) and vacuolized cells that detached from the intestinal wall into the lumen (arrowhead). Scale bar = 50 μm.

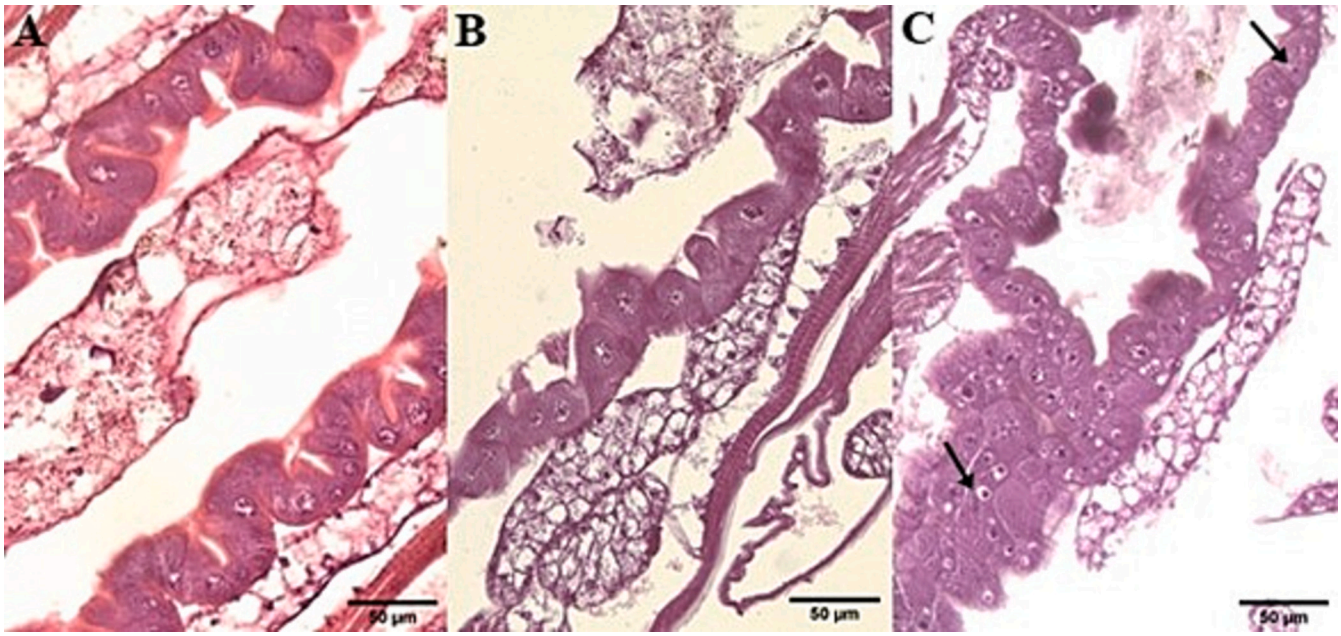

Fig. 3. Photomicrograph of midgut region III digestive cells of *C. riparius* from (A) control group; (B) MPs treatment; (C) LCFA treatment showing numerous vacuoles with potential apoptotic bodies (arrow). Scale bar = 50 µm.

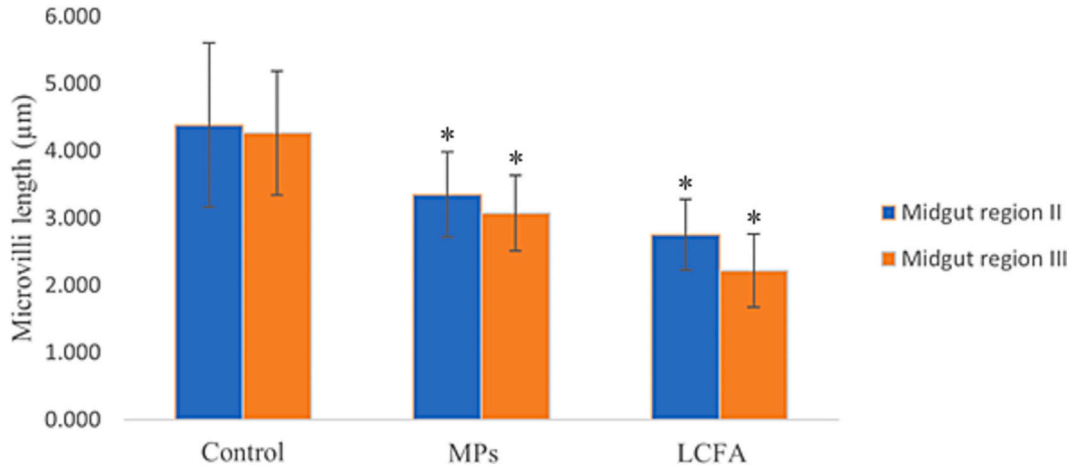

Fig. 4. Microvilli length (µm) of midgut region II and region III exposed to MPs and LCFA. The bars represent the standard deviation – SD (*-represents statistically significant difference, $p < 0.001$, Dunnett test).

treatment was not different from the control (Fig. 6).

3.4. Oxidative stress

The total protein content varied from 0.69 to 0.89 mg/mL of the tested sample (Table 2). The Man-Whitney test showed a significant difference in TBARS concentration in MPs treatment when compared to the control ($p<0.05$). Significantly higher AOPP concentrations were detected in both treatments when compared to the control (Man Whitney, $p<0.05$). SOD activity was significantly higher in both treatments when compared to the control (Man Whitney, $p<0.05$).

3.5. Hemoglobin concentration

A significantly higher concentration of hemoglobin was detected both in MPs and LCFA treatments when compared to the control (Man Whitney, $p<0.001$) (Fig. 7).

4. Discussion

This paper addresses two critical environmental concerns that have garnered global attention. Firstly, the study focuses on microplastic particles (MPs), which are under intense scrutiny due to their widespread presence in various ecosystems and potential implications for environmental health in the context of plastic global pollution. Secondly, the investigation targets fly ash, a byproduct of coal combustion in power plants that contains a mixture of potentially toxic substances. Understanding the impact of these emerging pollutants is of paramount importance for devising effective strategies to safeguard our environment. Various ecotoxicological analyses, done sporadically using different biomarkers and ecotoxicology experimental designs, revealed the toxic effects of MPs and LCFA on aquatic model organisms. (Malhotra et al., 2019; Palmer and Herat, 2021; Rodrigues et al., 2020; Stanković et al., 2020a). In the present study, we used several biomarkers known to be efficient in ecotoxicity testing from previous studies, compared them to the changes at the histological level, and

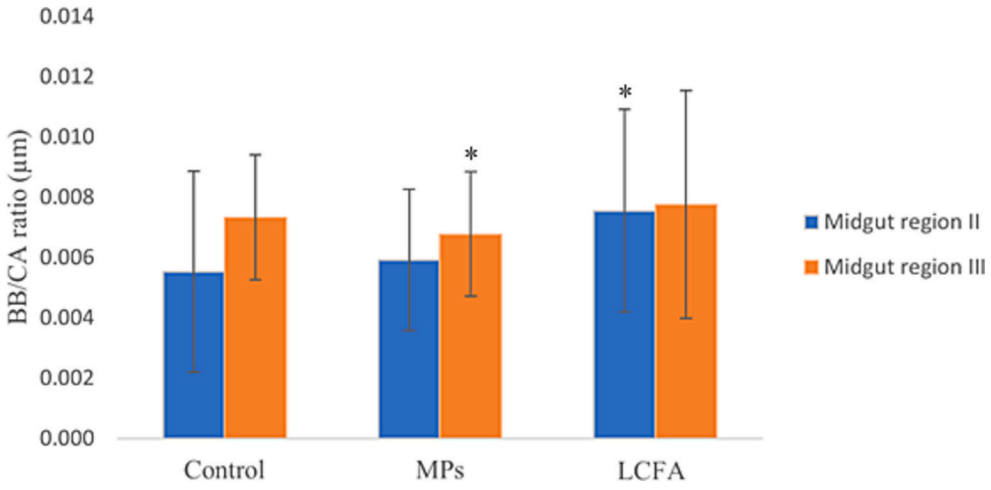

Fig. 5. BB/CA (µm) ratio of midgut region II and region III exposed to MPs and LCFA. The bars represent standard deviation – SD (*-represents statistically significant difference, $p \leq 0.001$, Dunnett test).

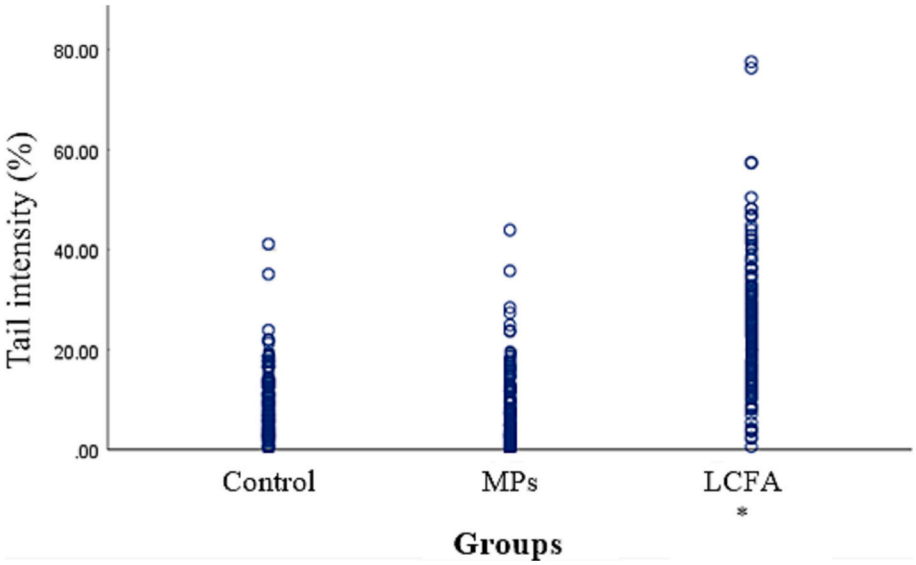

Fig. 6. The level of DNA damage expressed by tail intensity (TI %) measured in cell suspension of the whole *C. riparius* larvae exposed to MPs and LCFA (*-represents statistically significant difference, p<0.001, Dunnett test,).

Table 2
Oxidative stress parameters of *C. riparius* exposed to MPs and LCFA (TBARS-thiobarbituric acid reactive substances, AOPP-advanced oxidation protein product, CAT-catalase, SOD-superoxide dismutase).

	Control	MPs	LCFA
Total protein content (mg/mL)	0.89 ± 0.11	0.69 ± 0.08	0.71 ± 0.07
TBARS (umol/mg prot)	0.0062 ± 0.0005	0.0077 ± 0.0003*	0.0069 ± 0.0008
AOPP (umol/mg prot)	0.0069 ± 0.0007	0.00109 ± 0.0019*	0.0088 ± 0.0005*
CAT (U/mg prot)	0.1029 ± 0.0122	0.0938 ± 0.0307	0.1203 ± 0.0081
SOD (U/mg prot)	5.8629 ± 1.1471	7.8393 ± 1.2295*	7.3972 ± 0.5963*

* Statistical significance (Man Whitney, p<0.05) in treated groups compared to the control group.

provided comprehensive insight into the relationship between biomarkers suitable for assessing the toxic effect of tested contaminants. In addition, such experimental design enabled inter-calibration and the definition of the potential histopathological biomarkers in freshwater model organisms that could be used in further ecotoxicological assessment.

In our study, there was an evident vacuolization only in the LCFA treatment, which was detected by histological analyses. Histological changes induced by exposure to various chemicals can be detected using histopathology, a widely used method to identify morphological tissue alterations (Schuijt et al., 2021). It is mainly applied in in vivo toxicity assessments, and among aquatic species, mostly on fish (Bernet et al., 2001). Intensive vacuolization of midgut digestive cells was detected in regions II and III. Similarly, the chronic exposure of *Cerastoderma edule* to the pulverized fuel ash showed intensive vacuolization or complete atrophy of digestive glands after three months (Bowmer et al., 1994). Vacuoles that occurred in digestive cells of *C. riparius* larvae were presented as intracellular inclusions surrounded by a clear halo and resembled apoptotic bodies, similar to those seen in *Mytilus galloprovincialis* (Romero et al., 2011), suggesting that these cells were probably

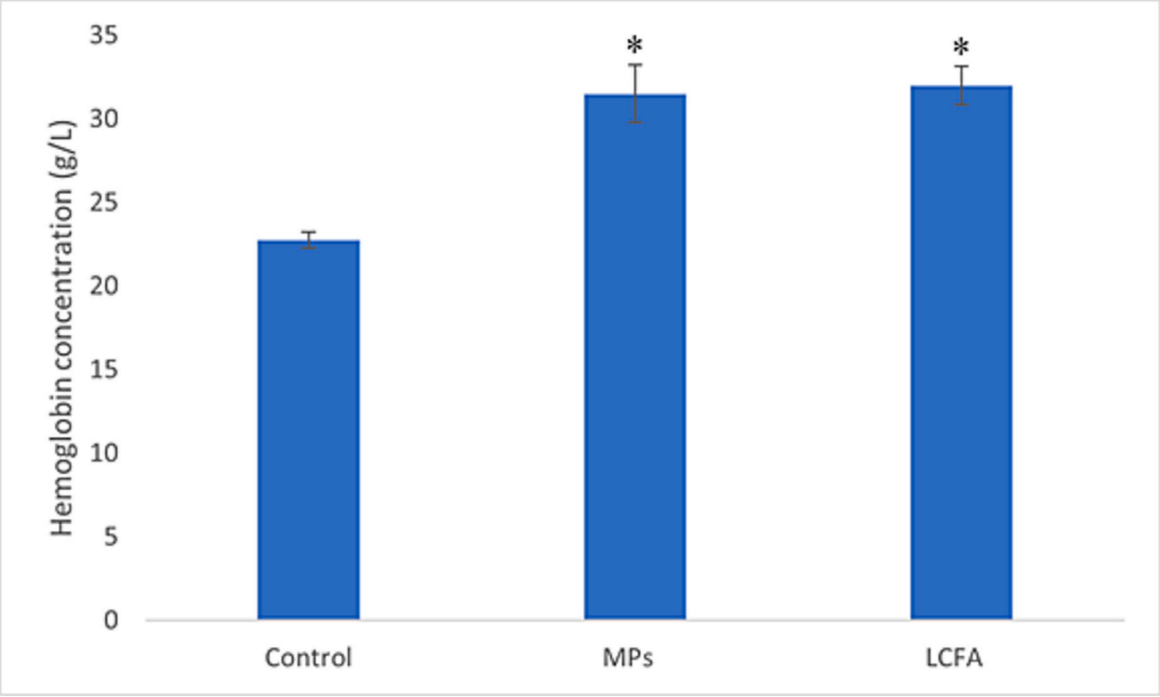

Fig. 7. Average concentration (with standard deviation) of hemoglobin in *C. riparius* larvae exposed to MPs and LCFA, presented in g/L of hemolymph. The bars represent the standard deviation – SD (*-represents statistically significant difference, $p < 0.001$, Man Whitney).

undergoing the process of cell death. Apoptosis is a form of cell death that is important for maintaining the homeostasis of the digestive epithelium and the stress responses, which was probably activated in larvae from our study. Denton et al. (2009) showed that the key mechanism involved in larval metamorphosis is autophagy. The digestive epithelium in the LCFA-treated larvae seemed to be undergoing a process of reorganization. The increased rate of apoptosis indicated by numerous apoptotic bodies and detached cells (exfoliation of epithelial cells) with acidophilic cytoplasm falling into the lumen can be explained as a reaction to toxic substances.

In the study of Ali et al. (2007) it was shown that fly ash has a pro-apoptotic effect in fish hepatocyte cells, where the resulting apoptosis is explained by oxidative stress, which was also observed in our present research. Invertebrates generally have the ability to cope with high levels of oxidative stress (Chaitanya et al., 2016), however, when the concentration of toxins exceeds the antioxidative enzyme's ability to manage the levels of generated reactive oxygen species (ROS), the oxidative stress can occur (Schuijt et al., 2021). The levels of antioxidant enzymes can be altered by exposure to chemicals which makes them suitable biomarkers for measuring the intensity of caused oxidative stress (Schuijt et al., 2021). The increased activity of antioxidant enzyme in the present study, SOD, indicate an activated antioxidant defense, which copes with oxidative stress and does not lead to a significantly increased rate of lipid peroxidation and membrane damage. On the other hand, due to the significantly increased genotoxicity exhibited by specimens in this treatment as well as increased AOPP, it can be assumed that the basis of apoptosis was predominantly DNA and protein damage.

Such a stimulating effect on remodeling, intensive apoptosis, and replacement of dead cells with a noticed vacuolization as a result of epithelial disturbance, was not noticed in the treatment with MPs, where only individual apoptotic cells were observed in the epithelia. MPs treatment, on the other hand, showed an increased rate of lipid peroxidation and a number of protein oxidation products, indicating oxidative damage to membranes of digestive cells and cellular proteins. The increase in SOD activity was higher than in the LCFA treatment, but no increase in CAT activity and DNA damage was noticed. Previous studies showed that MPs induce oxidative stress as a main mechanism of toxicity (Hu and Palić, 2020; Qiao et al., 2019). Except for the shortening of microvilli length and occasionally present apoptotic cells, no histopathological changes were observed in the larval midgut after MPs treatment. The size of the MPs prevents the cellular uptake of these particles, which probably is the reason why any visible intracellular histopathological changes weren't detected (Jovanović et al., 2018). On the other hand, MPs ingestion by *C. ripraius* and its potential to aggregate and block the gut passage was described by Silva et al. (2021), thus brush border shortening could be a consequence of direct mechanical damage. Additionally, the lack of nutritive particles in the midgut lumen, due to the MPs accumulation, could lead to the microvilli shortening, similar to that in *D. melanogaster* starvation study (Li-Byarlay et al., 2016). Since all investigated parameters related to LCFA treatment in this study showed significant alterations we proposed that fly ash is toxic to *C. riparius*.

Changes in BB/CA ratio and microvilli length were observed in both treatments. The posterior region of the insect midgut has long, thin, and regular microvilli in contrast to the anterior one (Caccia et al., 2019), which goes in favor of the midgut region II and III brush border selection as a potential histopathological biomarker. In order to determine if the length of the microvilli is dependent on the area of the cell that they were attached to, the BB/CA ratio was analyzed. A high standard deviation in the BB/CA ratio parameter, however, indicated that digestive cells could vary in size. One of the reasons for this occurrence could be the depth of the section during the histological preparation of the slides. Even though the digestive cells of regions II and III were described as cubical (Stojanović et al., 2021), they are not completely uniform in their cubic structure, and thus, the dimensions of the same cell may vary across different histological sections. The other explanation could be the natural variations in the size of intestinal epithelial cells. Consequently, it was concluded that BB/CA ratio cannot be used as a reliable bio-indicator in ecotoxicological studies.

When only the length of microvilli was observed, its tendency to shorten after exposure to MPs/LCFA was noticed. In the present study, it is proposed that the shortening of microvilli is a kind of defense mechanism against pollutants present in the intestinal lumen. Stojanović et al. (2021) reported that by reducing the absorption surface, the potential

for harmful components to penetrate the cells is minimized. A similar observation was made in a study involving *D. melanogaster* that was fed protease inhibitors, where a reduction in the length of microvilli was detected. Analyses of *D. melanogaster* intestine on TEM showed abnormal droplets or small vesicles formed on the top of the microvilli that eventually broke off causing the shortening of the brush border. Similar results were obtained in *D. melanogaster* larvae that were deprived of food (Li-Byarlay et al., 2016).

Available studies on MPs influence on freshwater organisms recorded the negative impact of MPs on the most common ecotoxicological endpoints for aquatic species (Ziajahromi et al., 2018). The genetic response to the presence of toxins, which refers to DNA damage that can be detected and measured as biomarkers, can be evaluated using the widely used comet assay method in both vertebrates and invertebrates, as well as primary producers (Schuijt et al., 2021). The main advantages of this method are its sensibility, simplicity, low cost, and applicability to almost any type of cell. This assay also has a few limitations which include the inability to detect aneugenic effects and epigenetic mechanisms that do not involve alterations of DNA sequences (Costa and Paulo Teixeira, 2014). In our study, comet assay didn't reveal any significantly higher tail intensity in MPs mixture treatment, containing PE, PVC, and PA particles. Among microplastic polymers, PE is considered one of the least hazardous (Lithner et al., 2011). It showed the lowest genotoxicity in a study with fish erythrocytes when compared to PET and PS (Jakubowska et al., 2020). The study on sub-organismal responses of *C. riparius* exposed to MPs by Muñiz-González et al. (2021) observed increased expression of genes related to DNA repair. Therefore, the DNA damage that may have occurred in the present study could have triggered repair mechanisms, and as a result, the comet assay didn't reveal any significantly higher tail intensity in treated larvae. On the other hand, alterations in oxidative stress parameters were evident in the MPs treatment. Higher concentrations of TBARS and AOPP as well as increased activity of SOD clearly indicated the MPs potential to generate ROS and induce oxidative stress. Previous studies with *C. riparius* have also observed increased levels of lipid peroxidation, which was explained by MPs potential to induce physical stress rather than their chemical toxic potential (Silva et al., 2021). Considering all altered parameters, it is possible that MPs generate ROS which causes degradation of lipids, through the process of lipid peroxidation indicated by higher levels of TBARS. Additionally, SOD levels also showed increased antioxidant activity. Interestingly, even though all other tested parameters of oxidative stress were modified by MPs exposure, CAT activity remained unchanged. Previous studies proposed that CAT activates in the first hours/first day of exposure, and then gradually decreases (Regoli et al., 2011). On the contrary, other studies suggest that CAT is not actively involved in oxidative response to the MPs exposure in invertebrates (Ribeiro et al., 2017). Since chironomids are invertebrates, the unchanged activity of CAT could be expected. A novel study demonstrated that the red form of Hb in chironomids had higher antioxidant activity and could eliminate hydrogen peroxide by degrading it to water and oxygen via peroxidase activity (Szczerkowska-Majchrzak and Jarosiewicz, 2020). This could also explain the higher Hb concentration in MPs treatment in the present research. Studies have confirmed the potential of invertebrate hemoglobin (Hb) as a great biomarker for environmental monitoring (Ha and Choi, 2008). It is shown that the function of Hb in *C. riparius* is to enable aerobic metabolic processes in hypoxic conditions (Grazioli et al., 2016). In addition to this role, Hb also has the potential to be a novel biomarker of toxin presence, as evidenced by detected larval discoloration and reduced Hb levels in heavy metal-exposed larvae (Majumdar and Gupta, 2012) and suggested Hb's buffering potential for *C. riparius* larvae to adapt to acid conditions (Jernelöv et al., 1981). The length of microvilli changes in response to physiological stress, and thus, the modification of brush border length can be viewed as a sub-organismal response that is related to biochemical responses.

The treatment with LCFA exhibited the highest level of DNA damage, which is expected given that LCFA is a blend of various metal oxides with known toxicity to living organisms. High levels of genotoxicity were detected by comet assay in earthworm (*Dichogaster curgensis*) coelomocytes, following an in vitro exposure, with the suggested mechanism of this effect being the potential of metals to generate ROS (Manerikar et al., 2008). Available freshwater ecotoxicological studies examined the influence of fly ash on ROS generation, mostly on fish. Acute exposure of *Channa punctata* to fly ash resulted in induced LPO in all examined tissues, including the kidney, liver, and gills, as well as increased CAT and GST activity, which suggests that fly ash has the potential to induce oxidative stress and cause DNA damage via ROS generation (Ali et al., 2004). The activity of antioxidant enzymes in the present study has also been elevated in LCFA treatment, following the increased concentration of AOPP and hemoglobin, which has, as mentioned before, antioxidant activity in the red form in chironomids. To summarize, LCFA induced prominent alterations of all analyzed biomarkers. This toxic effect was clearly noticeable on the tissue level with numerous vacuoles and potential apoptotic bodies following a decrease in microvilli length. In a study by Shah and Parveen (2022), vacuolization of hepatocytes of the fish liver followed by increased enzymatic antioxidants was detected as a result of pesticide exposure in contaminated rivers of India. The appearance of vacuoles was explained by the excessive work of the organ to eliminate toxicants from the fish's body. The insect gut is a multi-tasking system that participates in multiple functions including nutrition, immunity, and osmoregulation (Huang et al., 2015). Vacuolization of the midgut digestive cells could have a similar purpose as the one described in the fish liver or be a direct negative impact of the LCFA that cause cell death as described in a previous study with acute exposure of *C. riparius* (Stojanović et al., 2021), or maybe both.

Biochemical and cellular responses in organisms can give information about the health status of an analyzed individual. Several studies have previously described the use of a multi-marker approach, which combines biochemical and histopathological biomarkers, to assess the toxicity of contaminants (Noleto et al., 2021; Shah and Parveen, 2022).

These biomarkers are the early signs of injury caused by the presence of contaminants (Gusso-Choueri et al., 2015). A holistic approach showed an advantage for pollution assessment in different aquatic environments since it enhances the sensitivity and detects responses to environmental changes even at low concentrations of the contaminants (Gusso-Choueri et al., 2015).

5. Conclusion

Histological analyzes showed great sensitivity of the digestive cells in the midgut of *C. riparius*. Intensive vacuolization of the midgut region II and III followed by formation of the apoptotic body-like structures indicating direct or indirect toxicity caused by LCFA exposure. Brush border measurements revealed its tendency to shorten when exposed to MPs and LCFA. Following that, other sensitive sub-organismal biomarkers analyzed, that includes DNA damage, oxidative stress parameters and hemoglobin concentrations, also showed negative impact of tested contaminants. Different methodology of the present study that was used to intercalibrate mentioned sub-organismal biomarkers with the potential histopathological ones, showed that the length of microvilli has a great potential for use in standard ecotoxicological assessment as a histopathological biomarker since it showed high sensitivity to the presence of the MPs and LCFA. It was also concluded that brush border length isn't linked to the cell area that it belongs to, so the BB/CA ratio cannot be used as a reliable biomarker.

Supplementary material S1 associated with this article can be found, in the online version, at doi: https://data.mendeley.com/datasets/tdkzfhhb7t/1. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166042

CRediT authorship contribution statement

Jelena Stojanović: Conceptualization, Methodology, Investigation, Writing – original draft. **Dimitrija Savić-Zdravković:** Conceptualization, Validation, Investigation, Writing – review & editing. **Boris Jovanović:** Validation, Writing – review & editing. **Jelena Vitorović:** Writing – review & editing, Validation. **Jelena Bašić:** Methodology, Writing – review & editing. **Ivana Stojanović:** Methodology, Writing – review & editing. **Andrea Žabar Popović:** Investigation, Writing – review & editing. **Hatice Duran:** Methodology, Writing – review & editing. **Margareta Kračun Kolarević:** Methodology, Writing – review & editing. **Đurađ Milošević:** Conceptualization, Validation, Formal analysis, Writing – review & editing, Supervision, Project administration.

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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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The ethical statement is uploaded as a Supplementary material S2.

References

Ali, M., Parvez, S., Pandey, S., Atif, F., Kaur, M., Rehman, H., Raisuddin, S., 2004. Fly ash leachate induces oxidative stress in freshwater fish Channa punctata (Bloch). Environ. Int. 30, 933–938. https://doi.org/10.1016/j.envint.2004.03.004.

Ali, M., Rahman, S., Rehman, H., Bhatia, K., Ansari, R.A., Raisuddin, S., 2007. Pro-apoptotic effect of fly ash leachates in hepatocytes of freshwater fish (Channa punctata Bloch). Toxicol. in Vitro 21, 63–71. https://doi.org/10.1016/j.tiv.2006.08.011.

Andreeva, L.I., Kozhemiakin, L.A., Kishkun, A.A., 1988. Modification of the Method of Determining Lipid Peroxidation in a Test Using Thiobarbituric Acid. Lab. Delo, pp. 41–43.

Bae, Y.J., Ryoo, K.S., Cho, K., Baek, M.J., 2014. A study on biosorptive removal of Cd from wastewater using Chironomid larvae (Diptera: Chironomidae). J. Adv. Chem. 10, 2415–2425. https://doi.org/10.24297/jac.v10i3.2272.

Bashir, I., Lone, F.A., Bhat, R.A., Mir, S.A., Dar, Z.A., Dar, S.A., 2020. Concerns and threats of contamination on aquatic ecosystems. In: Hakeem, K.R., Bhat, R.A., Qadri, H. (Eds.), Bioremediation and Biotechnology: Sustainable Approaches to Pollution Degradation. Springer International Publishing, Cham, pp. 1–26. https://doi.org/10.1007/978-3-030-35691-0_1.

Bernabò, P., Gaglio, M., Bellamoli, F., Viero, G., Lencioni, V., 2017. DNA damage and translational response during detoxification from copper exposure in a wild population of Chironomus riparius. Chemosphere 173, 235–244. https://doi.org/10.1016/j.chemosphere.2017.01.052.

Bernet, D., Schmidt-Posthaus, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 2001. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J. Fish Dis. 22, 25–34. https://doi.org/10.1046/j.1365-2761.1999.00134.x.

Bowmer, T., Jenner, H.A., Foekema, E., van der Meer, M., 1994. The detection of chronic biological effects in the marine intertidal bivalve cerastoderma edule, in model ecosystem studies with pulverised fuel ash: reproduction and histopathology. Environ. Pollut. 85, 191–204. https://doi.org/10.1016/0269-7491(94)90086-8.

Caccia, S., Casartelli, M., Tettamanti, G., 2019. The amazing complexity of insect midgut cells: types, peculiarities, and functions. Cell Tissue Res. 377, 505–525. https://doi.org/10.1007/s00441-019-03076-w.

Chaitanya, R.K., Shashank, K., Sridevi, P., Chaitanya, R.K., Shashank, K., Sridevi, P., 2016. Oxidative stress in invertebrate systems. In: Free Radicals and Diseases. IntechOpen. https://doi.org/10.5772/64573.

Cherry, D.S., Guthrie, R.K., Sherberger, F.F., Larrick, S.R., 1979. The influence of coal ash and thermal discharges upon the distribution and bioaccumulation of aquatic invertebrates. Hydrobiologia 62, 257–267. https://doi.org/10.1007/BF00043543.

Costa, S., Paulo Teixeira, J., 2014. Comet assay. In: Wexler, P. (Ed.), Encyclopedia of Toxicology, Third edition. Academic Press, Oxford, pp. 1020–1023. https://doi.org/10.1016/B978-0-12-386454-3.01072-1.

Denton, D., Shravage, B., Simin, R., Mills, K., Berry, D.L., Baehrecke, E.H., Kumar, S., 2009. Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. Curr. Biol. 19, 1741–1746. https://doi.org/10.1016/j.cub.2009.08.042.

Góth, L., 1991. A simple method for determination of serum catalase activity and revision of reference range. Clin. Chim. Acta 196, 143–151. https://doi.org/10.1016/0009-8981(91)90067-M.

Grazioli, V., Rossaro, B., Parenti, P., Giacchini, R., Lencioni, V., 2016. Hypoxia and anoxia effects on alcohol dehydrogenase activity and hemoglobin content in Chironomus riparius Meigen, 1804. J. Limnol. 75 https://doi.org/10.4081/jlimnol.2016.1377.

Gusso-Choueri, P.K., Choueri, R.B., de Araújo, G.S., Cruz, A.C.F., Stremel, T., Campos, S., de Sousa Abessa, D.M., Ribeiro, C.A.O., 2015. Assessing pollution in marine protected areas: the role of a multi-biomarker and multi-organ approach. Environ. Sci. Pollut. Res. 22, 18047–18065. https://doi.org/10.1007/s11356-015-4911-y.

Ha, M.-H., Choi, J., 2008. Effects of environmental contaminants on hemoglobin of larvae of aquatic midge, Chironomus riparius (Diptera: Chironomidae): a potential biomarker for ecotoxicity monitoring. Chemosphere 71, 1928–1936. https://doi.org/10.1016/j.chemosphere.2008.01.018.

Harja, M., Teodosiu, C., Isopescu, D.N., Gencel, O., Lutic, D., Ciobanu, G., Cretescu, I., 2022. Using fly ash wastes for the development of new building materials with improved compressive strength. Materials 15, 644. https://doi.org/10.3390/ma15020644.

Horton, A.A., Walton, A., Spurgeon, D.J., Lahive, E., Svendsen, C., 2017. Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. Sci. Total Environ. 586, 127–141. https://doi.org/10.1016/j.scitotenv.2017.01.190.

Hu, M., Palić, D., 2020. Micro- and nano-plastics activation of oxidative and inflammatory adverse outcome pathways. Redox Biol. 37, 101620 https://doi.org/10.1016/j.redox.2020.101620.

Huang, J.-H., Jing, X., Douglas, A.E., 2015. The multi-tasking gut epithelium of insects. In: Insect Biochem. Mol. Biol., Insect Epitheliome, 67, pp. 15–20. https://doi.org/10.1016/j.ibmb.2015.05.004.

Jakubowska, M., Białowąs, M., Stankevičiūtė, M., Chomiczewska, A., Pažusienė, J., Jonko-Sobuś, K., Hallmann, A., Urban-Malinga, B., 2020. Effects of chronic exposure to microplastics of different polymer types on early life stages of sea trout Salmo trutta. Sci. Total Environ. 740, 139922 https://doi.org/10.1016/j.scitotenv.2020.139922.

Jernelöv, A., Nagell, B., Svenson, A., 1981. Adaptation to an acid environment in Chironomus riparius (Diptera, Chironomidae) from smoking hills, NWT, Canada. Holarct. Ecol. 4, 116–119.

Jovanović, B., Gökdağ, K., Güven, O., Emre, Y., Whitley, E.M., Kideys, A.E., 2018. Virgin microplastics are not causing imminent harm to fish after dietary exposure. Mar. Pollut. Bull. 130, 123–131. https://doi.org/10.1016/j.marpolbul.2018.03.016.

Kozbek, A.Y., Şahin, K., Sarı, E., Bedir, E., Yüce, F.G., Çitoğlu, S., Duran, H., 2022. Evaluation of amine functionalized thermal power plant solid waste for industrial wastewater remediation. Adsorpt. Sci. Technol. 2022 (12006), 1–14. https://doi.org/10.1155/2022/8335566.

Li-Byarlay, H., Pittendrigh, B.R., Murdock, L.L., 2016. Plant defense inhibitors affect the structures of midgut cells in Drosophila melanogaster and *Callosobruchus maculatus*. Int. J. Insect Sci. 8, IJIS.S28595 https://doi.org/10.4137/IJIS.S28595.

Lithner, D., Larsson, Å., Dave, G., 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Sci. Total Environ. 409, 3309–3324. https://doi.org/10.1016/j.scitotenv.2011.04.038.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maddela, N.R., Ramakrishnan, B., Kakarla, D., Venkateswarlu, K., Megharaj, M., 2022. Major contaminants of emerging concern in soils: a perspective on potential health risks. RSC Adv. 12, 12396–12415. https://doi.org/10.1039/D1RA09072K.

Majumdar, T.N., Gupta, A., 2012. Acute and chronic toxicity of copper on aquatic insect Chironomus ramosus from Assam, India. J. Environ. Biol. 33, 139–142.

Malhotra, N., Chen, J.-R., Sarasamma, S., Audira, G., Siregar, P., Liang, S.-T., Lai, Y.-H., Lin, G.-M., Ger, T.-R., Hsiao, C.-D., 2019. Ecotoxicity assessment of Fe3O4 magnetic nanoparticle exposure in adult zebrafish at an environmental pertinent concentration by behavioral and biochemical testing. Nanomaterials 9, 873. https://doi.org/10.3390/nano9060873.

Manerikar, R.S., Apte, A.A., Ghole, V.S., 2008. In vitro genotoxicity of fly ash leachate in earthworm coelomocytes. Toxicol. Environ. Chem. 90, 293–300. https://doi.org/10.1080/02772240701458451.

Minami, M., Yoshikawa, H., 1979. A simplified assay method of superoxide dismutase activity for clinical use. Clin. Chim. Acta 92, 337–342. https://doi.org/10.1016/0009-8981(79)90211-0.

Muñiz-González, A.-B., Silva, C.J.M., Patricio Silva, A.L., Campos, D., Pestana, J.L.T., Martínez-Guitarte, J.-L., 2021. Suborganismal responses of the aquatic midge Chironomus riparius to polyethylene microplastics. Sci. Total Environ. 783, 146981 https://doi.org/10.1016/j.scitotenv.2021.146981.

Noleto, K.S., de Oliveira, S.R.S., Lima, I.M.A., de Jesus, W.B., da Silva Castro, J., de Santana, T.C., de Lima Cardoso, R., Jorge, M.B., Santos, D.M.S., de Souza Torres-Júnior, J.R., Fortes Carvalho Neta, R.N., 2021. Biochemical and histological biomarkers in Crassostrea sp. (Bivalvia, Ostreidae) for environmental monitoring of a Neotropical estuarine area (São José Bay, Northeastern Brazil). Bull. Environ. Contam. Toxicol. 106, 614–621. https://doi.org/10.1007/s00128-021-03149-z.

OECD, 2004a. Test No. 218: Sediment-Water Chironomid Toxicity Using Spiked Sediment. Organisation for Economic Co-operation and Development, Paris.

OECD, 2004b. Test No. 219: Sediment-Water Chironomid Toxicity Using Spiked Water. Organisation for Economic Co-operation and Development, Paris.

OECD, 2018. Sediment-Water Chironomid Life-Cycle Toxicity Test (OECD TG 233). OECD, Paris. https://doi.org/10.1787/9789264304741-17-en.

Palmer, J., Herat, S., 2021. Ecotoxicity of microplastic pollutants to marine organisms: a systematic review. Water Air Soil Pollut. 232, 195. https://doi.org/10.1007/s11270-021-05155-7.

Poynton, H.C., Vulpe, C.D., 2009. Ecotoxicogenomics: emerging technologies for emerging contaminants1. JAWRA J. Am. Water Resour. Assoc. 45, 83–96. https://doi.org/10.1111/j.1752-1688.2008.00291.x.

Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., Lemos, B., 2019. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. Sci. Total Environ. 662, 246–253. https://doi.org/10.1016/j.scitotenv.2019.01.245.

Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011. Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. Aquat. Toxicol. 105, 56–66. https://doi.org/10.1016/j.aquatox.2011.06.014.

Ribeiro, F., Garcia, A.R., Pereira, B.P., Fonseca, M., Mestre, N.C., Fonseca, T.G., Ilharco, L.M., Bebianno, M.J., 2017. Microplastics effects in Scrobicularia plana. Mar. Pollut. Bull. 122, 379–391. https://doi.org/10.1016/j.marpolbul.2017.06.078.

Rodrigues, P., Silvestre, J.D., Flores-Colen, I., Viegas, C.A., Ahmed, H.H., Kurda, R., de Brito, J., 2020. Evaluation of the ecotoxicological potential of fly ash and recycled concrete aggregates use in concrete. Appl. Sci. 10, 351. https://doi.org/10.3390/app10010351.

Romero, A., Estévez-Calvar, N., Dios, S., Figueras, A., Novoa, B., 2011. New insights into the apoptotic process in mollusks: characterization of caspase genes in Mytilus galloprovincialis. PLoS One 6, e17003. https://doi.org/10.1371/journal.pone.0017003.

Scherer, C., Brennholt, N., Reifferscheid, G., Wagner, M., 2017. Feeding type and development drive the ingestion of microplastics by freshwater invertebrates. Sci. Rep. 7, 17006. https://doi.org/10.1038/s41598-017-17191-7.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

Schuijt, L.M., Peng, F.-J., van den Berg, S.J.P., Dingemans, M.M.L., Van den Brink, P.J., 2021. (Eco)toxicological tests for assessing impacts of chemical stress to aquatic ecosystems: facts, challenges, and future. Sci. Total Environ. 795, 148776 https://doi.org/10.1016/j.scitotenv.2021.148776.

Shah, Z.U., Parveen, S., 2022. Oxidative, biochemical and histopathological alterations in fishes from pesticide contaminated river Ganga, India. Sci. Rep. 12, 3628. https://doi.org/10.1038/s41598-022-07506-8.

Sherrard, R.M., Carriker, N.E., Greeley Jr., M.S., 2015. How toxic is coal ash? A laboratory toxicity case study. Integr. Environ. Assess. Manag. 11, 5–9. https://doi.org/10.1002/ieam.1587.

Silva, C.J.M., Patrício Silva, A.L., Campos, D., Machado, A.L., Pestana, J.L.T., Gravato, C., 2021. Oxidative damage and decreased aerobic energy production due to ingestion of polyethylene microplastics by Chironomus riparius (Diptera) larvae. J. Hazard. Mater. 402, 123775 https://doi.org/10.1016/j.jhazmat.2020.123775.

Smit, M.G.D., Bechmann, R.K., Hendriks, A.J., Skadsheim, A., Larsen, B.K., Baussant, T., Bamber, S., Sanni, S., 2009. Relating biomarkers to whole-organism effects using species sensitivity distributions: a pilot study for marine species exposed to oil. Environ. Toxicol. Chem. 28, 1104–1109. https://doi.org/10.1897/08-464.1.

Stanković, N., Kostić, I., Jovanović, B., Savić-Zdravković, D., Matić, S., Bašić, J., Cvetković, T., Simeunović, J., Milošević, D., 2020a. Can phytoplankton blooming be harmful to benthic organisms? The toxic influence of Anabaena sp. and Chlorella sp. on Chironomus riparius larvae. Sci. Total Environ. 729, 138666 https://doi.org/10.1016/j.scitotenv.2020.138666.

Stanković, J., Milošević, D., Savić-Zdraković, D., Yalçın, G., Yildiz, D., Beklioğlu, M., Jovanović, B., 2020b. Exposure to a microplastic mixture is altering the life traits and is causing deformities in the non-biting midge Chironomus riparius Meigen (1804). Environ. Pollut. 262, 114248 https://doi.org/10.1016/j.envpol.2020.114248.

Stanković, J., Milošević, D., Jovanović, B., Savić-Zdravković, D., Petrović, A., Raković, M., Stanković, N., Stojković Piperac, M., 2022. In situ effects of a microplastic mixture on the community structure of benthic macroinvertebrates in a freshwater pond. Environ. Toxicol. Chem. 41, 888–895. https://doi.org/10.1002/etc.5119.

Stojanović, J.S., Milošević, Đ.D., Vitorović, J.S., Zdravković, D.N.S., Stanković, N.R., Stanković, J.B., Vasiljević, P.J., 2021. Histopathology of Chironomus riparius (Diptera, Chironomidae) exposed to metal oxide nanoparticles. Arch. Biol. Sci. 73, 319–329. https://doi.org/10.2298/ABS210515025S.

Szczerkowska-Majchrzak, E., Jarosiewicz, M., 2020. A comparative study of the oxidative system in Chironomidae larvae with contrasting feeding strategies. Eur. Zool. J. 87, 463–474. https://doi.org/10.1080/24750263.2020.1810336.

Trestrail, C., Shimeta, J., Nugegoda, D., 2020. Sub-Lethal Responses to Microplastic Ingestion in Invertebrates: Toward a Mechanistic Understanding Using Energy Flux, pp. 247–283. https://doi.org/10.1201/9781003053071-19.

Uherek, C.B., Pinto Gouveia, F.B., 2014. Biological monitoring using macroinvertebrates as bioindicators of water quality of Maroaga stream in the Maroaga cave system, Presidente Figueiredo, Amazon, Brazil. Int. J. Ecol. 2014, e308149 https://doi.org/10.1155/2014/308149.

Upadhyay, S.K., Edrisi, S.A., 2021. Developing sustainable measures to restore fly-ash contaminated lands: current challenges and future prospects. Land Degrad. Dev. 32, 4817–4831. https://doi.org/10.1002/ldr.4090.

US EPA, O, 2013. Emerging Contaminants and Federal Facility Contaminants of Concern [WWW Document]. URL. https://www.epa.gov/fedfac/emerging-contaminants-and-federal-facility-contaminants-concern (accessed 4.26.23).

Witko-Sarsat, V., Friedlander, M., Capeillère-Blandin, C., Nguyen-Khoa, T., Nguyen, A.T., Zingraff, J., Jungers, P., Descamps-Latscha, B., 1996. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int. 49, 1304–1313. https://doi.org/10.1038/ki.1996.186.

Ziajahromi, S., Kumar, A., Neale, P.A., Leusch, F.D.L., 2018. Environmentally relevant concentrations of polyethylene microplastics negatively impact the survival, growth and emergence of sediment-dwelling invertebrates. Environ. Pollut. 236, 425–431. https://doi.org/10.1016/j.envpol.2018.01.094.