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ORIGINAL RESEARCH ARTICLE

Active biomonitoring in streams by using multimarker approaches of mussels

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ABSTRACT

BACKGROUND AND OBJECTIVES: Streams that pass through densely populated areas and business and industrial centers are continuously threatened by various pollutants, including metals and microplastics, originating from dispersed sources. Biomonitoring is necessary to evaluate the health of stream ecosystems, considering that streams are essential ecologically and for human life. A biomonitoring approach through multimarkers can provide a comprehensive picture of the condition of stream ecosystems. It can identify biomarkers that are sensitive and specific to the presence of certain types of pollutants. This study evaluates the ecosystem health of Code Stream, Yogyakarta, Indonesia, through active biomonitoring by transplanting mussels Anodonta woodiana into cages at three stations, representing mild (station 1), moderate (station 3), and severe (station 2) polluted ecosystem conditions based on human activities around the stream.

METHODS: The mussels were transplanted into the Code Stream. Then, on days 0, 3, 7, 14, 21, and 28, the organisms were taken, and their gills and mantle were dissected in the laboratory. The organs were analyzed for microplastic accumulation and characteristics, copper concentration, superoxide dismutase, catalase, acetylcholinesterase activities, metallothionein concentration, and deoxyribonucleic acid damage. Biomarkers sensitive to pollutants were evaluated by integrated biomarker response. The combined effects of the complexity of environmental factors on the biomarkers were analyzed by multiple-factor analysis.

FINDINGS: The Code Stream waters at all stations were polluted with microplastics and copper. The increase in the two pollutants in the mussel organs was a function of time, with no differences among stations. The abundance of microplastics and copper concentrations in the water was closely related to their accumulation in both organs. Exposure to various contaminants in the stream strongly increased the superoxide dismutase and catalase activities in both organs at the beginning of exposure in all stations, with the highest being at station 3. The acetylcholinesterase activity was strongly inhibited in the gills at station 2. The metallothionein concentration slightly increased, and the highest increase occurred in the gills at station 2. The deoxyribonucleic acid damage was more intense at stations 2 and 3. Integrated biomarker response analysis showed that deoxyribonucleic acid damage, catalase activity, and metallothionein concentration were biomarkers responsive to stream pollution. Multiple-factor analysis revealed that superoxide dismutase, catalase, and acetylcholinesterase activities were biomarkers that indicated the environmental pollution of Code Stream waters. Multimarker analysis confirmed that the pollution level at stations 2 and 3 was higher than at station 1.

CONCLUSION: Active biomonitoring can offer a more accurate and comprehensive view of the time-dependent link between exposure and biomarker response. This active biomonitoring strategy identified sensitive and specific biomarkers for the presence of metal and pesticide contaminants in stream ecosystems. The pollution of Code Stream waters harms oxidatively stressed mussels and may endanger human health via the food chain. This work contributes substantially to understanding pollution exposure and its effect on mussels. It develops pollution-sensitive biomarkers for routine stream health monitoring. Mitigation activities involving diverse stakeholders and public education on sustainable management efforts must continue to achieve sustainable development.

NUMBER OF REFERENCES	NUMBER OF FIGURES	NUMBER OF TABLES
84	10	1

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INTRODUCTION

Stream ecosystems are ecologically and socially remarkable because they provide habitats for animals and plants, water sources, and recreational areas for humans. Environmentally unoriented development can affect the health of ecosystems, especially streams and other aquatic ecosystems (Brettschneider *et al*., 2019). These aquatic systems are constantly threatened by various chemical pollutants (such as fertilizers, pesticides, plastics/microplastics, and heavy metals), which enter stream ecosystems from various dispersed sources, including urbanization processes and industrial, agricultural, recreational/tourism, and household activities. These anthropogenic activities affect changes in water quality and aquatic biota (Santana *et al*., 2018). Ultimately, these pollutants can threaten human health through the food chain. Stream biomonitoring is an effort to characterize the condition of stream ecosystems and the severity of biological degradation and for long-term trend analysis (Faquim *et al*., 2021). Active biomonitoring refers to the evaluation of the response of transplanted aquatic organisms, i.e., transplanting aquatic organisms from known low-pollution sites at pollution monitoring sites so that responses can be analyzed directly during the exposure period, to assess the impact of environmental changes in the aquatic system (Wepener *et al*., 2005). This biomonitoring reduces biotic variability by using standardized species with identical physiological properties, offers realistic environmental exposure, and incorporates various environmental elements affecting aquatic systems (Santana *et al*., 2018). Previous research has shown that the transplantation of mussels from low-pollution sites to high-pollution sites is a viable strategy for actively monitoring environmental change in aquatic ecosystems (Smolders *et al*., 2003). Biomonitoring approaches can be carried out by evaluating biological responses at the molecular or cellular level (Beghin *et al*., 2022). Biological responses at the molecular or cellular level (biomarkers) can provide sensitive early warning of environmental contaminants. Biomarkers can provide evidence of a response from direct exposure to environmental stressors (short-term response) (Adams *et al*., 2001; Nugroho *et al*., 2020). In stream ecosystems, a pollutant is generally mixed with other pollutants, so a single biomarker cannot provide a complete diagnosis of the condition of an ecosystem. A multi-biomarker (multimarker) approach enables an integrative picture of the effect of exposure to complex and dynamic pollutants on organisms. Georgieva *et al.* (2022) have conducted active biomonitoring research with a multi-biomarker strategy (antioxidative enzyme activities and deoxyribonucleic acid [DNA] damage) in three Bulgarian reservoirs by employing the mussel *Sinanodonta woodiana*. Georgieva *et al.* (2022) found a link between the metal pollution index and antioxidative enzyme activities, and that DNA damage is a sensitive biomarker. Mussels are ideal bioindicators because they are sessile, filter feeders, and long-lived organisms. Compared with other bioindicators, such as fish, mussels filter the water for food, so all materials in the water, including various contaminants, can enter the mussel's body (Dvoretsky and Dvoretsky, 2023). This organism can accumulate significant contaminants in its organs (Georgieva *et al*., 2022). Kim and Choi (2017) reported metal enrichment in transplanted mussel *Mytilus galloprovincialis* compared with the starting values and regional gradients consistent with dissolved and particulate metal concentrations in water. Mussels also attract interest in assessing human health concerns linked with water deterioration (Georgieva *et al*., 2022). The effectiveness of biomonitoring programs depends on selecting relevant biomarkers with high sensitivity and specificity to the presence of different types of pollutants in the stream ecosystem. For this reason, biomonitoring was conducted by assessing the organism's response to a pollution gradient along the stream course (Meng *et al*., 2023; Santana *et al*., 2018). The Code Stream is the mainstream in the Special Region of Yogyakarta, Indonesia. Most areas near the stream are highly populated with diverse residential and industrial activities, and garbage from these activities can pollute stream water. The dominance of multiple types of plastic waste from anthropogenic activities along the flow of the Code Stream is a source of microplastics (MPs) (Sabilillah *et al*., 2023). In an initial survey in 2023, high concentrations of copper (Cu) were found in the sediments of the Code Stream. The present study continues the passive biomonitoring conducted in 2022, and active biomonitoring has never been conducted in the Code Stream. This biomonitoring was conducted actively using the mussel *Anodonta woodiana* transplanted in cages in the stream,

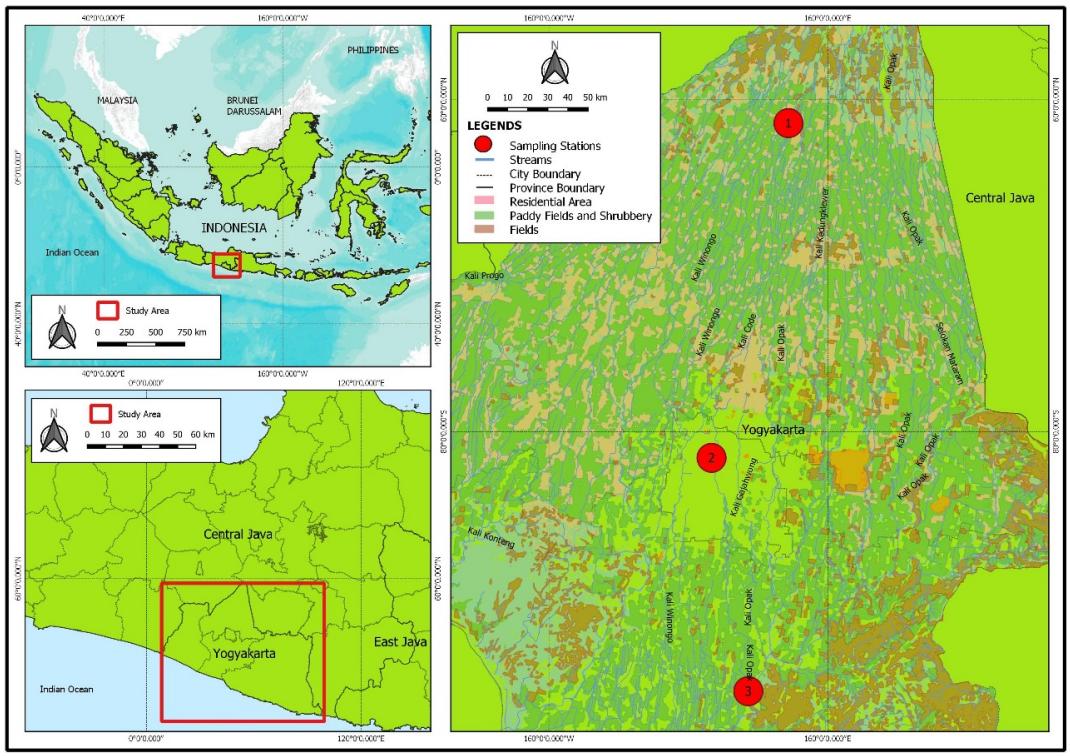


Fig. 1: Geographic location of the study area along with the sampling stations in Code Stream in the special region of Yogyakarta, Indonesia

following the pollution gradient. The freshwater mussel *A. woodiana* has been recognized as a reliable bioindicator for active biomonitoring (Chen *et al*., 2019). This mussel is exposed to not only MPs and Cu but also the complexity of environmental pollutants in each station. Active biomonitoring in the Code Stream was conducted through a multi-biomarker approach, which evaluated the superoxide dismutase (SOD), catalase (CAT), and acetylcholinesterase (AChE) activities; the metallothionein (MT) concentration; and the DNA damage in these mussels as a function of time and environmental condition at each station. Several biomarkers, including AChE, CAT and SOD, MT, and DNA damage, have been used in the biomonitoring of stream ecosystem health (Liu and Wang, 2012; Moussa *et al*., 2022). SOD and CAT activities are selected as biomarkers because both enzymes protect cells against oxidative stress due to exposure to pollution mixtures (Bigot *et al*., 2011). AChE is involved in the transmission of nerve impulses, whose activity can be affected by various types of exposure to contaminant mixtures (Aguirre-Martínez and Martín-Díaz, 2020; Catherine *et al*., 2016; Perić and Burić, 2019). MT maintains cellular metal homeostasis and responds to metal stress (Bigot *et al*., 2011; Mourgaud *et al*., 2002). Interpretation of these biomonitoring studies is expressed as integrated biomarker response (IBR), a graphical synthesis of different biomarker responses, and a numerical value that integrates all the responses. This active biomonitoring approach evaluates the combined effects of the complexity of environmental pollutants on the biomarkers of *A. woodiana* through multiple-factor analysis (MFA). The results of this study could reflect the pollution and health of the stream ecosystem and the potential risk of pollution to human health. The study aims to evaluate the ecosystem health of the Code Stream in Yogyakarta, Indonesia, through active biomonitoring using multimarker approaches. It was carried out in Code Stream in 2023.

MATERIALS AND METHODS

Study area and test organism

The Code Stream is located in the special region of Yogyakarta, Indonesia. It crosses Sleman, Yogyakarta,

and Bantul Regencies. The field study was conducted from May 2023, to June 2023, at three sampling stations (Fig. 1), representing lightly, moderately, and heavily polluted ecosystem conditions on the basis of human activities around the stream and confirmed by remote sensing. Station 1 (St1) is the upstream area of the Code Stream classified as a third-order stream, because it is a meeting of various springs that form a fast stream flow (7°38'19.5''S 110°24'11.5''E). This location was chosen because the population density around the site is relatively low, and no significant anthropogenic activities occur in the vicinity of the stream basin. Therefore, it is considered the station with the lowest pollution level. Station 2 (St2) is the middle area of the Code Stream located in the center of Yogyakarta City. This stream area belongs to the fourth-order stream with slower flow velocity and a stream width between 10–15 meters (m, 7°47'19.2''S 110°22'06.6''E). The area around the station is relatively densely populated and has various anthropogenic activities with high intensity. Visually, the pollution level at this station is relatively the highest. Station 3 (St3) is located downstream of the Code Stream (7°53'34.2''S 110°23'06.3''E). This station is the outflow of the Code Stream, and it belongs to the sixth-order stream with a lower slope and slower stream flow towards the Opak Stream. The area around the station is residential, but the density is relatively lower. Around St3, which is also an agricultural area, the pollution level is considered lower than that at St2. The mussels *A. woodiana* used in this study were obtained from the natural stream waters in the Pangandaran area, West Java. Mussels' developmental stage and age can influence their biochemical and physiological processes. Thus, mussels of comparable size and weight were employed to reduce this effect. The organisms measured 12 ± 2 centimeters (cm) in length and weighed 100 ± 10 grams (g).

Sample collection

At each location, 18 *A. woodiana* mussels were transplanted into 1 m × 2 m bamboo cages and placed 50 cm deep in the stream. Three mussels were taken from each station on days 0, 3, 7, 14, 21, and 28. The samples were placed in a cold box, transported to the laboratory, cleaned with distilled water, and stored in a freezer at −20 degrees Celsius (˚C) until further analysis. The mussels' gills and mantle were dissected and separated into five parts to analyze Cu (gills), MP, enzyme activities, MT, and DNA damage. Each portion was weighed to calculate the wet weight. For MP and Cu analysis in water, surface water was randomly sampled with a 1 liter (L) water sampler at each station (triplicate) and stored in 1000 milliliter (mL) glass bottles (Asare *et al*., 2018; McNeish *et al*., 2018). Glass bottles containing water samples were promptly capped to avoid contamination. The materials were placed in a cold box and transported to the laboratory for further analysis. Sediment samples were obtained from each station (triplicate) by using 1-L Ekman Dredge samplers following the procedure of Hidalgo-Ruz *et al.* (2012) to analyze MP and Cu in sediment. The samples were labeled and stored in 1 kilogram (kg) aluminum foil Ziplock bag.

Extraction of MP

MP extraction in mussel organs was carried out following the method of Adji *et al*. (2022). The gills and mantle were placed in a 50 mL Erlenmeyer flask (Pyrex), and 10 percent (%) potassium hydroxide (KOH) was added until the organs were fully submerged in the solution. The samples were then dried in an oven for 24 hours at 70 ˚C. They were filtered through a 0.45 micrometer (µm) filter paper (Whatman TM, UK). Each filter paper was placed in a Petri dish and labeled. The water sample was extracted by sifting it with a filter paper. The paper was placed in a Petri dish and labeled for MP observation of the sample. The sample Petri dish was promptly closed during data collection to avoid the MP from becoming contaminated by air. Field sampling, laboratory preparation, and MP analysis emphasized quality control and quality assurance. The presence of MP contamination during extraction was evaluated using a control filter paper. The number of replicates was 10. The sample on the Petri dish was quickly and permanently closed to prevent MP contamination from the air during field data collection. The sample preservation equipment was cleaned with distilled water and filtered using a 0.45 µm filter paper (Sabilillah *et al*., 2023).

Characterization of MP

MP particles were physically characterized by measuring the length of each particle in accordance with the method of Adji *et al*. (2022). MPs were divided into three categories: small (less than 1.5

millimeter: mm), medium (1.5–3.3 mm), and large (more than 3.3 mm). The colors and shapes of the MPs were observed (Li *et al*., 2021). MPs were classified into several shape categories (i.e., fibers, fragments, films, and pellets). The colors of MPs were categorized into red, blue, black, green, yellow, white/transparent, and other colors. Observations were performed using a microscope (Leica DM 100) with Image Raster 3. The polymer MP was determined via Fourier transform infrared spectroscopy (FTIR, Nicolet iS10). The particles utilized in the analysis were chosen randomly, with particle quantity, size, and diversity varying between samples.

Determination of Cu

The Cu in mussel gills was analyzed by referring to the method of Asare *et al*. (2018). Samples (1 g dry weight) were placed into a 50 mL Erlenmeyer flask (Pyrex). Next, 5 mL of concentrated sulfuric acid (H_2SO_4, Merck) and 10 mL nitric acid (HNO_3, Merck) were added and heated on a hot plate at 130 ˚C for 20 minutes. The sample was filtered with a 0.45 µm filter paper (Whatman TM, UK) in a 50 mL volumetric flask (Pyrex), and bi-distilled water was added to the limit. Cu content was determined using flame atomic absorption spectroscopy (FAAS) with a Cu detection limit of 0.1 micrometers per liter (µg/L). Cu concentration was expressed as milligrams per kg (mg/kg) dry weight. The Cu analysis on water and sediment samples followed the method of Adji *et al*. (2022). Water samples were filtered through a number 20 sieve to separate large particles. A 50 mL water sample was added with 1 mL concentrated HNO_3 and heated to a reduced volume of 20 mL on a hot plate at 105 °C–120°C. The sample was filtered with a 0.45 µm filter paper in a 50 mL volumetric flask, and bi-distilled water was added to the limit. The Cu concentration in water was determined by FAAS and expressed as mg/L. Sediment samples were dried at room temperature and sieved with a graded sieve to obtain fine particles. Sediment samples of as much as 0.5 g were placed into a 250 mL Erlenmeyer flask, added with 25 mL bi-distilled water, stirred using a stirring rod, added with 5–10 mL concentrated HNO_3, and stirred until evenly distributed. The samples were added with 3–5 boiling stones and covered with a watch glass. The Erlenmeyer flask was placed on a hot plate at 105 °C–120°C, up to a volume of 10 mL. Afterwards, 1–3 mL of concentrated perchloric acid ($HClO_4$) was added, and then the sample was reheated on a hot plate until a white smoke appeared and the solution became clear. The sample was filtered with a 0.45 µm filter paper in a 50 mL volumetric flask, and bi-distilled water was added to the limit. Cu concentration was determined by FAAS and expressed in mg/kg.

Sample extraction for biomarker analysis

Frozen tissue samples weighing 0.1 g were thawed and mixed with 500 microliter (µL) potassium chloride ([KCl, 150 millimoles per L: mmol/L]/phosphate buffer [50 mmol/L; potential of Hydrogen (pH) 7.4]) containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol, and 0.01% w/v phenylmethanesulfonyl fluoride (PMSF). The samples were homogenized in an ice bath with 12 sonicator strokes at 20 kilohertz (kHz) and 50 watts (W) of acoustic power before centrifugation at 4 °C for 30 minutes at 10,000 times gravity (x *g*). The supernatants were analyzed to determine the enzymatic activity and DNA damage (Nugroho and Frank, 2012).

For MT analysis, 0.1 g frozen tissue samples were thawed and mixed with 500 µL sucrose ([0.5 moles per liter: mol/L]/Tris hydrochloride [HCl, 20 mmol/L; pH 8.6]) buffer. Antiproteolytic agents leupeptin (6 micromoles per liter: µmol/L) and PMSF (0.5 mmol/L) were added, and β-mercaptoethanol (0.01%) was utilized as a reducing agent. The mixtures were sonicated in an ice bath for 12 strokes with a sonicator set to 20 kHz and an acoustic power of 50 W. The homogenate was centrifuged at 10,000 x *g* for 30 minutes at 4 °C. The supernatants were utilized to determine MT (Nugroho and Frank, 2012).

SOD activity

SOD activity was analyzed following the method of Marklund and Marklund (1974) in Kim *et al*. (2018) and expressed as units per milligram wet weight (1 unit is the amount of enzyme used to inhibit 50% auto-oxidation of pyrogallol per minute).

CAT activity

CAT activity was analyzed following the protocol of Aebi (1984), which was modified by Kasmiyati (2016) and measured with a microplate reader at 240 nanometers (nm) wavelength for 3 minutes (1-minute intervals). The expansion coefficient of hydrogen

peroxide (H_2O_2) was 40 millimolar per cm (mM/cm). Enzyme activity was expressed in µmol H_2O_2 per gram wet weight.

AChE activity

AChE activity was determined by colorimetric method following Ellman *et al*. (1961) and Tu *et al*. (2009) and expressed in nanomoles of acetylthiocholine iodide hydrolyzed per minute per mg wet weight.

MT concentration

The assay for MT concentration followed the protocol of Viarengo *et al*. (1999). A reduced glutathione (GSH) stock solution was prepared to plot a calibration curve to determine the concentration of MT, with 1 micromolar (µM) GSH = 0.55 µM MT. The MT concentration was expressed as nanomolar per gram (nM/g) wet weight.

DNA damage

DNA damage was analyzed by comet assay in reference to Izdihar *et al*. (2023). In the present study, the DNA damage was evaluated with the parameters of the percentage tail intensity (TI%, % of DNA in the tail), head intensity (HI, % of DNA in the head), and tail factor (TF, a measure for the degree of DNA fragmentation in a cell population) by using the Comet Score: Automatic Comet assay software series 2.0.0.38, which displayed the TI% and HI results. For TF, mussel nuclei were classified into five groups (A–E) on the basis of the tail's DNA amount. Category A exhibited 2.5% DNA in the tail, followed by categories B, C, D, and E, which exhibited cells with 12.5%, 30%, 67.5%, and 97.5% DNA tails, respectively (Focke *et al*., 2010). Category A damage suggested that the cells were primarily unharmed, whereas B–E represented higher levels of DNA fragmentation (Focke *et al*., 2010).

Data analyses

The variability in the activities of SOD, CAT, and AChE; the MT concentration; and the DNA damage (TI and HI) were tested by two-way ANOVA using SPSS (version 16.0, IBM Corp., New York, NY, USA), with station and exposure time as independent variables, followed by Dunnet testing ($p < 0.05$). A stress index called IBR was calculated to assess the health status of an organism or population by following the method of Bertrand *et al*. (2016) and Iturburu *et al*. (2018). IBR calculates all biomarkers into one index. The index calculates star plot areas, so IBR provides a comprehensive visualization of the response of all biomarkers to differences between exposures or stations in the Code Stream. The visualization could provide information on the health of the Code Stream ecosystem. MFA was conducted using R (version 4.2.2) with factorMineR, factorextra, and ggplot2 to assess the combined effects of various environmental variables on the biomarkers of *A. woodiana*.

RESULTS AND DISCUSSION

Microplastics in water

The water sample analysis revealed that MP contaminated the Code Stream waters at all stations. The mean number of MP particles was the highest in St3, followed by St2, and the lowest in St1 (Fig. 2a), and the means differed considerably among stations ($p < 0.05$). The low abundance of MP could be attributed to St1's remote location from residential areas. The presence of MP at St1 could be attributed to the activity of local fishermen. Garbage disposal from residential areas into St2 and St3 may be higher than into St1, resulting in substantial MP contamination at both stations. The significant quantity of MP particles detected in St3 could be influenced by the advection mechanism of stream water that transports MP from St2 to St3. Around this station is a residential area and a fishing ground for the local community. MP abundance can be affected by environmental factors, including seasons and currents (Rahmayanti *et al*., 2022). Similar findings were obtained in the Rhine Stream region, which is the most polluted urban area. MP abundance occurred in places very remote from the metro center (Mani *et al*., 2015). The present study's MP abundance in water was around 40%–140% higher than that of the passive biomonitoring in the Code Stream conducted in 2022. This finding shows that plastic waste management needs to be improved. Nearly 70% of MPs identified in the Code Stream were smaller than 1.5 mm in size (Fig. 2b). Several other places had similar results, such as the inlet and outlet of Jombor Swamp (Rahmayanti *et al*., 2022), the Brisbane Stream in Australia (He *et al*., 2020), and the Han Stream in South Korea (Park *et al*., 2020). These findings attest to the fragmentation and degradation of plastics into secondary MP particles (Rahmayanti *et al*., 2022). The large percentage of

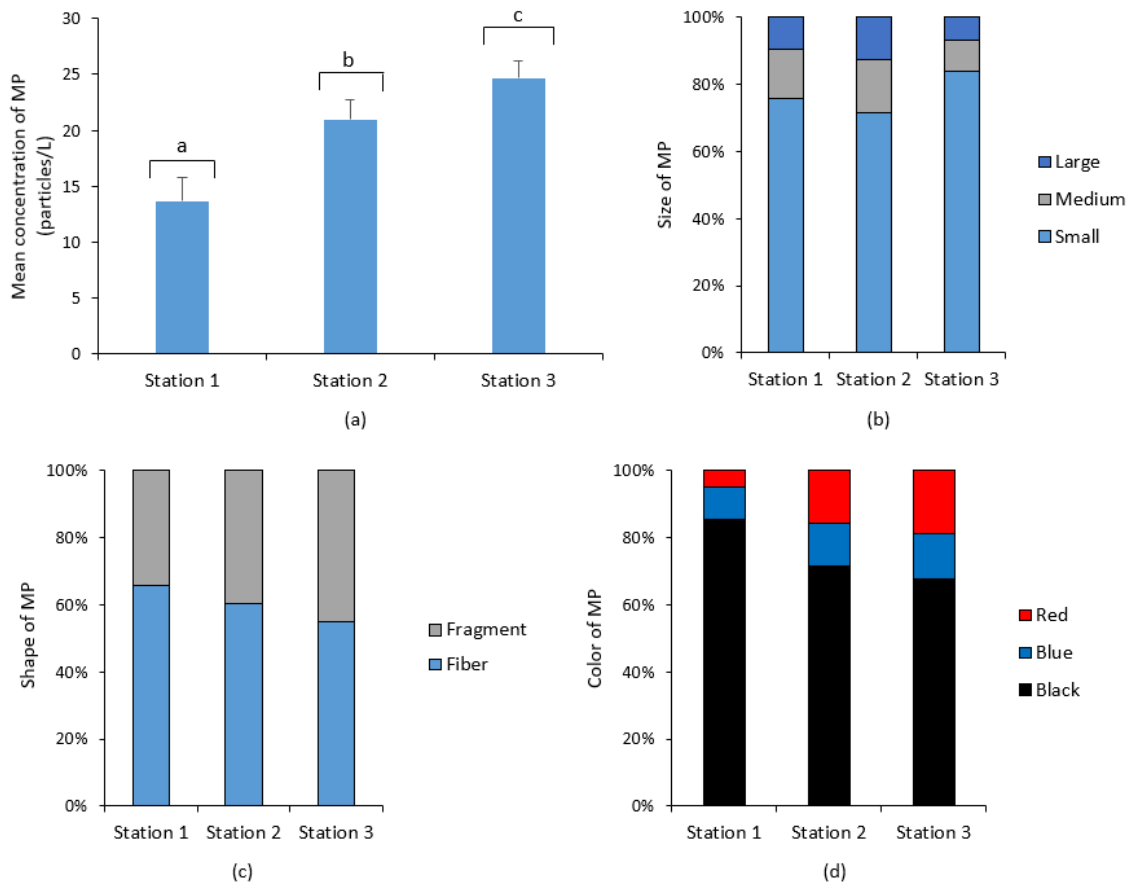


Fig. 2: (a) Mean, (b) size, (c) shape, and (d) color of MP found at three stations of Code Stream. Different letter in part (a) indicates significant difference among stations ($p < 0.05$)

small MPs (Fig. 2b) showed that the stream flow carries plastic debris from a long distance or for a long time, given that plastic takes a long time to disintegrate into small plastic particles (Sabilillah *et al*., 2023). In the present study, the MP particles that polluted stream water had various colours, including black, red, and blue (Fig. 2d), with black dominating all stations.

Fragments and fibers of MP were discovered at all three locations. Fibers comprised 65% of the MPs found at the three stations (Fig 2c). Similar results were reported by Su *et al*. (2018), who found that MPs in the form of fibers dominated the waters of the Yangtze Stream in China. Fiber contamination can be caused by high human activities around the stream such as fishing with fishing rods (Basri *et al*., 2021), washing clothes (Yang *et al*., 2021), and waste production from the household scale clothing industry or textile industry (Alam *et al*., 2019). The discovery of MP dominated by fragments was also reported by Babel *et al*. (2022) in 25 streams in Thailand. Rahmayanti *et al*. (2022) noted that the form of MP found at the inlet and outlet of Rawa Jombor was dominated by films and fibers. In addition, MPs in the form of films, fibers, and fragments dominated the Pearl Stream, China (Fan *et al*., 2019). According to Clere *et al*. (2022), fibers are more flexible than fragments, making them easier to transport by currents and more challenging to capture. The discovery of numerous types of MPs demonstrates that plastics degrade and fragment into smaller particles with varying shapes depending on the source and type of trash polluting the waters.

MP accumulation in mussels

The transplanted *A. woodiana* exposed to all three stations' waters showed increased MP accumulation in the gills and mantle (Figs. 3a and 3b). The MP accumulation showed a linear increase from day 0 to day 28. The MP abundance in both organs showed significant differences on certain exposure days with day 0 (Dunnet analysis, $p < 0.05$). The highest average

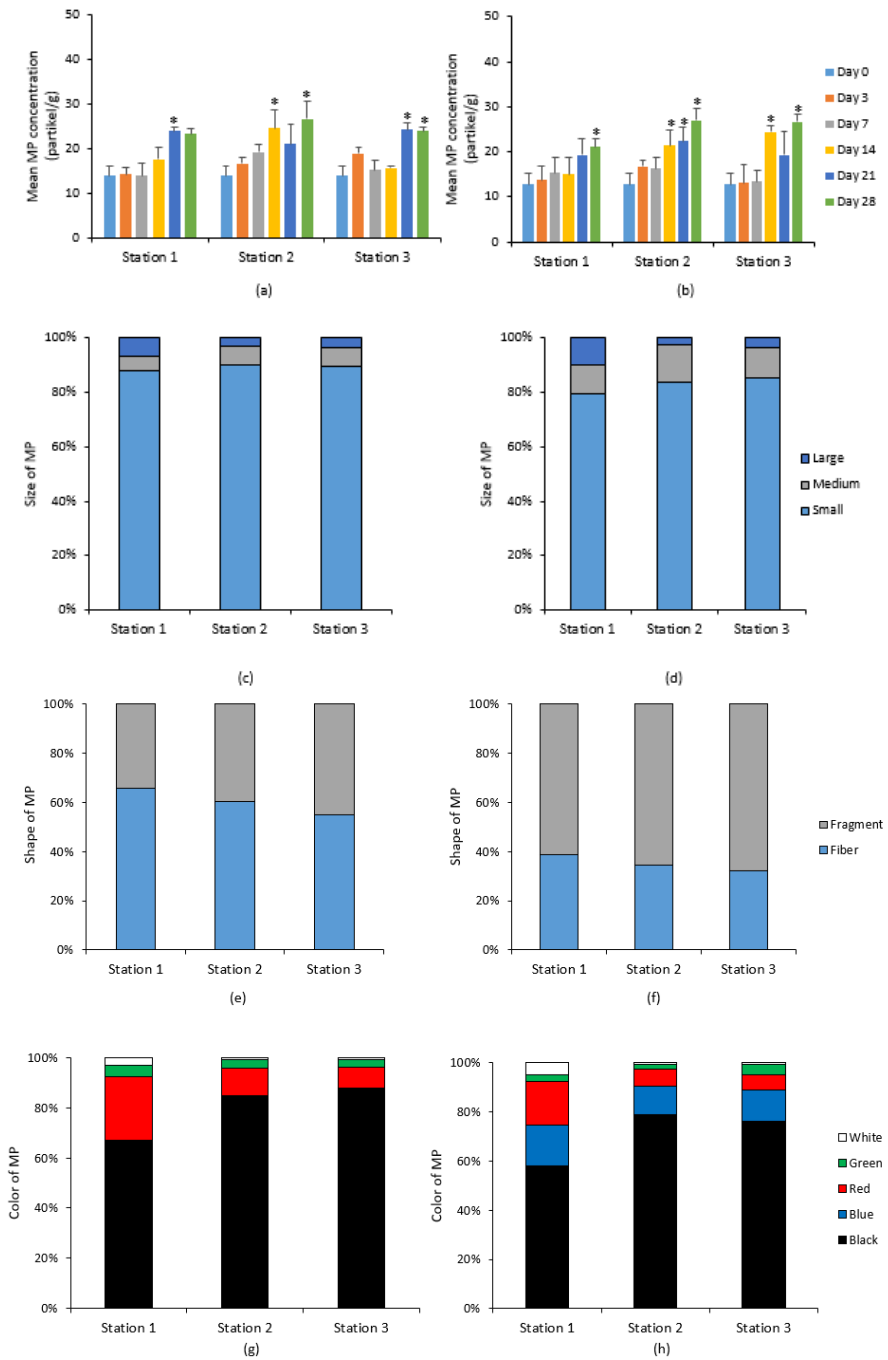


Fig. 3: (a) Mean of MP in the gills, (b) mean of MP in the mantle, (c) size of MP in the gills, (d) size of MP in the mantle, (e) shape of MP in the gills, (f) shape of MP in the mantle, (g) color of MP in the gills, and (h) color of MP in the mantle of *A. woodiana* at the three stations of Code Stream. The asterisk symbol at parts (a) and (b) indicates significant difference between the days of exposure and day 0 ($p < 0.05$)

accumulation of MP occurred on day 28, and it was two times the accumulation of MP compared with the lowest on day 0, adding 10–12 particles. The present study's results align with those of Kolandhasamy *et al*. (2018), who reported accumulation of MP in the gills and mantle of mussel *Mytilus edulis*. The present study also showed an increase in MP in Code Stream waters (Fig. 1a), followed by an increase in MP concentration in *A. woodiana*. This finding confirmed that mussels can record the pollution level, especially MP, in streams and thus can be used as bioindicators of pollution in stream ecosystems. The dynamics of MP in both organs can be affected by the length of exposure, water filtration rate, and the process of MP elimination from the mussel body (Barboza *et al*., 2020; Rahmayanti *et al*., 2022). The MP abundance in mussels did not alter significantly among stations. However, the MP concentrations in water increased from St1 to St3, with significant difference. Mussels' water filtering process, which brings water in and out of the mussel body, may have a role in the dynamics of MP accumulation. A laboratory study by Li *et al*. (2019) showed that mussels are suitable model organisms for studying MP accumulation and toxicity. Qu *et al*. (2018) confirmed the results of the present study, that is, MP accumulation in mussels is closely related to human activities and a positive correlation exists between MP accumulation in mussels and surrounding waters. On day 0, MP was found in the mussels, indicating MP contamination of the stream waters where the mussels were collected before being transplanted. Compared with passive biomonitoring, this active biomonitoring study can show the trend of MP accumulation in *A. woodiana*, along with the length of exposure time and exposure location (station). The accumulation of MPs in mussel organs can have a direct adverse effect, because various types of toxic pollutants can be attached to the surface of MP. Furthermore, MP enters the mussel's body and is distributed to organs or tissues; contaminants interact with these organs or tissues, and ultimately, toxic symptoms can occur (Kolandhasamy *et al*., 2018). Several studies have shown that MP has the potential to cause adverse effects, including endocrine disruption, energy disruption, oxidative stress, immune dysfunction, neurotransmission disorders, and even genotoxicity, on organisms (Avio *et al*., 2015; Lee *et al*., 2013; Rochman *et al*., 2014). In addition, plastic polymers can be toxic to organisms.

The form of MP found in mussel organs consisted of fragments and fibers. In contrast to the MPs in water, almost 65% of MPs in mussels were in fragment form (Figs. 3e and 3f). Among the sizes of MPs found in these two organs, nearly 80% were in small category (< 1.5 mm, Figs. 3c and 3d). The MP colors found in the mussel's gills and mantle were black, blue, red, green, and white (Figs. 3g and 3h), with black being the most dominant color. This result is similar to that of Sabilillah *et al*. (2023), who discovered that the MP colors in fish organs consisted of green, black, and red, with the dominance of small fiber shapes. Mussels actively filter water to obtain nutrients and indirectly ingest various pollutants and suspended particles (Inoue *et al*., 2021).

The analysis of MP polymers by FTIR showed that polymers of polyethylene terephthalate (PET), acrylonitrile butadiene styrene, polycarbonate, and latex were detected in the water and the gills and mantles of mussels (Fig. 4). The polymer type of MP was determined through matching with the absorption bands in the study of Jung *et al*. (2018). The polymer types found in the water (Fig. 4a) showed similarities with the polymers found in the gills (Fig. 4b) and mantle (Fig. 4c), indicating that the accumulation of MP in the mussels originated from the Code Stream waters. The accumulation of MP in these mussel organs may adversely affect the mussels. Choi *et al*. (2022) found that *M. galloprovincialis* mussels exposed to PET with concentrations of 0.0005, 0.1, 1, 10, and 100 mg/L for 32 days showed an increase in antioxidative enzyme (SOD and CAT) activities and related neurotoxicity (AChE) in the digestive glands and gill tissues. The exposure caused digestive tubule atrophy and decreased the sex hormones estradiol and testosterone in these organisms. Long-term exposure to MP, although not lethal, can cause reproductive failure in mussels (Choi *et al*., 2022).

Cu accumulation in mussels

Exposure of *A. woodiana* to the waters of the three stations led to an increasing trend of Cu in the gills during the exposure period (Fig. 5), but the Cu concentrations among the stations did not differ significantly ($p > 0.05$). The highest Cu concentration was found on day 28 at St2, two times higher than on day 0 ($p < 0.05$). The accumulation of Cu in the mussels was related to Cu pollution in the waters of the three

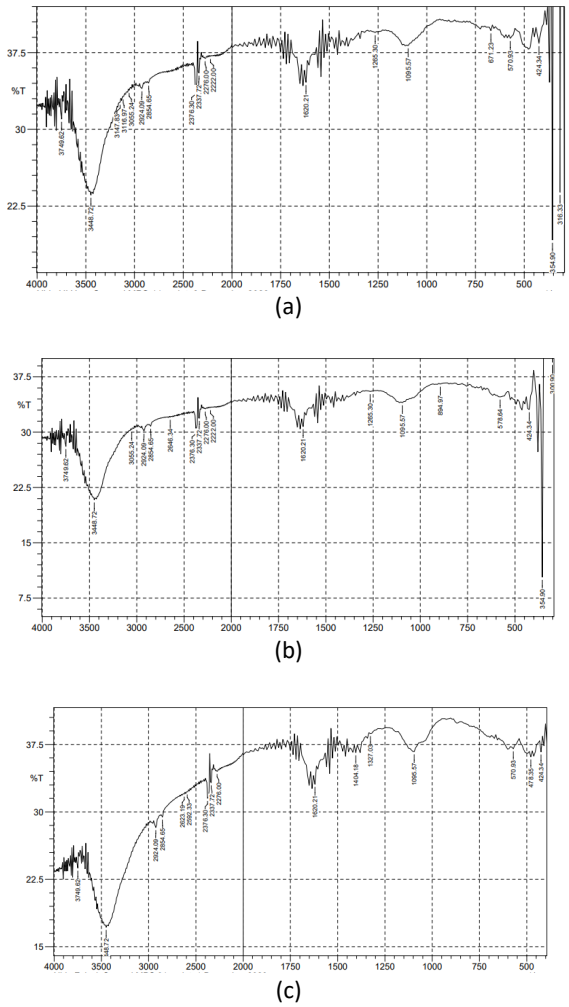


Fig. 4: FTIR spectra of microplastic samples in (a) water, (b) gills, and (c) mantle of *A. woodiana* from three stations of Code Stream

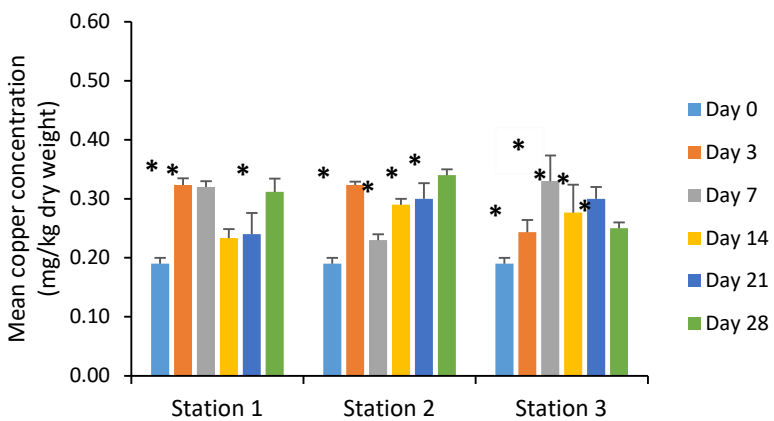


Fig. 5: Mean Cu in the gills of *A. woodiana* at three stations. The asterisk symbol (*) indicates significant difference between exposure days and day 0 ($p < 0.05$)

Table 1: Concentration of Cu in water and sediment at three stations of Code Stream

Station	Cu in water (mg/L)	Cu in sediment (mg/kg)
1	0.27–0.31	12.89–14.74
2	0.32–0.35	9.7–16.11
3	0.29–0.32	7.02–8.24

Code Stream stations (Table 1). According to Chen *et al*. (2023), mussels are filter-feeder organisms; when water is being filtered, it enters the mussel shell and then through the gills, and the Cu from the water can be accumulated in this organ through diffusion, active transport, or endocytosis. Mussel gills are used as an organ to study Cu bioaccumulation. Similar results were found in the research of Chen *et al*. (2019), who showed the accumulation of cadmium (Cd), aluminum (Al), and Cu in the gills of *A. woodiana* in the waters of Lake Taihu, China. In the present study, the dynamics of Cu concentration in the mussels during the exposure period (Fig. 5) indicated the accumulation and excretion process in the mussels. Through this active biomonitoring, the accumulation of Cu in this organism is a record of the pollution levels in the Code Stream during the exposure period (temporal scale), thus reflecting the trend or dynamic of Cu pollution in this stream. The passive biomonitoring conducted in the Code Stream by Sabilillah *et al*. (2023) determined only the level of metal accumulation (cadmium and lead) in fish and the occurrence of metal pollution in these waters at the time of sampling; the metal metabolism in the body of these organisms was not evaluated. Cu pollution in streams or other waters that exceed the threshold could directly and indirectly affect aquatic organisms (Velusamy *et al*., 2014). At high concentrations, Cu accumulation in mussel organs can cause adverse effects such as DNA damage and disruption of amino acid metabolism (Chalghmi *et al*., 2016; Chen *et al*., 2023). The level of metal exposure can be determined from the response of mussels, including GSH, S-transferase, CAT, AChE, and SOD activities; lipid peroxidation, heat shock protein 70, MT, and DNA damage levels; and amino acid metabolism (Chalghmi *et al*., 2016; Chen *et al*., 2023).

The highest concentrations of Cu in the water and sediment were found at St2 and St1, respectively (Table 1). St1 is close to the active mountain Merapi, so the high concentration of Cu in the sediment can be caused by metals entering through natural sources (Fadlillah *et al*., 2023). Moreover, the difference in the level of Cu concentration at each station in water and sediment is influenced by the level of activity and the habits of the community around the stream, such as garbage disposal, application of pesticides and agricultural fertilizers, and disposal of industrial waste (Asare *et al*., 2018). In the present study, the concentration of Cu in the Code Stream waters far exceeded the threshold allowed for biota life, which is around 0.008 mg/L (WHO, 2023). Based on the EPA-USA (2004) sediment pollution classification guidelines, the Cu threshold is 49.98 mg/kg. So, in the present study, the Cu concentration in the substrate was still below the allowable limit.

Biomarkers

The transplanted *A. woodiana* showed biochemical responses upon exposure to the environmental conditions of all three stations. The organisms were exposed to not only MP and Cu but also a complex of environmental pollutants. A significant increase in SOD activity occurred in the gills and mantle from day 3 of exposure, indicating oxidative stress in the mussel. Afterwards, the activity was relatively high throughout the exposure period ($p < 0.05$, Figs. 6a and 6b). The increase in SOD activity in the gills was slightly higher than that in the mantle, and the activity of this enzyme in St3 was relatively higher than in other stations. Significant changes in SOD activity were observed in the mantle between St1 and St2 compared with St3 ($p < 0.05$, Fig. 6b), whereas no significant differences were found in the gills ($p > 0.05$). SOD is the first line of defense, so when triggers for increased oxidative stress exist in the aquatic environment, its activity increases at the beginning of exposure. An increase in this enzyme indicates an increase in oxidative stress conditions, so it catalyzes the decomposition of superanion free radical ($O_2^{\cdot-}$) into oxygen (O_2) and H_2O_2 to reduce oxidative stress (Klimova *et al*., 2020). In cells, metals are involved in several reaction mechanisms that produce reactive oxygen species (ROS). These reactive species oxidize

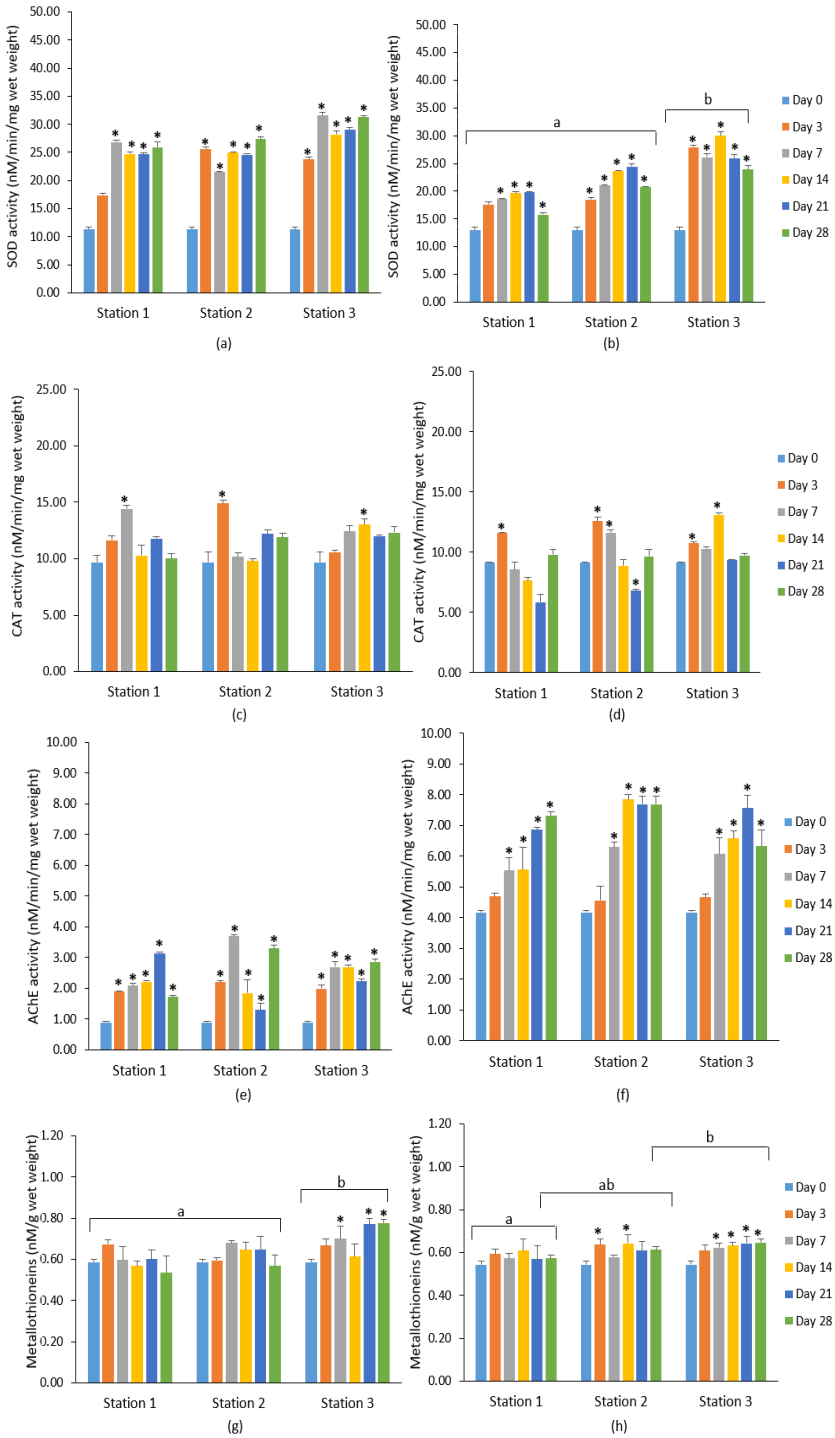


Fig. 6: Levels of (a) SOD in the gills, (b) SOD in the mantle, (c) CAT in the gills, (d) CAT in the mantle, (e) AChE in the gills, (f) AChE in the mantle, (g) MT in the gills, and (h) MT in the mantle of *A. woodiana* at three stations of the Code Stream during 28 days of exposure. The asterisk symbol (*) indicates significant difference between exposure days and day 0, and a different letter indicates significant difference between stations ($p < 0.05$)

lipids, proteins, and DNA, leading to changes in cell structure and mutagenesis. Transition metals (such as trivalent iron and divalent copper) participate in the Haber–Weiss cycle, generating hydroxyl radical ($^{\cdot}OH$) from $O_2^{\cdot-}$ and H_2O_2. In addition, metals without redox capacity (such as divalent cadmium, divalent lead, and divalent mercury) increase pro-oxidant status (Pinto *et al*., 2003). In the present study, the increase in SOD activity was followed or balanced by an increase in CAT activity (Fig. 6c and 6d). The enzyme CAT catalyzes the decomposition of H_2O_2 into H_2O and O_2 (Klimova *et al*., 2020; Wei *et al*., 2021). In transplanted *A. woodiana*, the CAT activity tended to fluctuate, but the highest increase in activity generally occurred at the beginning of exposure. The CAT activity increased drastically, especially on day 3, confirming the oxidative stress state. The increase in this enzyme activity indicated the decomposition of H_2O_2. The CAT activity at St3 was higher than at St1 but not significantly different ($p > 0.05$). Similar with SOD activity, the CAT activity in the gills was relatively higher than in the mantle. The activities of SOD and CAT in *A. woodiana* confirmed the occurrence of metal pollution in the Code Stream. CAT is a sensitive biomarker and considered as one of the earliest responses to pollution exposure. Many studies related to CAT activity have been conducted, such as exposure to organic pesticides and metals in mollusk species (Binelli *et al*., 2015; Manduzio *et al*., 2005). Tang *et al*. (2020) reported that exposure to MP with polyethylene (PE) or PET polymers did not cause significant fluctuations in CAT activity in oysters. The combined exposure of the polymers caused a significant increase in CAT activity due to the pathogenic bacteria on the surface of MP. Wei *et al*. (2021) reported a rapid increase in SOD and CAT activities in *M. galloprovincialis* in various tissues (digestive glands, gills, gonads, and muscles) exposed to MP. Regoli and Giuliani (2014) suggested that when organisms are under oxidative stress conditions, the activity of antioxidative enzymes, including CAT, tends to increase, thus reducing the ROS produced excessively and preventing further cell damage. AChE activity is an effect biomarker commonly used to detect physiological stress in the presence of pesticide pollution in waters (Rank *et al*., 2007). AChE is sensitive to metals, detergents, and complex mixtures of pollutants (Tu *et al*., 2009). It plays a role in nerve impulse transmission, and its inhibition is a biomarker of neurotoxicity (Fulton and Key, 2001). The AChE activity in *A. woodiana* fluctuated during exposure, especially in the gills at St2 (Fig. 6e and 6f). In the mantle, the activity tended to be higher than in the gills, and it increased linearly at all stations. Similar results were shown in a study that injected sewage extracts into mussels, increasing AChE activity (Gagné *et al*., 2010). The AChE activity, especially in the gills at St2, increased until day 7 then decreased on day 14 and reached its lowest level on day 21. This decrease indicates the inhibition of AChE activity, which may indicate the presence of pesticide contaminants and a complex mixture of pollutants in the Code Stream, mainly at St2. The increase in AChE activity on day 28 suggests the recovery of the enzyme (Matozzo *et al*., 2019; Tu *et al*., 2009). This enzyme activity was inhibited in the gills because it is the first organ exposed to contaminants in water. It is also the main site of absorption of xenobiotics due to their large surface area and permeability (Tu *et al*., 2009). Fulton and Key (2001) showed that organophosphate insecticides produce toxicity by inhibiting the AChE enzyme in the nervous system of aquatic organisms. MT is a low-molecular-weight, cysteine-rich protein that plays a significant role in the homeostasis of essential metals, such as Cu and zinc (Zn), and it is involved in metal detoxification (Catherine *et al*., 2016). MT induction is considered a biomarker of metal contamination and widely used as a tool in biomonitoring programs (Viarengo *et al*., 2007). The present study showed a slight increase in MT concentration during the exposure period. The highest MT concentration was found in the gills on day 28 at St3, indicating a higher level of metal contamination at St3 during exposure than at St1 and St2 (Fig. 6h). The MT concentrations between day 0 and other exposure days in St2 and St3 showed significant differences ($p < 0.05$). However, the MT concentrations between days in St1 did not show any significant difference ($p > 0.05$), indicating that the level of metal pollution at that station was relatively lower than at the other stations. Similar results were shown in the study of Catherine *et al*. (2016), which indicated that low MT concentrations in the mussels from the Eastern Mediterranean and the Black Sea could be attributed to low metal concentrations in these regions. This active biomonitoring allows the biomarker responses of *A. woodiana* and the environmental conditions of the Code Stream

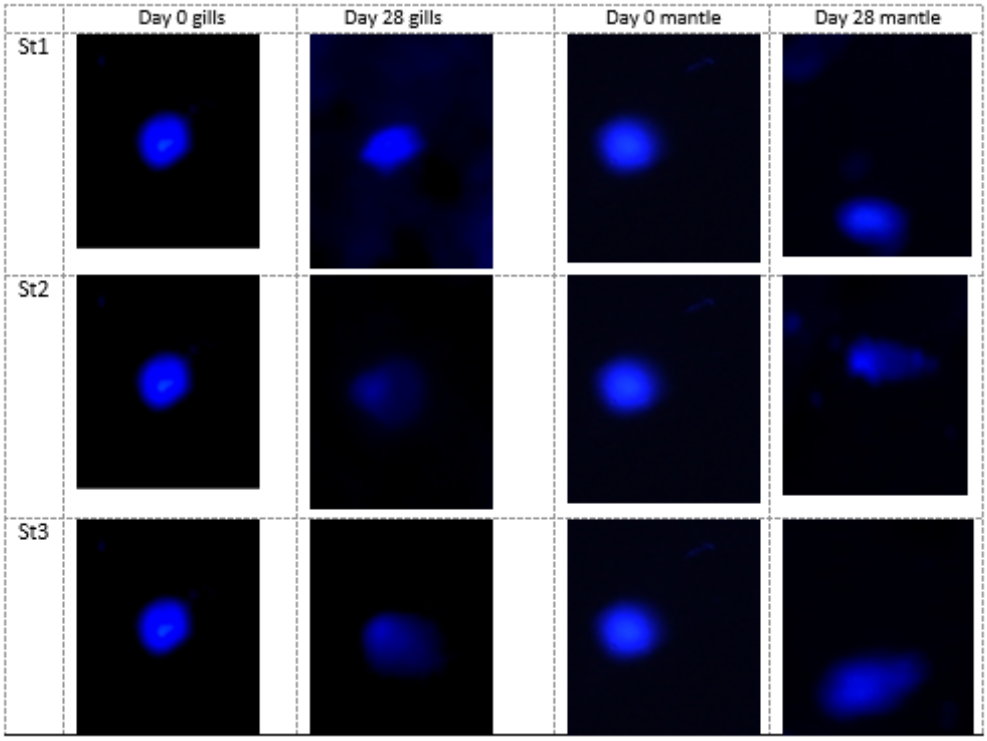


Fig. 7: Comets of DNA in the gills and mantle at the three stations of Code Stream after exposure for 0 and 28 days

waters to be examined on a temporal scale, thereby providing an overview of the dynamics of changes in the stream. This multimarker analysis confirmed that the pollution level at St2 and St3 was higher than at St1.

DNA damage

The 28-day exposure of transplanted *A. woodiana* to the waters of the Code Stream stations caused DNA damage, especially at St2 and St3, as indicated by the formation of comets (Fig. 7). DNA damage could form cells resembling comets with tails due to their movement towards the anode during electrophoresis. DNA damage in the gills and mantle of these mussels can occur through several mechanisms: One is through increased oxidative stress conditions caused by heavy metal accumulation in mussels (Chavan *et al*., 2018). Increased activities of SOD and CAT confirm the occurrence of oxidative stress.

In this study, DNA damage was assessed by measuring TI (%), HI (%), and TF (Focke *et al*., 2010; Knopper and Mcnamee, 2008). During the exposure period, the TI in the gills and mantle of mussels exposed at waters at St2 and St3 was slightly higher than at St1, with TI being the highest at St3. The TI in the gills was relatively higher than in the mantle. By contrast, exposure at all three stations caused a slight decrease in HI, with the strongest decline occurring at St3, followed by St2 and St1. The results of HI analysis (Figs. 8c and 8d) showed the lowest value in the gills on day 28 at St3, which is about 6% lower than on day 0. The TI values tends to be higher, indicating the displacement of DNA fragments to form comets, so the HI tends to decrease. This indicator shows an effect of exposure to the environmental conditions of the stations in the Code Stream that affect DNA. The extent of DNA damage was evaluated by TF analysis, which is a measure of the degree of DNA fragmentation in a cell population. Upon exposure to the Code Stream waters, most cells of *A. woodiana* were determined to belong to group B (DNA in tail 12.5%), followed by group C (30%). The number of cells belonging to group D (67.5%) tended to increase at St2 and St3. The highest intensity of DNA damage

was found in the mantle (Fig. 8b) on day 28 at St3, significantly different from the control on day 0 ($p < 0.05$). This finding may indicate that the pollution level at both stations was higher than at St1. These results also indicate the high sensitivity of DNA damage parameters as a function of time and environmental conditions (station). Kolarević *et al*. (2013) showed the sensitivity of comet assay test on *S. woodiana* in active biomonitoring of Cd and Zn metals in the Velika Morava Stream, Serbia. A study in Lake Igapo, Brazil, showed that the comet assay in *T. rendalli* has a high sensitivity that is worth using as a tool in water pollution monitoring and environmental health risk assessment (Lemos *et al*., 2005).

The mean TI values (Fig. 8a and 8b) indicated that the intensity of DNA in the tail increased significantly in the gill and mantle organs. The increase in TI% tended to be linear from day 0 to day 28. The increase in TI% in both organs showed significant differences between exposure days ($p < 0.05$). Greater TI% values indicate higher DNA fragmentation (Izdihar *et al*., 2023). The highest TI% value was found in the mantle organ on day 28 in St3, significantly different from that on day 0 ($P < 0.05$). These results indicate that TI% increases

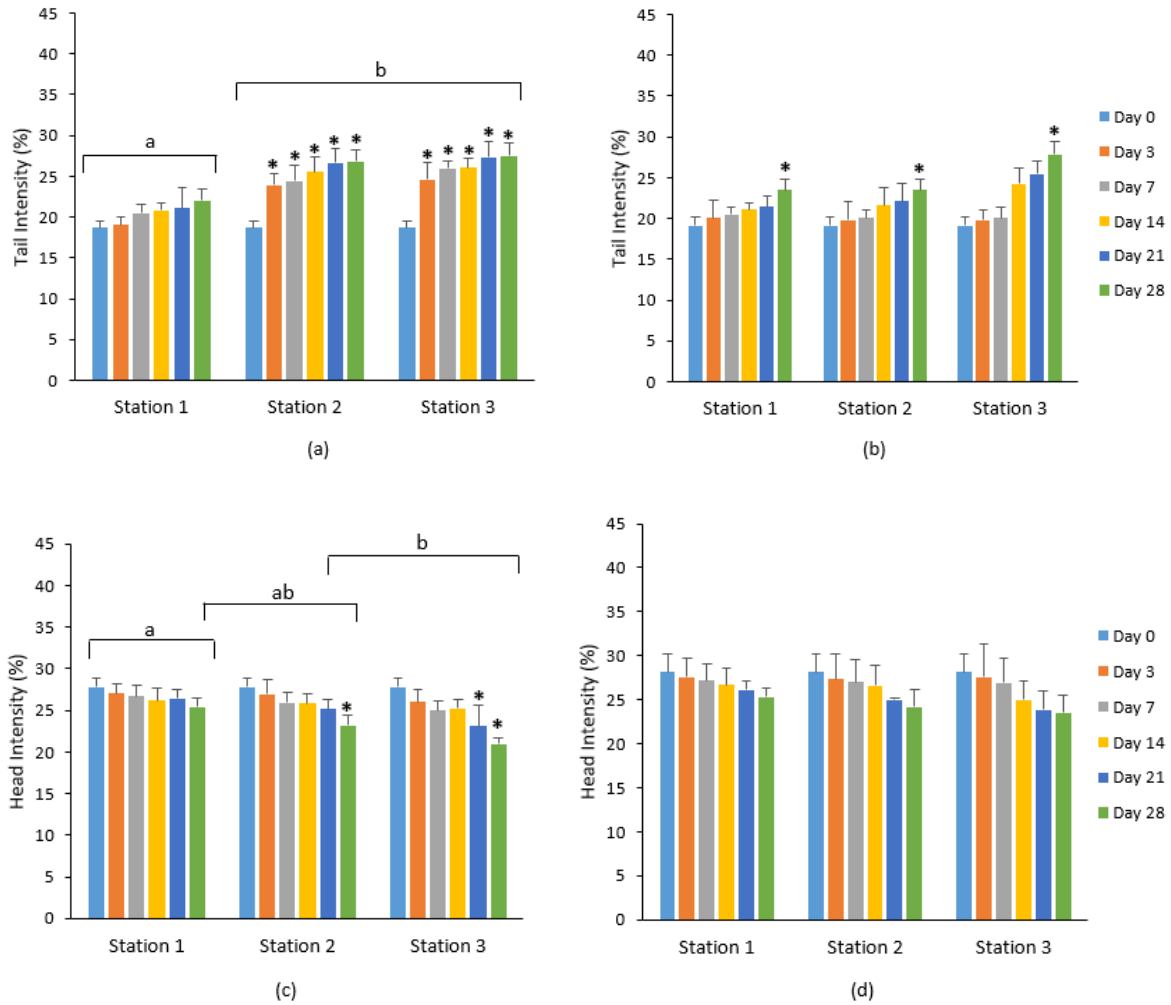
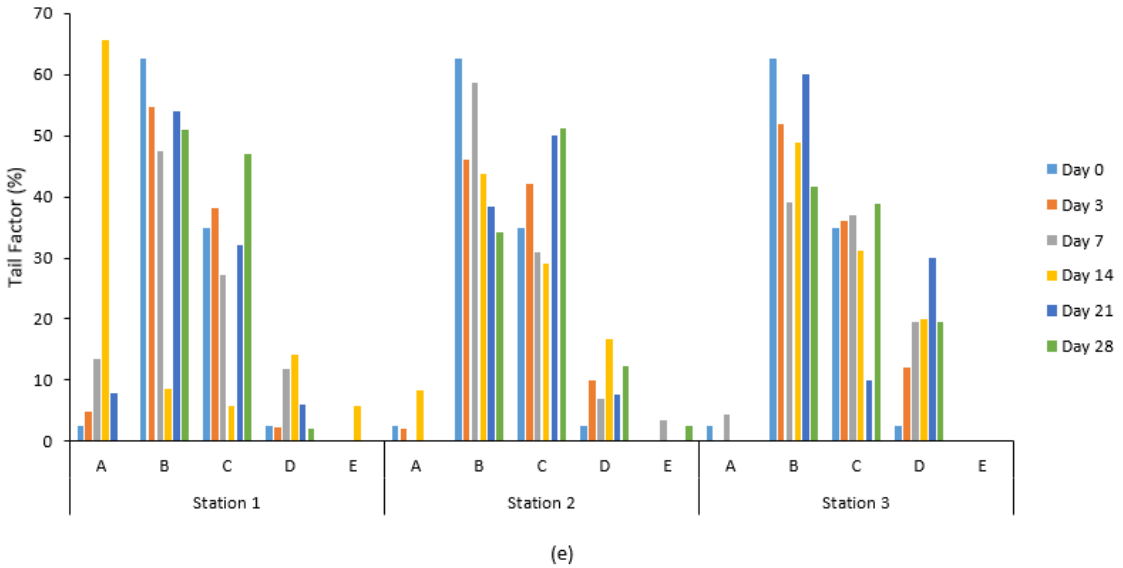
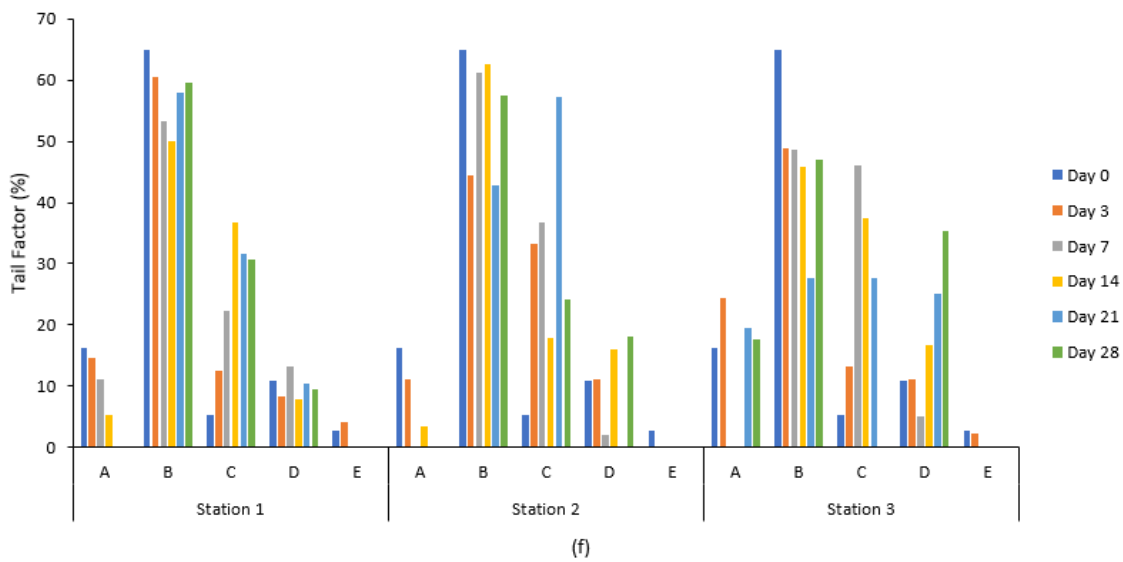


Fig. 8: (a) Tail intensity in the gills, (b) tail intensity in the mantle, (c) head intensity in the gills, (d) head intensity in the mantle, (e) tail factor in the gills, and (f) tail factor in the mantle of *A. woodiana* at three stations of the Code Stream during 28 days of exposure. The asterisk symbol (*) indicates significant difference between exposure days and day 0, and a different letter indicates significant difference between stations ($p < 0.05$) (parts (a)–(d)



(e)



(f)

Continued Fig. 8: (a) Tail intensity in the gills, (b) tail intensity in the mantle, (c) head intensity in the gills, (d) head intensity in the mantle, (e) tail factor in the gills, and (f) tail factor in the mantle of *A. woodiana* at three stations of the Code Stream during 28 days of exposure. The asterisk symbol (*) indicates significant difference between exposure days and day 0, and a different letter indicates significant difference between stations ($p < 0.05$) (parts (a)–(d)

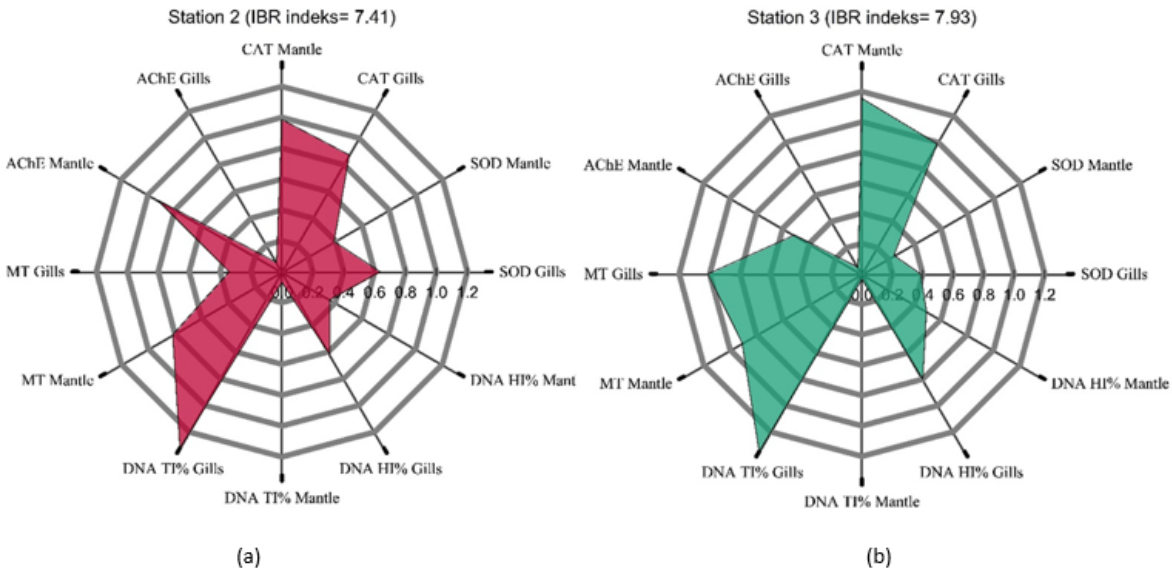


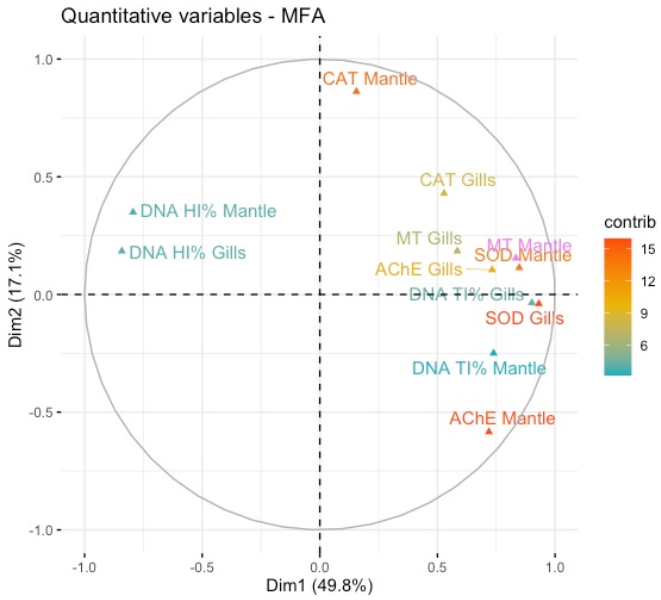
Fig. 9: Star plots (IBR) for biomarker responses in *A. woodiana* at (a) station 2 and (b) station 3

as a function of time, similar to a research conducted by Chavan *et al*. (2018), which showed an increase in TI% of *Perna viridis* clams transplanted on the East Coast of India almost two times on day 45 compared with the control. An increase in TI% indicates an increase in the frequency of DNA damage in a cell (Hazlina *et al*., 2019). The results of DNA damage suggested that the organism's defense mechanism was not able to maximally overcome the toxic effects of pollutants that enter the body, resulting in DNA damage (biomarker effect). The continuous condition of stream pollution is thought to be one of the factors that affect the ability of these organisms to detoxify. The rate of detoxification cannot keep up with the rate of absorption of contaminants.

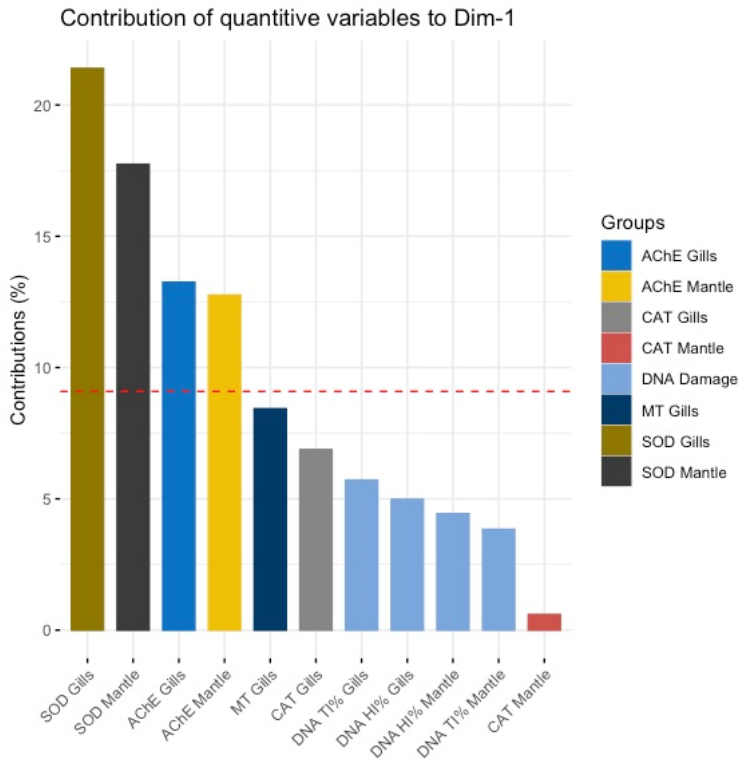
IBR

The IBR star plot analysis combined all biomarker parameters to assess the overall health status of mussels from different groups. The star plot shows the contribution of various biomarkers to the overall IBR values, and it may indicate other sources of exposure at each observation station. The analysis of IBR star plots showed that DNA damage, CAT activity, and MT concentration had the highest values among the selected parameters at St2 and St3. The analysis revealed that these three parameters were the most responsive to exposure to environmental conditions in the Code Stream (Figs. 9a and 9b).

The analysis of the combined IBR index of St3 (7.93) resulted in more significant stress on *A. woodiana* than the combined IBR index of St2 (7.41). This finding suggests that the environmental conditions at St3 were more stressful than those in the other study sites. Similar results were shown in the study of Aguirre-Martínez and Martín-Díaz (2020) in the Gulf of Cadiz through IBR analysis of star plots. They concluded that mussels experienced more significant stress in La Puntilla than in El Trocadero. A study by Georgieva *et al*. (2022), who conducted active biomonitoring using transplanted *S. woodiana* in three reservoirs in Bulgaria, showed that Zhrebchevo Reservoir was a site with relatively low IBR values, indicating that the pollution status was low at this sampling site. These results and those of other similar studies suggest that the IBR index values can linearly indicate the level of environmental pollution. MFA is a multivariate statistical method used to analyze and interpret data sets simultaneously with multiple variables, known as blocks. This technique is beneficial when dealing with complex data containing several variables that must be analyzed together. The MFA process involves the simultaneous analysis of relationships between different sets of variables, thus allowing the identification of underlying patterns and the interpretation of complex interrelationships. By

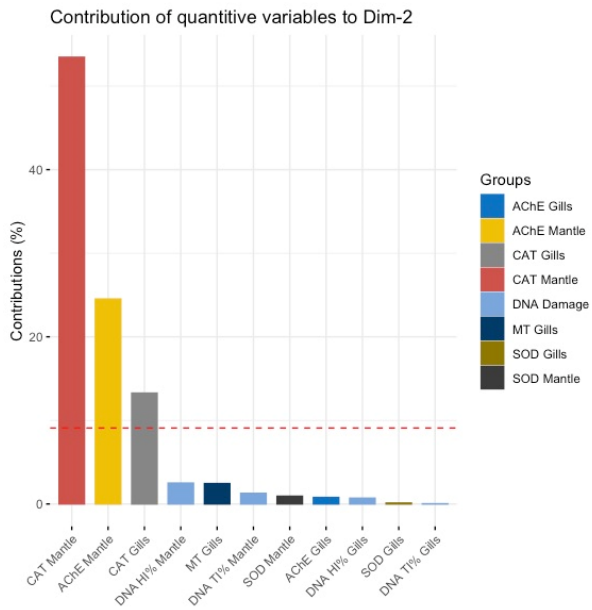


(a)



(b)

Fig. 10: (a) Multiple factor analysis from biomarker response, (b) contribution of biomarker response in dimension 1, and (c) contribution of biomarker response in dimension 2



(c)

Continued Fig. 10: (a) Multiple factor analysis from biomarker response, (b) contribution of biomarker response in dimension 1, and (c) contribution of biomarker response in dimension 2

integrating and analyzing multiple sets of variables, MFA provides a comprehensive understanding of the interactions and associations in the data, facilitating the identification of key drivers and underlying structures (Uher *et al.*, 2018). The MFA results showed that two dimensions were formed. Dimension 1 has a variance percentage value of 49.8%, and dimension 2 has a variance percentage value of 17.1%. High contribution values are variables located close to the circumference of the circle. By contrast, low contribution values are located close to the center of the circle, indicating that the variable is not perfectly represented in the main component (Fig. 10a). The CAT gills, CAT mantle, SOD mantle, AChE gills, MT gills, DNA HI% gills, and DNA HI% mantle were included in dimension 1, whereas the SOD gills, AChE mantle, DNA Ti% gills, and DNA TI% mantle were included in dimension 2. MT mantle was not included in any dimension because its correlation with each dimension was small.

In dimension 1, the SOD activity in the gills is the biomolecule that has the highest contribution value, meaning that this enzyme activity is the biomolecule most affected by exposure to the Code Stream environmental conditions, followed by the SOD activity in the mantle and the AChE activity in the gills (Fig. 10b). In dimension 2, the CAT activity in the mantle is the biomolecule that has the highest contribution value, so this enzyme activity is the most responsive during exposure to environmental conditions of the Code Stream, followed by the AChE activity in the mantle and the CAT activity in the gills (Fig. 10c). These results align with those of Georgieva *et al*. (2022), who showed a significant correlation trend between pollution levels and biomarker responses by using *S. woodiana* transplanted in three reservoirs in Bulgaria. Other results showed a relationship between metal levels and biochemical biomarkers in *Crassostrea gasar* (Ferreira *et al*., 2019). Ferreira *et al*. (2019) investigated the relationship between organic contaminants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and linear alkyl benzene) and molecular and biochemical markers in transplanted oysters. In addition, the results of the present study are in agreement with those of Capolupo *et al*. (2016), who followed the trend of correlation between CAT activity, DNA damage, and caffeine concentration in Mediterranean mussels (*M. galloprovincialis*), which were affected by caffeine at environmentally relevant concentrations. Biomarkers

that are sensitive and specific to environmental conditions in the Code Stream can then be employed in rapid assessment analyses, thus allowing the environmental status of the Code Stream waters to be promptly determined. The findings of biomonitoring activities serve as an evaluation tool for the health of the stream ecosystem. Periodic biomonitoring, followed by appropriate mitigation actions, could help maintain the quality of water resources used as raw materials for drinking water. Biomonitoring is predicted to result in a clean and healthy stream, and river ecotourism provides an opportunity to empower people along the river.

CONCLUSION

MP and Cu pollution occurred at all observation stations. The discovery of MP in the Code Stream waters indicates that plastic degradation occurred along the stream. Waste disposal by people lacking environmental sustainability awareness remains a major factor in Cu and MP pollution in the Code Stream. Most MPs were small (< 1.5 mm), black in colour, and fibrous, mainly consisting of PET polymers. The Cu contamination in the waters, sediments, gills, and mantles of the mussels was far from the maximum limits set by WHO in the aquatic environment. The variations in Cu and MP concentrations in mussel organs are a function of length of exposure, aquatic environmental conditions (stations), feeding behavior, water filtration rate, and elimination processes. Exposure to various contaminants in the stream strongly increased the SOD and CAT activities in both organs at the beginning of exposure in all stations, with the highest being at St3. Inhibition of AChE activity occurred strongly in the gills at St2. MT concentration slightly increased, with the highest increase occurribf in the gills at St2. DNA damage was more intense at St2 and St3 than at St1. This multimarker approach revealed the occurrence of oxidative stress, AChE enzyme inhibition, induction of protein synthesis, and genotoxic effects in the transplanted *A. woodiana* after 28-day exposure to the waters of the Code Stream, including metal and pesticide. The results of DNA damage analysis indicated that the defense mechanisms of these organisms were not able to maximally overcome the toxic effects of pollutants that enter the body, resulting in DNA damage (biomarker effects). The continuous condition of stream pollution is thought to be one of the factors that affect the ability of these organisms to detoxify. The rate of detoxification cannot keep up with the rate of absorption of contaminants. The results of IBR analysis showed that DNA damage, CAT activity, and MT concentration are sensitive and promising biomarkers in stream ecosystem biomonitoring. Based on MFA analysis, SOD, CAT, and AChE activities are biomarkers that indicate the occurrence of environmental pollution of the Code Stream. As a comprehensive interdisciplinary framework, sustainable waste management is required to solve complicated concerns. Improving the environment requires expanding the breadth and effectiveness of waste management and stewardship. Good policies for managing stream ecosystems require the participation of multiple linked stakeholders. Mitigation initiatives involving several parties, community involvement, and ongoing education must be up to date. The results of this study could serve as a solid scientific foundation for policymaking by the authorities about the management and control of pollution in stream ecosystems and a reliable source for active biomonitoring that regularly uses sensitive biomarkers to assess the health of streams.

AUTHOR CONTRIBUTIONS

T. Ukasha performed sampling, determination of Cu, and biomarker analysis. N.U.H. Faisal performed the extraction of MP. B.K. Adji prepared a map of sampling stations. A.P. Nugroho supervised the project and contributed to managing and developing research ideas, verifying study methods, and reviewing the manuscript. All authors provided critical feedback and helped shape the study, analysis, and manuscript.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest regarding the publication of this work. The authors have witnessed ethical issues, including plagiarism, informed consent, misconduct, data fabrication and falsification, double publication and submission, and redundancy.

ABBREVIATIONS

%	Percent
˚C	Degree Celcius
µg/g	Microgram per gram
µg/L	Microgram per liter
µL	Microliter
µm	Micrometer
µmol	Micromoles
µmol/L	Micromoles per liter
x g	Times gravity
ABS	Acrylonitrile-butadiene-styrene
AChE	Acetylcholinesterase
Al	Aluminum
ASChI	Acetylthiocholine iodide
CAT	Catalase
Cd	Cadmium
cm	Centimeter
Cu	Copper
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FAAS	Flame atomic absorption spectrometer
Fig	Figure
FTIR	Fourier-transform infrared spectroscopy
g	Gram
GSH	Glutathione
H_2O_2	Hydrogen peroxide
H_2O	Water
H_2SO_4	Sulfuric acid
HCl	Hydrogen chloride
$HClO_4$	Perchloric acid
HI	Head intensity
HNO_3	Nitric acid
IBR	Integrated biomarker response
i.e.	Id Est (that is)
KCl	Potassium chloride
kg	Kilogram
kHz	Kilohertz
KOH	Potassium hydroxide
L	Liter
m	meter
MFA	Multiple Factor Analysis
mg	Milligram
mg/kg	Milligram per kilogram
mg/L	Milligram per liter
mL	Milliliter

mm	Millimeter
mM/cm	Millimolar per centimeter
mmol/L	Millimoles per liter
mol/L	Moles per liter
MP	Microplastics
MT	Metallothionein
nm	Nanometer
nM/g	Nanomolar per gram
O_2	Oxygen
$O_2^{\bullet-}$	Superanion free radical
$^{\bullet}OH$	Hydroxyl radical
Particles/L	Particles per liter
PC	Polycarbonate
PE	Polyethylene
PET	Polyethylene terephthalate
pH	Potential of Hydrogen
PMSF	Phenylmethanesulfonyl fluoride
ROS	Reactive Oxygen Species
rpm	Revolution per minute
SOD	Superoxide dismutase
St	Station
St1	Station 1
St2	Station 2
St3	Station 3
TF	Tail factor
TI	Tail intensity
Tris-HCl	TRIS Hydrochloride
W	Watt
w/v	weight per volume
Zn	Zinc

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