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Esterhuizen, Maranda

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In vivo oxidative stress responses of the freshwater basket clam *Corbicula javanicus* to microplastic fibres and particles

Maranda Esterhuizen[a,b,c,*], Lucille Buchenhorst[d,e], Young Jun Kim[b], Stephan Pflugmacher[c]

[a] *University of Helsinki, Ecosystems and Environment Research Programme, Faculty of Biological and Environmental Sciences, Niemenkatu 73, 15140 Lahti, Finland, And Helsinki Institute of Sustainability Science (HELSUS), Fabianinkatu 33, 00014, Helsinki, Finland*

[b] *Korea Institute of Science and Technology Europe (KIST Europe) Forschungsgesellschaft mbH, Joint Laboratory of Applied Ecotoxicology, Environmental Safety Group, Universität des Saarlandes Campus E7 1, 66123, Saarbrücken, Germany*

[c] *University of Manitoba, Clayton H. Riddell Faculty of Environment, Earth, and Resources, Wallace Building, 125 Dysart Road, Winnipeg, MB R3T 2N2, Canada*

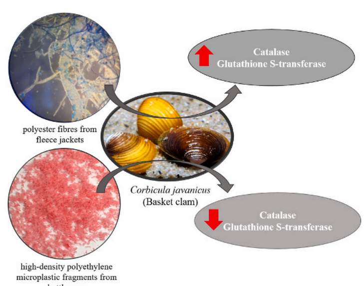
[d] *Technische Universität Berlin, Institute of Ecology, Chair Ecological Impact Research & Ecotoxicology, Ernst-Reuter-Platz 1, 10587, Berlin, Germany*

[e] *Stockholm University, Department of Ecology, Environment and Plant Sciences, Svante Arrhenius väg 20A, 11418, Stockholm, Sweden*

HIGHLIGHTS

- Polyester fibres caused increased CAT and GST activities in the clams.
- Polyethylene fragments caused decreased CAT and GST activities.
- Yellow polyethylene fragments caused more significant inhibition than blue and red.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics have been detected in several aquatic organisms, especially bivalves such as clams, oysters, and mussels. To understand the ecotoxicological implication of microplastic accumulation in biota, it is crucial to investigate effects at the physiological level to identify knowledge gaps regarding the threat posed to the environment and assist decision-makers to set the necessary priorities. Typically, xenobiotics elicit an over-production of reactive oxygen species in organisms, resulting in oxidative stress and cellular damage when not combated by the antioxidative system. Therefore, the present study aimed to establish the impacts of micro-plastic particles and fibres on the freshwater basket clam *Corbicula javanicus*. We measured the oxidative stress responses following microplastic exposure as the specific activities of the antioxidative enzymes glutathione *S*-transferase and catalase. When exposed to polyester fibres from the fleece jackets, the enzyme activities increased in the clams, while the enzyme activities decreased with high-density polyethylene microplastic fragments from bottle caps. All the exposures showed that the adverse effects on the antioxidative response system were elicited, indicating the negative ecotoxicological implications of microplastic pollution.

* Corresponding author. University of Helsinki, Ecosystems and Environment Research Programme, Faculty of Biological and Environmental Sciences, Niemenkatu 73, 15140, Lahti, Finland.

E-mail address: maranda.esterhuizen@helsinki.fi (M. Esterhuizen).

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1. Introduction

Microplastic (MP) pollution awareness is growing due to the considerable rise in environmental monitoring and assessment (Hung et al., 2021). MPs, which are considered such when the particles are 5 mm and smaller (Arthur et al., 2008), occur in the environment due to weathering of plastic materials over time (Zhang et al., 2021a) or are released into the environment, for example, from domestic effluent water contaminated with fibres from washing clothing made of synthetic materials (Carney Almroth et al., 2008; Scopetani et al., 2020). Synthetic textiles have been identified as a major source of MP fibres released via laundering (Henry et al., 2019). One of the most commonly detected MP shapes in the environment is fibres (Browne et al., 2011), i.e., 91% of all MP particles detected in surface waters worldwide are fibres (Barrow et al., 2018). Rebelein et al. (2021) reviewed that the threat fibres pose to aquatic organisms has to date been greatly underestimated.

Due to their small sizes and prevalence in aquatic environments, MPs are likely to be ingested by aquatic organisms. For the marine environment, ingestions were already proved to occur with vertebrates such as seals (Eriksson and Burton, 2003), invertebrates such as sea cucumbers (Graham and Thompson, 2009), and crustaceans (Murray and Cowie, 2011), as well as bivalves (reviewed by Ward and Shumway, 2004) such as clams (Baechler et al., 2019) and mussels (von Moos et al., 2012; Van Cauwenberghe et al., 2015; Li et al., 2018).

Considering that aquacultural industries build their farms for cultivation in naturally occurring water bodies, the shellfish are exposed to all contaminants and pollution present in the ambient water, including MPs and associated chemicals (Avio et al., 2015). Especially concerning is that shellfish likely bioaccumulate large quantities of MPs due to the mode and unspecificity of filter-feeding (Cole et al., 2013; Setälä et al., 2016). A recent study by Fabra et al. (2021) also showed that MP particles covered by a biofilm are ten times more readily taken up.

Mytilus edulis, or the blue mussel, is popular in the seafood industry and consumed by humans globally. Numerous laboratory studies have demonstrated the ingestion of MP particles by the blue mussel, and information about MP concentrations in both wild and cultured populations also has been growing (Brown et al., 2008; De Witte et al., 2014). Other bivalves have also been observed to ingest MPs, such as the brown mussel (Perna perna), the oyster (Crassostrea gigas), Pacific oysters (*Crassostrea gigas*), and Pacific razor clams (*Siliqua patula*) (Santana et al., 2016; Baechler et al., 2019; Martinelli et al., 2020; Patterson et al., 2021). Baltic clams (*Limecola balthica*) can transfer MPs from the sediment surface to a depth of 5 cm via bioturbation, during which ingestion occurs, but not internal accumulation (Näkki et al., 2021). In these bivalves, the amount of ingested MPs reported vary substantially (0.6–178 particles/organism); however, the differences in the quantification methods should be considered in such comparisons. The plastic types the bivalves in China most commonly took up were polyethylene, polyethylene terephthalate, and polyamide, varying in size from 5 μm to 5 mm (Li et al., 2015). Over 99% of the ingested MP particles in Pacific oysters and razor clams were fibres (Baechler et al., 2019).

Interestingly, Woods et al. (2018) found that bivalves such as mussels could expel the majority of the MP particles they encounter. The same study showed that the organism would excrete most of the ingested plastic once in an environment devoid of plastics. However, MP is still being detected in tissue from wild mussels, likely, as their environment is never completely clear of MP anymore, thus not allowing for depuration.

Considering constant exposure and the likelihood of ingestion, it is essential to understand the possible effects of MP particles on aquatic biota at molecular, organism, population, and ecosystem levels. Bivalves serve as a food source for many organisms in the aquatic ecosystems, as well as humans, and have the potential to transfer MPs to higher trophic levels in food webs, including humans (Farrell and Nelson, 2013; Van Cauwenberghe et al., 2014). Several studies regarding the uptake and translocation of MP in bivalves exist; however, studies on the effects of MP at a physiological and morphological level seem to be focused on mussels (Hamm and Lenz, 2021) and oysters (Green, 2016). Information regarding the effects of MP on clams, another popular global food, is emerging (Davidson and Dudas, 2016) but still limited. This study, therefore, intended to expand on the available knowledge regarding the effect of MP on clams by investigating the oxidative stress responses. The present study aimed to investigate the effects on the physiology of the freshwater clam *Corbicula javanicus,* specifically the biotransformation and the oxidative stress enzyme systems. Therefore, the activities of the enzymes glutathione S-transferase (GST) (biotransformation) and catalase (oxidative stress) were investigated after exposing the clams to high-density MP fragments generated from bottle caps and fibres from fleece jackets, both ranging in size to represent environmental exposure. Bottle caps made of high-density polyethylene were selected based on their abundance in the environment (Walther et al., 2018) due to the throwaway culture associated with plastics. Polyester (polyethylene terephthalate, PET) fleece was chosen as this synthetic material dominates the textile market at present (Schöpel and Stamminger, 2019) and among fibres detected in nature (Singh et al., 2020).

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma Aldrich (Darmstadt, Germany) unless specified otherwise. The freshwater basket clam, *C. javanicus,* was purchased from FRAKU Aquaristik and cultivated in Java, Indonesia, before being imported to Germany. The organisms were acclimatised in the laboratory for three weeks before the exposure experiments. The clams were kept in a glass tank with a volume of 100 L, filled with modified a medium containing de-ionised water, $CaCl_2$ (0.136 mol), $NaHCO_3$ (0.123 mol) and sea salt (0.318 mol) (Stein, 1973). The clams were fed twice a week with spirulina powder, and the medium was renewed once per week.

Three new, unused, black polar fleece jackets made of 100% polyester (PET) were purchased from the local supermarket. MP fibres were obtained by washing the three jackets individually by hand, without detergent, in 10 L of tap water. The resulting tap water was vacuum filtered through filter paper with a pore size of 0.45 μm. The fibres per washed jacket were counted using a Neubauer haemocytometer and an Olympus bright field microscope at 100× magnification (Fig. 1A). On average, $16.3 \times 10^5 \pm 4.1 \times 10^5$ fibres per jacket (in 10 L of water) were obtained. The obtained fibres were diverse, ranging in fibre length and width.

Bottle caps in three colours were obtained by purchasing soft drinks from the local supermarket, removing the high-density polyethylene (HDPE) caps (seals removed) and washing them in tap water. The MP fragments were obtained by grating the lids separated by colour, i.e., red, blue, and yellow, resulting in irregularly shaped particles. The fragment sizes varied from 5 mm to 1 μm (size range obtained by sieving). The particles had different sizes and forms to represent the diversity of MP particles existing in the environment (Fig. 1B).

2.2. Exposure setup

2.2.1. Polyester (PET) fibres

For exposures to the MP fibres, five clams per replicates ($n = 3$) were exposed to the number of fibres per jacket per wash (average $16.3 \times 10^5 \pm 4.1 \times 10^5$) suspended in 2 L of synthetic medium (8.1×10^4 fibres per litre) against a negative control for 24 h. During this time, the clams were not fed with spirulina powder, nor was the medium changed.

2.2.2. HDPE fragments

For exposures to the MP fragments, the clams were individually exposed in 250 mL beakers to three concentrations of the MP fragments, 0.01, 0.1 and 1.0 mg/L (w/v) per colour (red, yellow, and blue) for 24 h (n = 3) against a negative control. The lowest exposure concentration (0.01 mg/L) was selected based on the reported MP fragment concentration in freshwater lakes in Asia and Europe which are 2561 MP particles/m^3 (Cera et al., 2020) and 0.013 mg/L using the Besseling et al. (2019) conversion (Esterhuizen and Kim, 2021). The 10-fold and 100-fold higher concentrations were selected to account for predicted increases in these environmental concentrations.

For all exposures, the ambient temperature was 22 °C ± 1 °C. After the exposures, the tissue of each organism was collected, shock frozen in liquid nitrogen, subsequently stored at −80 °C.

2.3. Enzyme preparation and measurement

The enzymes were extracted according to Pflugmacher, 2004, with minor modifications. Clam tissue, amounting to 1.5 g FW, was homogenised in 0.1 M sodium phosphate buffer (pH 6.5) consisting of 20% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1.4 mM dithioerythritol (DTE). To remove the cell debris, the samples were centrifuged at 10,600×*g* for 10 min at 4 °C (Eppendorf Centrifuge 5417 R, Hamburg, Germany). Proteins in the supernatant were concentrated through ammonium sulfate precipitation to a final saturation of 80% and centrifuged at 20,800×*g* for 60 min at 4 °C. The obtained pellet was suspended in 20 mM sodium phosphate buffer (pH 7.0) and desalted by gel filtration using Sephadex columns (NAP-5, Amersham GE Healthcare, Uppsala, Sweden).

GST (EC 2.5.1.18) was determined photometrically by monitoring the conjugation rate of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH at 340 nm (extinction coefficient Ɛ = 9,6 L/mmol·cm) according to Habig et al. (1974). Catalase (CAT) (EC 1.11.1.6) activity was determined according to Claiborne (1985), where the decrease of H_2O_2 was measured as a decrease in absorption at 240 nm (extinction coefficient Ɛ = 0.0361 L/mmol·cm). All enzyme activities were normalised according to their protein content, measured at 595 nm after incubation with Bradford reagent (Bradford, 1976), and expressed as katal per mg protein.

2.4. Statistical analysis

IBM® SPSS® statistics 25 (2018) was the selected software for statistical analysis. Descriptive analysis based on the mean and standard deviations (SD) was performed. The homogeneity and normality of the data were assessed via histograms and Levene's test of homogeneity. For the exposures with fibres, independent samples T-tests were performed. CAT activity with exposure to the three colours of MP fragments was tested for significance via the one-way analysis of variance (ANOVA) and Tukey posthoc test (Levene's test: $p = 0.168$). The GST activities were compared with the non-parametric Kruskal-Wallis test followed by pair-wise comparisons (Levene's test: $p = 0.004$). An alpha value of 0.05 was used to identify significant differences among the controls and treatments.

3. Results and discussion

3.1. Polyester (PET) fibres

The discharge of MP fibres from synthetic textiles during washing and the effect of different washing conditions have been extensively studied (i.a., Sillanpää and Sainio, 2017; Carney Almroth et al., 2018; Zambrano et al., 2019; Cesa et al., 2020; Özkan and Gündoğdu, 2021). The results have been difficult to compare due to the reporting of different units that are nonconvertible, using different materials, as well as the methodologies (reviewed by Gaylarde et al., 2021). In our study, an average of $16.3 \times 10^5 \pm 4.1 \times 10^5$ polyester microfibres were released per jacket into 10 L of tap water with handwashing. Carney Almroth et al. (2018) reported 7360 polyester fibres per square meter of fleece per litre of water. Assuming a jacket consists of approximately three square meters of material, the amount of fibres obtained in this study equates to 54,334 fibres/m^2/L, nearly ten times more than previously reported which could be due to the quality of the garments or the intensity of the handwashing.

Asian clams (*Corbicula fluminea*) were previously shown to take up polyester (PET) microfibres (4 fibres/g) in significantly higher amounts than six other polymers tested (Li et al., 2019). Size played a significant role in the uptake as the highest amount of fibres were taken up in the size range of 100–250 μm. Considering that exposure could be direct contact to fibres and leachates as well as uptake, the physiological effect in terms of the effect on the oxidative stress system in clams was evaluated.

Exposing the basket clam *C. javanicus* to the polyester microfibres (8.1×10^4 fibres/L) for 24 h elevated the CAT activity (Fig. 2A) by 75.8% compared to the control ($t\ (8.394) = -5.146$, $p = 0.0008$). Similarly, exposure to the polyester microfibres led to a 39.4% increase in the GST activity compared to the control ($t\ (12.796) = -6.146$, $p = 0.00004$).

Oxidative stress is accepted as the imbalance between reactive

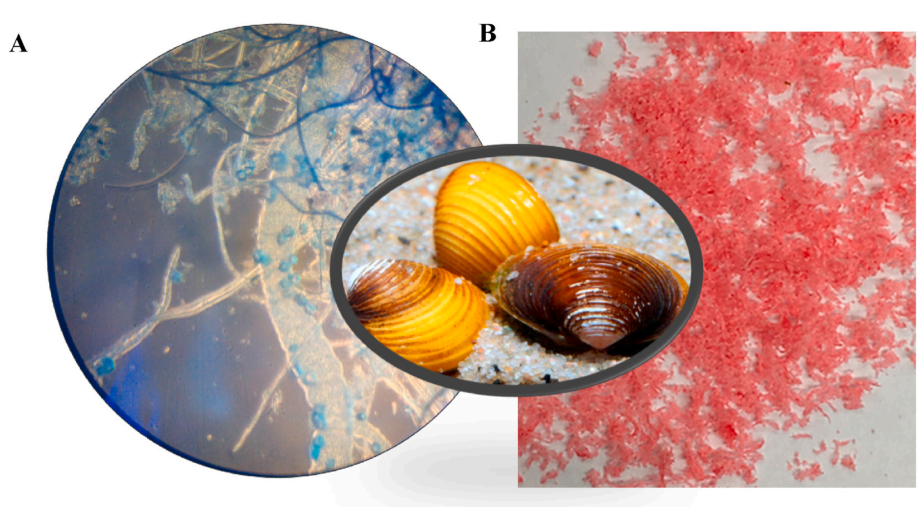

Fig. 1. A) Microplastic fibres obtained by washing microfibre polyester (polyethylene terephthalate) fleece jackets; viewed at 100× magnification on a bright-field Olympus microscope. B) Image of the red HDPE MP fragments obtained via grating before sieving. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

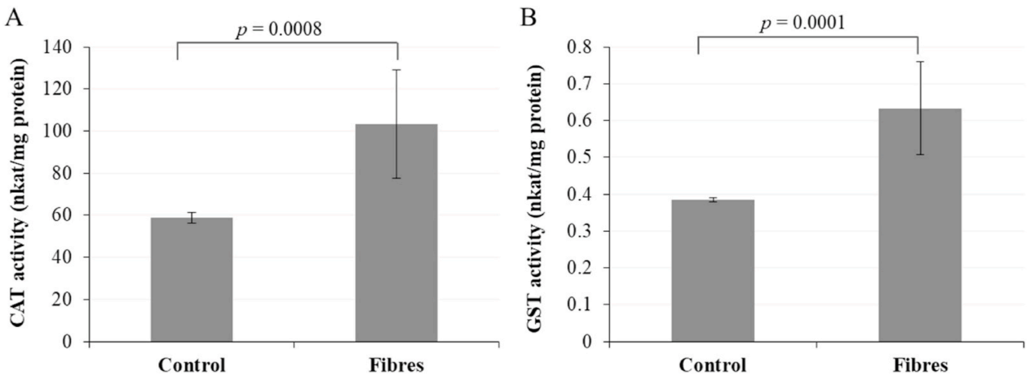

Fig. 2. *In vivo* (A) catalase (CAT) and (B) glutathione S-transferase (GST) activities in the basket clam, *Corbicula javanicus* exposed to 8.1×10^4 fibres per litre. The bars represent the average enzyme activity ± standard deviation (n = 3).

oxygen species (ROS), generated via normal cellular metabolism and in response to xenobiotics, and the antioxidative response system's ability to combat ROS to prevent cellular damage (Kaur et al., 2014). Oxidative stress responses to microfibre exposure have not previously been investigated in clams; however, studies on *Caenorhabditis elegans* (roundworms) (Liu et al., 2021) and snails (Song et al., 2019) have been published. With *C. elegans*, only exposure to a specific size of PET microfibres (250 μm) caused a significant increase in ROS and thus promoted oxidative stress (Liu et al., 2021). The present study included an extensive size range specifically for maximum uptake and adverse effects on oxidative stress. With terrestrial snails, *Achatina fulica*, the decrease in glutathione peroxidase activity and total antioxidant capacity paired with lipid peroxidation indicated oxidative stress associated with PET microfibre exposure (Song et al., 2019). In the present study, both tested antioxidative enzymes' activities increased in response to microfibre exposure; however, the exposure period of 24 h was short (24 h) relative to the other two discussed studies, which were much longer (e.g., 28 days for the snails and a generation for the worms). Typically, ROS is generated during the oxidation, reduction and hydrolysis of xenobiotics, causing increases in the activities of the antioxidative enzymes. Our data suggest that polyester fibres or chemicals leaching from these fibres are metabolised as xenobiotics.

3.2. HDPE fragments

In Fig. 3, the clams were exposed to three different concentrations of HDPE separated by colour, i.e., red, blue, and yellow MP ranging in size from 5 mm to 1 μm. The CAT activity was reduced by 53.6% with exposure to 0.1 mg/L MP ($F_{(9, 25)} = 4.532$; $p = 0.006$) but not with exposure to red HDPE MP particles at a concentration of 0.01 mg/L ($p = 0.458$) or 1 mg/L ($p = 0.093$) (Fig. 3A). The same trend was observed when exposing the clams to the blue HDPE particles, i.e., with 0.1 mg/L,

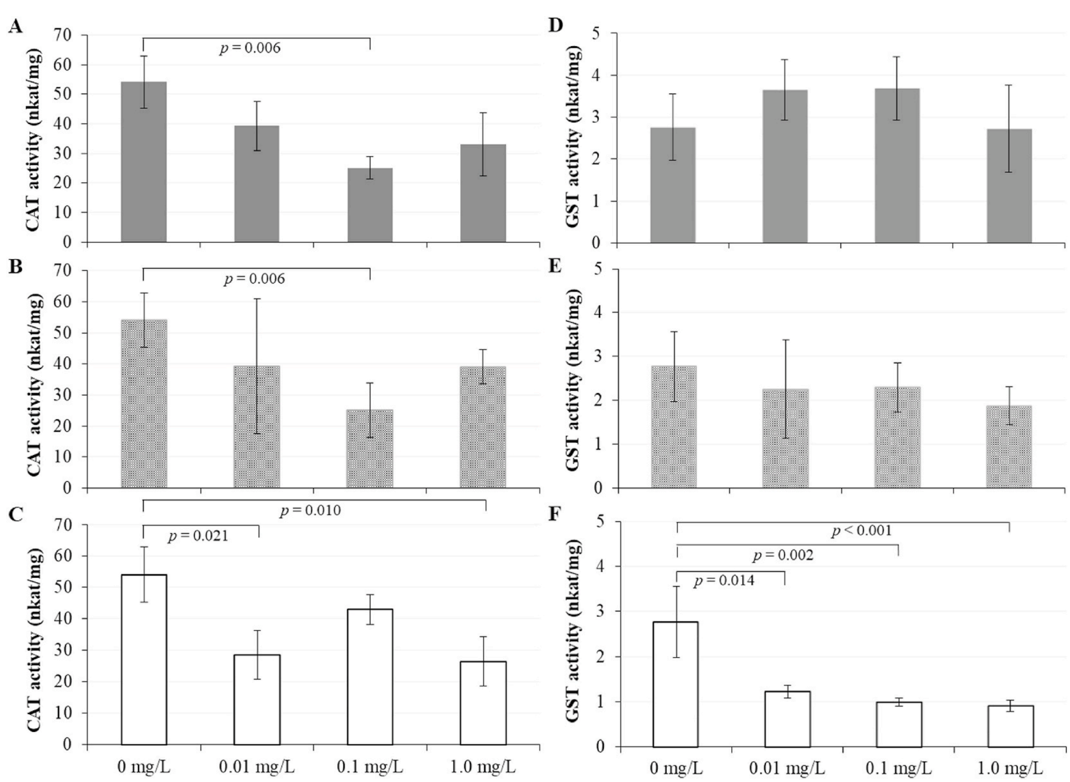

Fig. 3. *In vivo* catalase (CAT; A-C) and glutathione S-transferase (GST; D-F) activities in the basket clam, *Corbicula javanicus* exposed to red (A and D), blue (B and E), and yellow (C and F) microplastic fragments at three concentrations (0.01 mg/L, 0.1 mg/L, and 1 mg/L). The bars represent the average enzyme activity ± standard deviation (n = 3).

the CAT activity was inhibited by 58.8%, but not with affected with exposure to 0.01 mg/L ($p = 0.454$) or 1 mg/L ($p = 0.4583$) (Fig. 3B). With yellow HDPE MP fragments, concentrations of 0.1 mg/L ($p = 0.021$) and 1 mg/L ($p = 0.01$) resulted in lowered CAT activity compare to the control (Fig. 3C).

The GST activity was not affected by exposure to either red (Fig. 3D) or blue (Fig. 3E) HDPE MP at any of the exposure concentrations ($p > 0.05$). However, with exposure to yellow HDPE MP (Fig. 3F), significant inhibitions of the GST activity (on average by 61.5% ± 5.5%) were observed for all exposure concentrations ($p < 0.05$).

To the best of our knowledge, the effect of MP colour on the antioxidative enzymes has not previously been considered. Considering CAT activity, the colour of the MP did not play a significant role ($p > 0.05$). Regarding GST, there was no difference in the response of the activities when exposed to either red or blue ($p > 0.05$). However, exposure to yellow HDPE MP caused significant reductions in the activities compared to red and blue for all three concentrations tested ($p < 0.05$). This finding may imply that colourants and additives associated with yellow HDPE may be more toxic than the other two dyes.

Varying results have been reported regarding the effect of different MP types on the antioxidant enzymes in clams. For example, in Manila clams (*Ruditapes philippinarum*), irregular-shaped PET, at a concentration of 0.125 mg/L had no effect on the antioxidative system, whereas, at a concentration of 12.5 mg/L, GST in the digestive gland was significantly inhibited, SOD, CAT, and GPx activities were unaffected, but lipid peroxidation was observed (Parolini et al., 2020). By exposing the clam *Mactra veneriformis* to 1 mg/L 150 μm polystyrene, the SOD and GST activities were significantly inhibited (Zhang et al., 2021b). However, no information was found regarding the exposure of clams to irregular-shaped HDPE particles. In *R. philippinarum* exposed to polyethylene microbeads (25 μg/L), the CAT activity was unaffected (Sıkdokur et al., 2020). Nevertheless, the concentration applied was significantly lower (4-fold) than the concentration of HDPE particles that resulted in the inhibited CAT activity in the present study.

Several studies have reported inhibition of the antioxidative enzymes following exposure to oil-based MPs in mussels (Magara et al., 2019; Avio et al., 2015; Paul-Pont et al., 2016) and shrimp (Hsieh et al., 2021). Magara et al. (2019) previously hypothesised that, depending on the size, oil-based MPs could accumulate and cause physical damage within an organism triggering inflammation and enabling the greater accumulation of ROS. The reduced enzyme activities likely could be attributed to ROS damage either at protein (damage to enzymes) or gene level (reduced expression). However, the possibility of enzyme inhibiting compounds leaching from plastics should be investigated.

4. Conclusion

The data demonstrates that the type and shape of MP play a major role in how the baseline antioxidative enzyme activities could be affected and how they are affected. Different organisms also diverged in their response to the MP. The currently available information is still too limited to generalise how MP, the various types, colours, and sizes, could affect the oxidative status of aquatic organisms exposed to MP at environmental concentrations.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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Credit author statement

Maranda Esterhuizen, Conceptualisation, Data curation, Investigation, Methodology and Formal analysis, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing, Lucille Buchenhorst, Investigation, Methodology and Formal analysis, Young Jun Kim, Conceptualisation, Investigation, Methodology and Formal analysis, Funding acquisition, Writing – review & editing, Stephan Pflugmacher, Conceptualisation, Investigation, Methodology and Formal analysis, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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