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## **Toxicity assessment of environmental MPs and NPs and polystyrene NPs on the bivalve *Corbicula fluminea* using a multi-marker approach**

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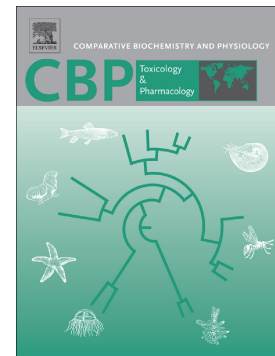
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Toxicity assessment of environmental MPs and NPs and polystyrene NPs on the bivalve *Corbicula fluminea* using a multi-marker approach

Oïhana Latchere<sup>a,\*</sup>, Coraline Roman<sup>a</sup>, Isabelle Métails<sup>a</sup>, Hanane Perrein-Ettajani<sup>a</sup>, Mohammed Mouloud<sup>a</sup>, Didier Georges<sup>a</sup>, Agnès Feurtet-Mazel<sup>b</sup>, Julien Gigault<sup>cd</sup>, Charlotte Catrouillet<sup>de</sup>, Magalie Baudrimont<sup>b</sup>, Amélie Châtel<sup>a</sup>

<sup>a</sup>Université Catholique de l'Ouest, laboratoire BIOSSE, 3 place André Leroy, Angers, France

<sup>b</sup>Université de Bordeaux, UMR EPOC 5805, équipe Ecotoxicologie Aquatique, Station marine d'Arcachon, Place Du Dr Peyneau, 33120, Arcachon, France

<sup>c</sup>Université Laval, Département de Biologie, Pavillon Alexandre-Vachon, 1045, Av. de La Médecine, Local 2064, Québec, Québec, G1V0A6, Canada

<sup>d</sup>Univ. Rennes, CNRS, Géosciences Rennes - UMR 6118, F-35000 Rennes, France

<sup>e</sup>Université de Paris, Institut de physique du globe de Paris, CNRS, F-75005 Paris, France

\*Corresponding author : Oïhana LATCHERE. Email address : oihana.latchere@uco.fr. Postal address : 3 place André Leroy, 49100 Angers, France.

## Abstract

Small plastic particles, microplastics (MPs) and nanoplastics (NPs) represent a major threat in aquatic environments. Freshwater organisms are exposed to MPs and NPs, particularly in industrial and urban areas. The present study aimed to compare the toxicity between polystyrene NPs (PS NPs) and environmental microplastics (ENV MPs) and nanoplastics (ENV NPs) generated from macro-sized debris collected in the Garonne River on the freshwater bivalve *C. fluminea*. The organisms were exposed to the different plastic particles at three environmentally relevant concentrations: 0.008, 10, and 100  $\mu\text{g L}^{-1}$  for 21 days. The biological responses of organisms were assessed using a multi-biomarker approach from the sub-individual to the individual level. The results demonstrated that: i) ENV NPs triggered more effects on detoxification processes and immune response, confirming that using manufactured NPs for laboratory exposure can lead to misleading conclusions on the risks posed by plastic particles; ii) effects of ENV MPs were less marked than ENV NPs, emphasizing the importance of testing a size continuum of plastic particles from NPs to MPs; iii) some effects were only observed for the low and/or intermediate concentrations tested, underlining the importance of using environmentally relevant concentrations. In light of these results, laboratory studies should be continued by exposing aquatic species to environmental

MPs and NPs. The properties of these particles have to be characterized for a better risk assessment of environmental plastic particles.

Keywords: microplastics, nanoplastics, bivalve, *Corbicula fluminea*, environmental plastics.

## Introduction

Due to their ubiquity and propensity to degrade, plastics are pollution-threatening to terrestrial and aquatic environments (MacLeod et al., 2021). Plastics are identified as common debris in the marine environment, representing 60 to 80% of all waste (Derraik, 2002). Small plastic particles, microplastics (MPs), and nanoplastics (NPs) have been identified in marine and freshwater environments. Depending on their origins, these MPs and NPs are classified according to their size and considered primary or secondary. Plastic particles smaller than 5 mm have been commonly described as MPs. The NPs have been defined as particles ranging in size from 1 to 1000 nm and exhibiting colloidal behavior (Gigault et al., 2018). Primary plastic particles are intentionally produced as small particles, while secondary ones are generated by the breakdown of macro-sized plastic debris through physical, biological, and chemical processes (Andrady 2015).

While rivers are the major contributors of plastic inputs to the oceans (Lebreton et al., 2017; Moore et al., 2011), little is known concerning the biological effects of MPs and NPs in these environments compared to marine ones (Li et al., 2020). The toxicity of MPs and NPs can be due to physical damage, leaching of additives present in the plastic particles, or sorption and desorption processes of pollutants or pathogens from the surface of the plastics. In freshwater environments, organisms are closer to the source of MPs and NPs and the use of chemicals than in the marine environment (Dris et al., 2015; Meng et al., 2020). They may be particularly vulnerable to the risk of MPs and NPs in industrial and urban areas (Meng et al., 2020). Studies have been conducted in the laboratory to understand the effects of MPs and NPs on freshwater organisms such as algae (Besseling et al., 2014; Bhattacharya et al., 2010), bivalves (Magni et al., 2018; Rochman et al., 2017), and fish (Rainieri et al., 2018; Rochman et al., 2013). However, exposure to freshwater organisms in the laboratory does not allow for a conclusive assessment of the risk posed by MPs and NPs (Triebkorn et al., 2019). Indeed, in most of these studies, standard MPs and NPs are used and are not representative of the plastic particles found in the environment in terms of shape, composition, surface charge,

adsorbed chemicals, and biofilms (Gigault et al., 2016; Phuong et al., 2016). Thus, it becomes necessary to carry out more realistic experiments from an environmental point of view by using plastic particles from the environment for laboratory exposures and characterizing them (Latchere et al., 2021).

The Asiatic clam *Corbicula fluminea* (Müller, 1774) is a widespread macroinvertebrate described as a key species in many rivers and shallow waters (Viergutz et al., 2012). This clam is a sessile benthic species with a very effective filtration capacity (Viergutz et al., 2012), which makes it particularly exposed to the ingestion of MPs and NPs. In addition, this species is also likely to ingest plastic particles in the sediment and contaminated preys. It is widely used to assess the toxicity of freshwater and sediments. Previous studies have shown that markers such as bioaccumulation, biochemical process alterations, and behavioral endpoints in this species are relevant for studying responses to pollutants. (Guo and Feng, 2018).

The present study aimed to evaluate the toxicity of both environmental MPs and NPs and standard PS NPs in two tissues (gills and visceral mass) of *Corbicula fluminea* at environmentally relevant concentrations. Biological responses of organisms were assessed using a multi-biomarker approach from the sub-individual level (biochemical biomarkers of detoxification processes and immune system, genotoxicity) to the individual level (burrowing behavior and condition index). These biological responses are discussed according to the size and metals associated with the environmental MPs and NPs.

## Materials and methods

### *Collection, preparation, and characterization of field-derived MPs and NPs*

Plastic wastes were collected with pliers on the right bank of the Garonne River at low tide, near the Langoiran bridge (44°42'14.56"N, 0°24'3.91"W). Various oxidized plastic debris were sampled, rinsed in the laboratory with ultrapure water, and dried at 45°C for 48h before micro and nanoplastic solutions were prepared.

### *Environmental micro and nanoplastics production*

Environmental microplastics (ENV MPs) and nanoplastics (ENV NPs) were generated from macro-sized plastic debris according to the protocol described by Blanco et al. (2021). Briefly, NPs and MPs were produced by coupling agitation and sonification in aquatic media. The mean size was  $235 \pm 70$  nm for ENV NPs, and the size range was between 1.2 and 300  $\mu\text{m}$  for ENV MPs. ENV NPs and ENV MPs were characterized in terms of composition, size, shape, and surface properties by Pyrolysis (Pyrolyzer PY-3030 Frontier Lab) coupled to gas chromatography-mass spectrometry (Py-GC-MS) (5977B, Agilent Technologies). Dynamic Light Scattering (DLS, Vasco Flex, Cordouan Technologies) and Asymmetrical flow field flow fractionation (A4F, Eclipse, Wyatt Technologies) coupled to static light scattering (SLS, Dawn Heleos, Wyatt Technologies) and Inductively-coupled-plasma-mass spectrophotometry (ICP-MS, 7700x, Agilent Technologies) were used to characterize size distribution and elemental composition thanks to the separation methods previously optimized (Gigault et al., 2017). Plastic analysis showed that ENV NPs and ENV MPs were mainly composed of polyethylene (PE) (95%) (Supplementary Information, Figure 1). They were anisotropic, polydisperse in size (Supplementary Information, Figure 2), and possessed high levels of carboxylic groups on their surface. Zeta potential of the ENV NPs was -36.2 mV. Carboxylated polystyrene nanobeads (200 nm) were used as reference material (Polysciences).

#### *Acidic digestion and ICP-MS measurements*

To optimize the total digestion, 100 mg of microplastics and nanoplastics powder were acid-digested (12  $\text{NHNO}_3$  sub-grade) using a multistep procedure with a microwave oven (MW7000 system from Anton-Paar; increasing ramp of the temperature of  $6.6^\circ\text{C}$  per minute until reaching  $250^\circ\text{C}$ , then 25 min at  $250^\circ\text{C}$  under 140 bar of pressure). The solution of three tubes was mixed, evaporated at  $90^\circ\text{C}$ , and solubilized in 0.37  $\text{NHNO}_3$  before ICP-MS measurements. Metal concentrations were measured by ICP-MS from Agilent Technologies (7700x Model, Agilent) (Supplementary Data Table A). The digestion and analysis processes were validated using reference materials (ERM-EC 680 and ERM-EC 681) from the Joint Research Centre of the European Commission (JRC, Ispra, Italy).

#### *Suspensions of microplastics*

For each type of plastic particle (ENV MPs, ENV NPs, and PS NPs), stock suspensions at 1 and  $0.1 \text{ g L}^{-1}$  were prepared in ultrapure (milliQ, Millipore) water at pH 7. A working solution

at  $0.1 \text{ mg L}^{-1}$  was obtained for each type of plastic particle by three serial dilutions of the stock suspension at  $0.1 \text{ g L}^{-1}$  in ultrapure water (milliQ, Millipore), as performed in Revel et al. (2019). A specific volume of the stock suspension ( $1 \text{ g L}^{-1}$  or  $0.1 \text{ g L}^{-1}$ ) or the working solution ( $0.1 \text{ mg L}^{-1}$ ) was distributed in the aquaria to obtain the final concentrations of 0.008, 10, and  $100 \text{ } \mu\text{g L}^{-1}$ . No surfactant was used during MPs and NPs preparation to prevent any additional effects. Each solution was well mixed before being added to the aquaria. All MPs and NPs solutions were prepared and added to the aquaria every 3 days, just after a water change, to maintain the same concentration during the exposure.

#### *Corbicula fluminea collection and laboratory exposure assay*

Individuals of *Corbicula fluminea* were hand-collected in the lake of Biscarrosse-Parentis (France). Clams were transported to the laboratory in boxes with sediment from the collection site. Bivalves were immediately transferred into aquaria (30L) containing 27L of tap water in a temperature-controlled room at  $15^\circ\text{C}$  for an acclimatization period of 7 days. Photoperiod was maintained at 12:12. A total of 600 clams were equally divided among ten glass aquariums. The water was renewed entirely every three days. Clams were fed once a week with microalgae of the genus *Scenedesmus* (GreenSea).

Clams were exposed for 21 days to manufactured polystyrene nanoplastics (PS NPs, 200 nm, Polysciences), field-derived microplastics (ENV MPs, 1.2-300  $\mu\text{m}$ ), and field-derived nanoplastics (ENV NPs,  $235 \pm 70 \text{ nm}$ ) at the following concentrations:  $0.008 \text{ } \mu\text{g L}^{-1}$ ,  $10 \text{ } \mu\text{g L}^{-1}$  and  $100 \text{ } \mu\text{g L}^{-1}$ . According to Revel et al. (2020), the three tested concentrations were chosen to expose clams to environmentally relevant concentrations of MPs and NPs, which are representative of coastal regions and gyres (Goldstein et al., 2013). The experimental conditions are abbreviated in the results and discussion sections: for example, ENV MPs 10 is used for environmental microplastics at  $10 \text{ } \mu\text{g L}^{-1}$ . One group of individuals was used as a control (no added plastic particles). The use of plastic material was avoided during the experiment to prevent any plastic contamination.

#### *Clam sampling for analysis*

After 7 days of exposure to the ten conditions, 8 individuals per condition were sampled: 5 for biomarker analysis and 3 for genotoxicity. At the end of the experiment, at 21 days, 18 individuals per condition were sampled: 5 for biomarker analysis, 3 for genotoxicity, 10 to

determine burial rate and the condition index. Gills and visceral mass were dissected and frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further enzymatic activity analysis.

#### *Biochemical biomarkers*

Enzyme activities (catalase - CAT, Glutathione-S-transferase - GST, and phosphatase acid - AcP) were quantified in gills and digestive gland tissues. First, the tissues were thawed, weighed, and homogenized on ice in Tris buffer at pH 7.2 (Tris 0.8M (Trizma-base Sigma® T6791); NaCl 2M (Sigma® S9888); DTT 100 mM (Sigma® 8349-12-3); CIP according to supplier protocol (Protease Inhibitor Cocktail, Sigma® P8340)). The homogenates were centrifuged at 9000 g at  $4^{\circ}\text{C}$  for 30 min. The amount of total protein was quantified using the Bradford method (Bradford 1976). Catalase (CAT) activity was measured spectrophotometrically at 240 nm ( $\epsilon = 0.04 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ ; UviLine® 9600), following the dismutation of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (Claiborne et al. 1985). Specific activity was expressed in  $\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein. Glutathione-S-Transferase (GST) activity was measured spectrophotometrically at 340 nm ( $\epsilon = 9.6 \text{ mM}^{-1}\cdot\text{cm}^{-1}$  TECAN® Sunrise) by observing the conjugation of the 1-chloro-2,4-dinitrobenzene (CDNB) and the L-glutathione reduced (GSH) (Habig et al. 1974). Specific activity was expressed in  $\text{nmoles}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein. Phosphatase acid (AcP) activity was determined spectrophotometrically at 340 nm ( $\epsilon = 18.3 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ ; TECAN® Sunrise; Phosphatase acid Assay kit Sigma® CS0740), measuring the appearance of p-nitrophenol (pNP) by the hydrolysis of p-nitrophenylphosphate (4-NPP) and expressed in  $\text{nmoles}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein.

#### *Genotoxicity (comet assay)*

The comet assay is a method for measuring the amount of DNA damage present in cells. This test was carried out as previously described (Barranger et al. 2014; Akcha et al. 2000). After exposure to the 10 conditions, gills and digestive glands were dissected and then immersed in a solution of dispase II ( $2 \text{ U}\cdot\text{mL}^{-1} - 30 \text{ min} - 37^{\circ}\text{C}$ ). Cell viability was checked using the Trypan Blue test. Cells were resuspended in low melting point agarose 0.5%, and the mixture was placed on microscope slides, previously covered with agarose 0.8%. After gel solidification, slides were submerged in a lysis buffer (NaCl 2.5M (Sigma® S9888), EDTA 1M (Sigma® E5134), Tris 0.001M (Trizma-base Sigma® T6791), Triton (Sigma® TritonX 100)) overnight at  $4^{\circ}\text{C}$ . Following lysis, slides were washed 3 times 5 minutes with PBS 1X,



and the DNA denaturation was carried out (15 min), followed by the electrophoresis (10 min – 300 mA). At the end of the electrophoresis, slides were rinsed 3 x 5 min in a neutralization buffer (Tris-base 0.4 M (Trizma-base Sigma® T6791) pH 7.5) and then dehydrated for 10 min with 70% ethanol. DNA is revealed by adding 30 µL of ethidium bromide and observed using an optical fluorescence microscope (Olympus BX60, x 40) equipped with a CDD camera (Lucas-S, Andor Technology) and a Komet 6 image analysis system (Kinetic Imaging Ltd). 150 nuclei were analyzed per slide, and DNA damage was expressed in the percentage of the DNA present in the Comet tail (% Tail DNA) for each nucleus.

#### *Condition index*

At the end of the exposure, 10 clams per condition were sampled, and the soft tissues were removed. Tissues and shells were oven-dried at 70°C for 48h. After 48h, dried tissue and shells were individually weighted to determine the Condition Index (CI). It was calculated according to the following formula (Walne, 1970).

$$\text{Condition Index (CI)} = \frac{\text{Dried tissues weight (g)}}{\text{Dried shell weight (g)}} \times 100$$

#### *Burrowing test*

Burrowing tests were performed as described in Bonnard et al. (2009) at T21. The burrowing experiments were conducted in glass crystallizers containing 5 cm of artificial sediments (90% sand, 9% kaolinite, 1% calcium carbonate) supplemented with salt water (2 cm above the sediment). The sediment was hand prepared and placed in the crystallizers five days before the experimentation. The burial behavior was evaluated by placing 10 individuals per condition at the surface of the sediment in the dark and observing the number of them that burrowed at frequent intervals. The interval was every 5 minutes within the first hour, every 10 minutes in the second hour, and every 20 minutes from the third to the sixth hour. Percentages of unburrowed individuals over time were determined for each condition.

#### *Statistical analysis*

The normality of data distribution was tested using the Shapiro-Wilk test, and the homogeneity of variances among groups was tested using the Bartlett test. If the data did not meet the conditions for parametric tests, Kruskal-Wallis tests were used to test for differences between the treatments. If the overall test was significant, a Dunn procedure was performed to determine which means were significantly different. If the data followed the conditions for the application of parametric tests, an ANOVA was used to test for differences between the treatments. If the overall test was significant, a Tukey post hoc test was performed to determine which means were significantly different. For the burrowing test, percentages of unburrowed individuals were first ln-transformed for linearization, and then the regression coefficients of the leastsquare regression lines were compared using analysis of covariance (ANCOVA). In all cases,  $p$  values  $\leq 0.05$  were considered statistically significant, using XLSTAT 2019 (version 21.4.63762).

## Results

### *Oxidative stress and detoxification*

After 7 days of exposure to PS NPs 10, ENV NPs 0.008 and 100, and ENV MPs 0.008, a higher activity of catalase (CAT) was measured in the gills compared to the non-exposed clams (control) (Figure 1A). At 21 days post-exposure, repression of CAT activity was measured for organisms exposed to ENV NPs 10 and 100 compared to the controls (Figure 1B). In the visceral mass, the exposure to PS NPs 100 induced a significant reduction of the CAT activity compared to the control (Figure 1C). No effect of the different plastic particles was observed after 21 days of exposure in the visceral mass (Figure 1D).

The glutathione-S-transferase (GST) activity increased significantly in the gills of *Corbicula fluminea* after 7 days of exposure to PS NPs 10, ENV MPs 10, and ENV NPs 0.008 compared to the control (Figure 2A). After 21 days of exposure, GST activity was higher than the control for the organisms exposed to PS NPs 0.008 and 10, ENV NPs 100, and ENV MPs 0.008 and 100. In contrast, we observed a decrease in GST activity in organisms exposed to ENV NPs 0.008 and 10 compared to all the other plastic conditions and the controls. In the visceral mass, fewer effects were observed with lower GST activity after an exposure of 7 days to PS NPs 100 and ENV NPs 0.008 compared to the controls (Figure 2C) and after 21 days to ENV NPs 10 and 100 (Figure 2D).

### *Biochemical endpoint*

After 7 days, a significant increase in acid phosphatase (AcP) activity was measured in the gills of clams exposed to the low and intermediate concentrations of PS NPs (0.008 and 10  $\mu\text{g L}^{-1}$ ) compared to the non-exposed clams (Figure 3A). Conversely, AcP activity was significantly lower for organisms exposed to ENV NPs 10 and 100 than in all other conditions, including the control. At 21 days post-exposure, an increase in AcP activity was only observed for PS NP 10 and ENV MP 100 conditions compared to the control (Figure 3B). In the visceral mass, a significant increase in AcP activity was observed for the organisms exposed to ENV 0.008 and 10, and ENV MPs 10. After 21 days of exposure, no significant differences were observed between the control and the different plastic conditions.

### *Genotoxicity*

The exposure for 7 days to the lowest concentrations (0.008 and 10  $\mu\text{g L}^{-1}$ ) of PS NPs induced a significant increase of DNA damages in gills compared to the control while the level of genotoxicity was slightly decreased when clams were exposed to ENV NPs 100 (Figure 4A). After 21 days of exposure, all the plastic conditions impacted the samples with a significant increase in DNA damage compared to the control condition (Fig.4B). In the visceral mass, no increase in DNA damage was measured compared to the control condition after 7 and 21 days of exposure (Figure 4C and D).

### *Condition index (CI)*

No significant differences were observed between conditions, with a mean CI of the organisms comprised between 3.02 and 3.42 (Table 1).

### *Burrowing test*

The results of the burrowing test for *Corbicula fluminea* after 21 days of exposure to the ten conditions are reported in Table 2. Burrowing kinetics were significantly impaired in clams exposed to PS NPs 10 compared to the control. Organisms exposed to PS NPs 10 had a significantly higher burrowing speed (slope -0.0018) than the control (-0.0048) and all the

other plastic conditions. No concentration effect was measured for PS NPs, ENV NPs, or MPs.

## **Discussion**

There is a concern that misleading conclusions may be drawn regarding the toxicity of plastic particles to aquatic organisms from laboratory experiments using standard MPs and NPs (Latchere et al., 2021; Phuong et al., 2016). One of the questions from laboratory experiments is whether the toxic effects measured on aquatic organisms exposed to standard MPs and NPs reflect the reality encountered in the environment (Latchere et al., 2021; Phuong et al., 2016). Indeed, the properties of standard MPs and NPs differ from those identified in the environment in terms of shape, composition, surface charge, adsorbed contaminants, or biofilms covering their surface (Gigault et al., 2016, 2018; Phuong et al., 2016). To date, very few studies have been carried out to compare the toxicity of pristine plastic particles with those derived from the environment (Arini et al., 2022a, 2022b; Baudrimont et al., 2019; Lebordais et al., 2021b, 2021a; Métais et al., 2023). Pioneering studies revealed that the toxic effects of plastic particles could be underestimated using standard particles compared to those derived from the environment on the algae *S. subspicatus* (Baudrimont et al., 2019) and the oyster *I. alatus* (Arini et al., 2022a). The present study aimed to provide new insights into comparing the toxicity of standard and environmental plastic particles on the freshwater bivalve *C. fluminea*. Results of this study demonstrated that: i) ENV NPs triggered more effects on detoxification processes and immune response and fewer effects on burrowing activity than PS NPs, confirming that the use of standard NPs can lead to misleading conclusions on the risks posed by plastic particles; ii) effects of ENV MPs were less marked than ENV NPs, emphasizing the importance of testing a size continuum of plastic particles from NPs to MPs; iii) some effects were only observed for the low and/or intermediate concentrations tested, underlining the importance of using environmentally relevant concentrations.

### *Detoxification system*

Oxidative stress is a major mechanism involved in toxicity and stress caused by MPs and NPs (Hu and Palić, 2020; Li et al., 2022). Among the different enzymes of the antioxidant system, catalase (CAT) and glutathione-S-transferase (GST) are involved in the first line of defense to

eliminate reactive oxygen species (ROS) (Prokić et al., 2019). The catalase catalyzes the conversion of hydrogen peroxide into water and molecular oxygen. In our study, CAT activity was increased in the gills after 7 days of exposure to the three different types of plastic particles (PS NPs, ENV NPs, and ENV MPs) for at least one of the concentrations tested. This is consistent with another study demonstrating an increase in the CAT activity in the soft tissues of *C. fluminea* after 7 days of exposure to PS MPs at concentrations from 0.01 to 1 mg L<sup>-1</sup> (Fu et al., 2022). Similar results were also reported by Parra et al. (2021), demonstrating an increase in CAT activity in the gills of *C. fluminea* after a 7-day exposure to PS MPs. In addition, Ribeiro et al. (2017) reported an increase in CAT activity in the gills of *S. plana* exposed to PS MPs after 21 days (14 days of exposure and 7 days of depuration). On the contrary, a decrease in CAT activity was evidenced in the gills after a 21-day exposure to only 10 and 100 µg L<sup>-1</sup> ENV NPs. A reduction in CAT activity has also been reported in the mussel *Mytilus spp.*, exposed to PS MPs for 7 days (Paul-Pont et al., 2016). In the visceral mass, the minimal effect of the different particles tested was measured on the enzymatic activity of catalase at the two sampling times. Only the PS NPs at the highest concentration tested induced an increase in catalase activity after 7 days of exposure. This result is consistent with a previous study demonstrating an increase in CAT activity in the digestive glands of the mussel *Mytilus spp.* exposed to 0.008 and 10 µg L<sup>-1</sup> of PE and PP MPs (Revel et al., 2019).

The GST enzymes play a significant role in protecting against toxicants by catalyzing the conjugation of reduced glutathione (GSH) with metabolites and reactive electrophiles. Our study revealed an increase in GST activity in gills after a 7-day exposure to PS NPs 10, ENV NPs 0.008, and ENV MPs 10. Similar results were obtained by Ribeiro et al. (2017) in clam *S. plana* exposed to PS MP<sub>10</sub> for 14 days. Conversely, the GST activity was inhibited for 0.008 and 10 µg L<sup>-1</sup> ENV NPs in the gills. This suggests a more substantial effect of the ENV NPs compared to PS NPs and ENV MPs in gills, as observed for the catalase activity. This inhibitory effect can substantially reduce ROS detoxification capacities and lead to injuries in cells and tissues (Li et al., 2022). In the visceral mass, inhibition of the GST activity was depicted in the organisms exposed to 10 µg L<sup>-1</sup> PS NPs and 0.008 µg L<sup>-1</sup> ENV NPs after 7 days of exposure and to 10 and 100 µg L<sup>-1</sup> ENV NPs after 21 days of exposure. These results agree with a previous study showing a decrease in GST activity in the digestive gland of *S. plana* exposed to PS MP<sub>10</sub> for 3 and 21 days (Ribeiro et al., 2017). In addition, a decrease in GST activity was also reported in the digestive gland of mussels exposed to various types of

standard MPs (Abidli et al., 2021; Avio et al., 2015; Paul-Pont et al., 2016; Revel et al., 2019). Our study did not demonstrate any effect of the ENV MPs on GST activity in the visceral mass, unlike the two types of NPs (PS and ENV). The small size of NPs compared to MPs could make them more likely to be internalized and accumulated, which could partly explain their higher toxicity. Indeed, a study demonstrated that the accumulation of PS NPs was size-dependent in the tissues of the mussel *M. galloprovincialis*, with a higher internalization of NPs for the smallest particles (Sendra et al., 2020). Finally, the concentrations of certain chemical elements such as arsenic or chromium are much higher on the ENV NPs than in the ENV MPs in our study (S.I Table A). This could also explain the greater response of GST activity in organisms subjected to ENV NPs compared to ENV MPs.

### **Biochemical endpoint**

Acid phosphatase (AcP) is a widely used biomarker to assess the impacts of contaminants on the immune system (Jayakumar et al., 2009). Indeed, lysosome functions can be disrupted when organisms face pollutants (Wootton et al., 2003). In our study, an increase in AcP activity was reported in gills of organisms exposed to 0.008 and 10  $\mu\text{g L}^{-1}$  PS NPs at 7 days of exposure and 10  $\mu\text{g L}^{-1}$  PS NPs and 100  $\mu\text{g L}^{-1}$  ENV MPs at 21 days of exposure in comparison to the control condition. Such an increase in AcP activity has also been reported in the hemolymph of mussel *Mytilus spp.* exposed to 0.008 and 10  $\mu\text{g L}^{-1}$  of a mixture of PE and PP MPs for 10 days (Revel et al., 2019). In addition, the gene *mytilin B* involved in the immune system is up-regulated in the gills of the mussel *M. galloprovincialis* after acute exposure (24h) to PE MP (1 to 50  $\mu\text{m}$ ) at a concentration of  $1.5 \times 10^7$  beads per liter (D  tr  e and Gallardo-Esc  rate, 2017). In the present study, a decrease in AcP activity was shown in the gills after 7 days in the organisms exposed only to the ENV NPs (10 and 100  $\mu\text{g L}^{-1}$ ). This inhibition of the immune response by ENV NPs could be linked to a reduced energy budget and cause severe damage to the gills in the long term. Indeed, Gardon et al. demonstrated that pearl oysters exposed to PS MPs for two months showed limited immune response capacity hampered by a lower energetic budget (Gardon et al., 2020).

### **Genotoxicity**

Plastic particles can induce genotoxic damage such as DNA strand breaks or nuclear alterations. These damages can be caused by direct interaction of the plastic particle with the

genetic material and/or indirect damage through the production of ROS due to the toxicity of the plastic particles, their additives, or chemicals adsorbed on their surface (Magdolenova et al., 2014). Our study demonstrated DNA damage in gills after exposure to 0.008 and 10  $\mu\text{g L}^{-1}$  PS NPs and 100  $\mu\text{g L}^{-1}$  ENV NPs at 7 days and for all plastic conditions and regardless of the concentration tested at 21 days. Different studies have also reported such damages in bivalves exposed to PS MPs (Avio et al., 2015; Ribeiro et al., 2017) and plastic microfibers (Alnajjar et al., 2021). In our study, the damage could result partly from chemical compounds used as additives for the three types of particles tested or adsorbed on the surface of the environmental MPs and NPs. In this sense, we have measured very high concentrations of metals in ENV MPs and NPs (S.I Table A).

### **Condition index**

The CI is a widely used tool to evaluate the bivalves' physiological state, growth, and sexual maturity and is effective as an indicator of environmental stresses (Filgueira et al., 2013; Galvao et al., 2015; Zeng and Yang, 2021). No alteration of the CI was demonstrated for the different plastic particles tested. Our results agree with previous studies that have shown no effect of plastic particles on the body condition index of different bivalves (Bour et al., 2018; Joyce and Falkenberg, 2022; Métais et al., 2023; Ribeiro et al., 2017; Von Moos et al., 2012). Interestingly, a shift in energy allocation from reproduction to organism maintenance and structural growth was evidenced in oysters exposed to PS MPs (Sussarellu et al., 2016). Such a change in energy flow could explain the lack of effect on the condition index. However, this lack of effect is likely related to the duration of our exposure, which might not be long enough to trigger a decrease in the condition index, as pointed out by Shang et al. (2021).

### **Burrowing activity**

Behavioral biomarkers such as the burial rate are relevant tools to assess the impacts of various contaminants on aquatic organisms (Amiard-Triquet, 2009; Pan et al., 2012). In addition, behavioral effects have been reported at low realistic concentrations of pollutants similar to those measured in the environment (Amiard-Triquet, 2009). The sensitivity of these biomarkers can thus help assess the ecological risk of MPs and NPs. Burrowing activity is crucial for sediment-dwelling clams such as *C.fluminea*. Its impairment can increase predation

risks or lead to the translocation of the organisms by water flow to unsuitable habitats (Cooper and Bidwell, 2006; Tallqvist, 2001). In the present study, ENV MPs and NPs did not induce effects on burrowing activity after a 21-day exposure. Our results are consistent with a previous study demonstrating no effect of a 4-week exposure to PE MPs at environmentally relevant concentrations on the burrowing behavior of two sediment-dwelling bivalves, *E. tenuis* and *A. nitida* (Bour et al., 2018).

On the other hand, a significant alteration of the burrowing kinetic was demonstrated in clams exposed to PS NPs at  $10 \mu\text{g L}^{-1}$ , presenting a higher burrowing speed than controls. On the contrary, polychaetes *H. diversicolor* exposed to PS NPs (100 nm) at concentrations of 0.005 to  $0.5 \text{ mg L}^{-1}$  for 28 days exhibited altered burrowing behavior, taking longer to burrow in the sediment than the controls (Silva et al., 2020). This alteration in burrowing behavior could come from an inhibition of the neurotransmitter system by PS NPs. Indeed, studies have reported inhibition of acetylcholinesterase activity following exposure to PS MPs in the clam *S. plana* (Ribeiro et al., 2017) and the mussel *M. galloprovincialis* (Avio et al., 2015). To better understand the effects of plastic particles on the higher burrowing speed observed for PS NPs, other parameters could be analyzed as done by Montalvão et al. (2019) after video-recording: burial depth, latency to foot emission and to start excavation, and the number of excavation cycles.

### **Using environmental plastic particles for better risk assessment of plastic particles**

Our study compared the effects of two types of plastic particles: PS NPs and environmental MPs and NPs. These particles do not have the same properties concerning their shape, composition, surface charge, additives, and adsorbed pollutants. Our results demonstrated that ENV NPs might induce stronger effects than PS NPs in the detoxification processes (CAT and GST) and immunity response (AcP). In particular, a repression of the activity of these enzymes was only measured for organisms exposed to ENV NPs in the gills and the visceral mass. This may indicate a toxic effect of NPs on these enzymes by their surface reactivity, the oxidative stress generated, or the release of chemical compounds adsorbed on their surface. Another study aimed to compare the effects of PS NPs and environmental NPs at the molecular level in oyster *I. alatus* (Arini et al., 2022). The authors suggest that the environmental NPs may be more toxic than the standard PS NPs (Arini et al., 2022a). In addition, higher growth inhibition was measured in freshwater microalgae *S. subspicatus* exposed to environmental NPs compared to manufactured NPs (Baudrimont et al., 2019).



In general, it is recognized that NPs have stronger toxicity due to their smaller particle sizes and higher specific surface area. In our study, the effects of environmental NPs were more marked than those of environmental MPs, especially in enzymatic responses. This may indicate a higher toxicity of ENV NPs than ENV MPs in the detoxification processes and immune system. This could be partly explained by the higher concentrations of some metals and metalloids measured in the ENV NPs compared to the ENV MPs. In addition, Yin et al. (2021) compared the effects of MPs and NPs in the literature and concluded that NPs cause more significant oxidative stress and inflammation than MPs in the intestine. However, very few studies compare the effects of MPs and NPs under the same conditions. It is, therefore, necessary to pursue research efforts in this direction to better understand the similarities and differences in the mechanisms of toxicity of MPs and NPs on the same batch of individuals and under the same exposure conditions.

Our study only observed some effects of plastic particles at low and/or intermediate concentrations. This may be due to the aggregation of plastic particles occurring at high concentrations and decreasing the particle's bioavailability. Therefore, it appears appropriate to use relatively low concentrations close to those measured in the environment in future laboratory studies.

MPs and NPs toxicity can also arise from their potential roles as chemical vectors. Different persistent organic pollutants and toxic metals are adsorbed on MPs in the environment (Antunes et al., 2013; Mato et al., 2001). Indeed, additives in the particles and/or chemical compounds can be adsorbed on their surface (Brennecke et al., 2016; Ivar Do Sul and Costa, 2014). Additives and adsorbed chemicals can be transported and bioaccumulated in different tissues, leading to various toxicological effects (Gomiero et al., 2018; Guilhermino et al., 2018; Rainieri et al., 2018). Toxic effects were thereby observed in several bivalves (Latchere et al., 2021), including oxidative stress and lipid peroxidation damage in *C. fluminea* exposed to a mixture of MPs and mercury (Oliveira et al., 2018). However, most studies compare the toxic effects of organisms exposed to virgin plastic particles with or without added chemicals. Our study measured metal and metalloid concentrations in environmental MPs and NPs. The results revealed very high concentrations of oxidative stress, immune response, and genotoxicity observed in *C. fluminea*.

Interestingly, Baudrimont et al. (2019) measured higher concentrations of trace metals in environmental NPs compared to standard NPs. In their study, these authors also reported higher growth inhibition of microalgae *S. subspicatus* exposed to environmental NPs than standard ones (Baudrimont et al., 2019). In addition, two studies using the same plastic particles as in our study demonstrated that PS NPs did not present the same toxicity as compared to ENV MPs and NPs in the mussel *Mytilus edulis* and clam *Scrobicularia plana* (Métais et al., 2023; Roman et al., 2023). However, there is a knowledge gap on the mechanisms of toxicity of environmental MPs and NPs, and further investigations are needed to explore their potential joint toxicity with chemicals.

### **Conclusion**

We demonstrated in this study that exposures to ENV NPs triggered more effects than PS NPs on detoxification processes and immune responses in clam *C. fluminea*. Effects of exposure to ENV MPs were less pronounced than with ENV NPs, indicating a potential higher toxicity of small particles on detoxification and the immune system. These results demonstrate the interest in using environmental plastic particles rather than standard ones. In light of this study, laboratory experiments should continue with the exposure of aquatic species to environmental MPs and NPs. Their properties have to be characterized for a better risk assessment of environmental plastic particles.

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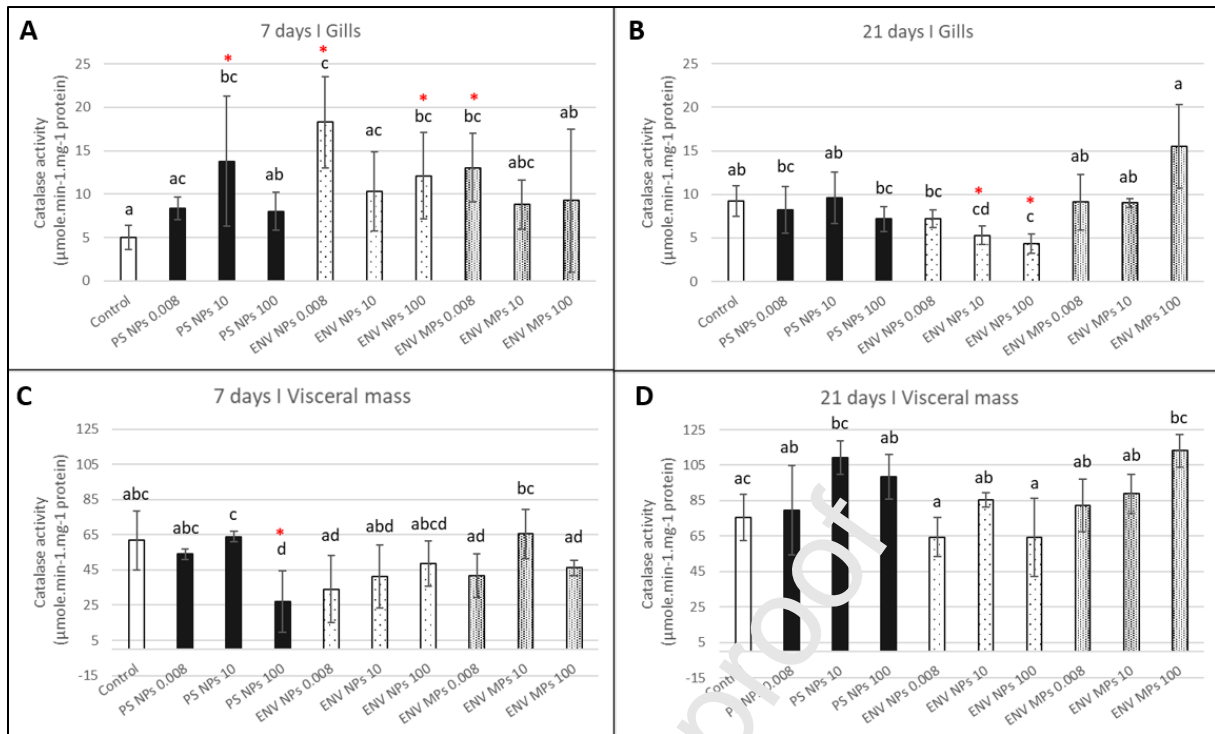
**Table 1** Condition Index (mean and standard deviation)

	<b>Concentrations (<math>\mu\text{g L}^{-1}</math>)</b>	<b>CI means</b>	<b>CI SD</b>
<b>Control</b>	0	3.03	0.79
<b>PS NPs</b>	0.008	3.02	0.80
	10	3.15	0.72
	100	3.15	0.72
<b>ENV NPs</b>	0.008	3.36	0.92
	10	3.39	0.90
	100	3.42	0.88
<b>ENV MPs</b>	0.008	3.14	0.70
	10	3.20	0.68
	100	3.36	0.92

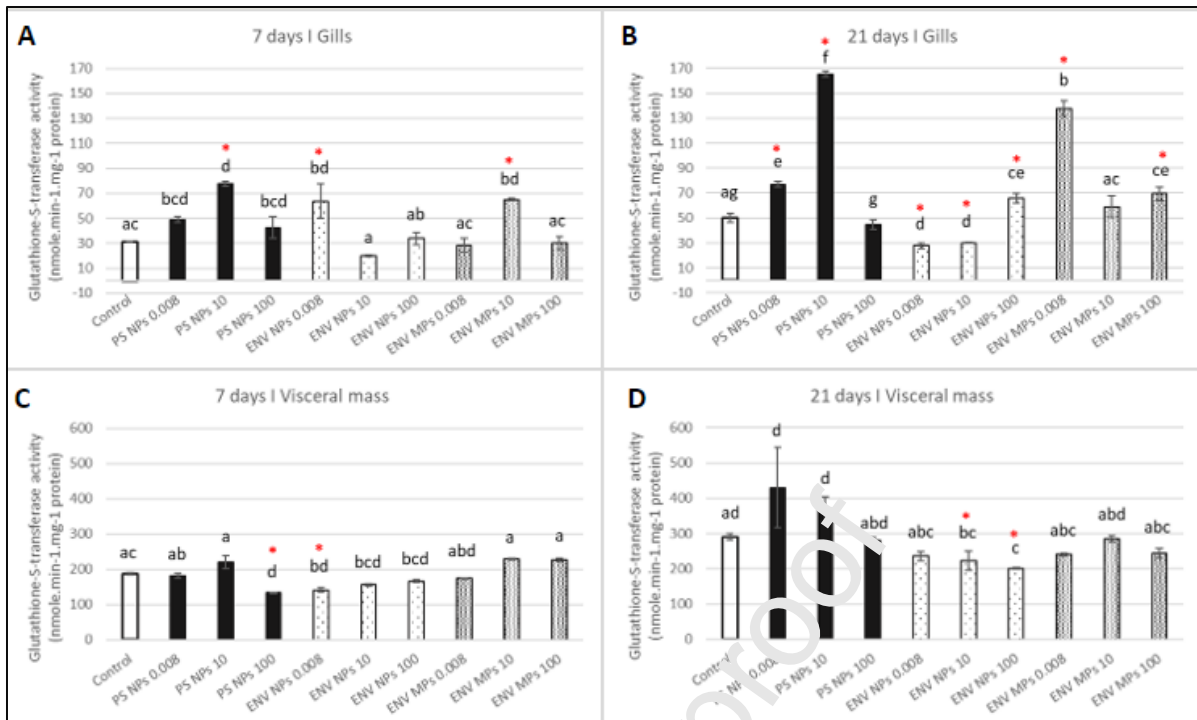


**Table 2** Slopes of burrowing tests for *Corbicula fluminea* in control conditions and after exposure to standard polystyrene nanoplastics (PS NPs) and environmental micro- (ENV MPs) and nanoplastics (ENV NPs) at 0.008, 10, and 100  $\mu\text{g L}^{-1}$ , (n=10). *Superscripts letters showed significant differences between conditions and the ones in bold showed for significant differences between control and plastic conditions,  $p < 0.05$* ).

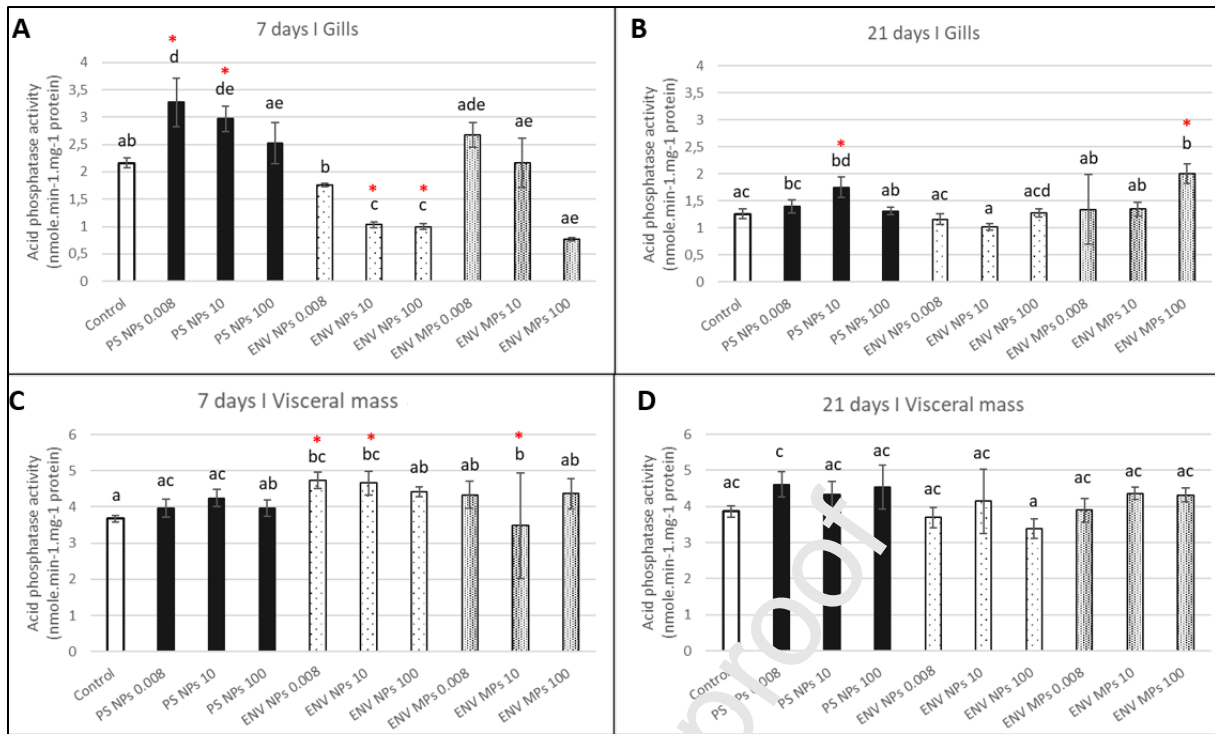
	Concentrations ( $\mu\text{g L}^{-1}$ )	Slopes
<b>Control</b>	0	-0,0048 <sup>b,c</sup>
<b>PS NPs</b>	0.008	-0,0071 <sup>b</sup>
	<b>10</b>	<b>-0,0018<sup>a</sup></b>
	100	-0,0101 <sup>b,c</sup>
<b>ENV NPs</b>	0.008	-0,0081 <sup>b,c</sup>
	10	-0,127 <sup>c</sup>
	100	-0,0099 <sup>b,c</sup>
<b>ENV MPs</b>	0.008	-0,0063 <sup>b,c</sup>
	10	-0,0085 <sup>b,c</sup>
	100	-0,009 <sup>b,c</sup>



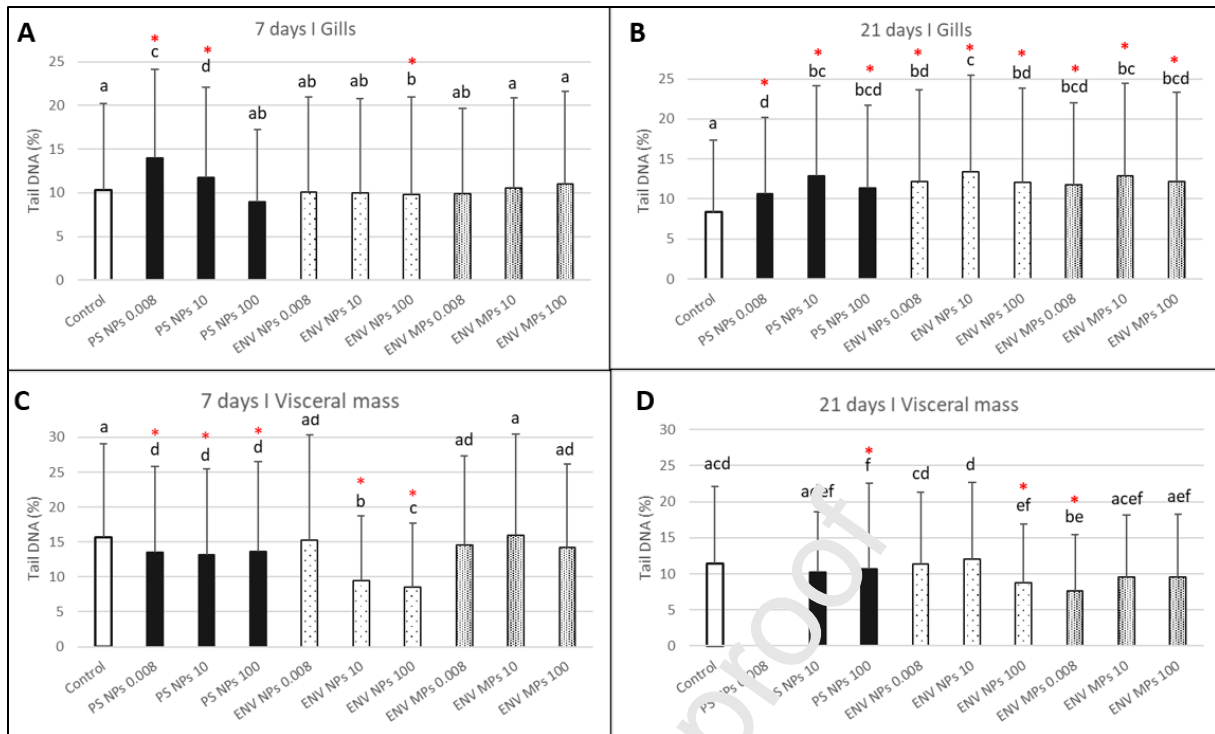
**Fig. 1** Enzymatic activity of CAT in gills (A, B) and visceral mass (C, D) of *Corbicula fluminea* after 7 (A, C) and 21 days (B, D) exposure to standard polystyrene nano- (PS NPs) and environmental micro- (ENV MPs) and nano- (ENV NPs) plastics at 0.008, 10 and 100  $\mu\text{g L}^{-1}$  (mean  $\pm$  SD; n=5; letters showed significant differences between conditions; red star showed significant differences between plastic condition and control,  $p < 0.05$ )



**Fig. 2** Enzymatic activity of GST in gills (A, B) and visceral mass (C, D) of *Corbicula fluminea* after 7 (A, C) and 21 days (B, D) exposure to standard polystyrene nanoplastics (PS NPs) and environmental micro- (ENV MPs) and nanoplastics (ENV NPs) at 0.008, 10 and 100  $\mu\text{g L}^{-1}$  (mean  $\pm$  SD; n=5; letters showed significant differences between conditions; red star showed significant differences between plastic condition and control,  $p < 0.05$ )



**Fig. 3** Enzymatic activity of AcP in gills (A, B) and visceral mass (C, D) of *Corbicula fluminea* after 7 (A, C) and 21 days (B, D) exposure to standard polystyrene nanoplastics (PS NPs) and environmental micro- (ENV MPs) and nanoplastics (ENV NPs) at 0.008, 10 and 100  $\mu\text{g L}^{-1}$  (mean  $\pm$  SD; n=5; letters showed significant differences between conditions; red star showed significant differences between plastic condition and control,  $p < 0.05$ )



**Fig. 4** DNA integrity in gills (A, B) and visceral mass (C, D) of *Corbicula fluminea* after 7 (A, C) and 21 days (B, D) exposure to standard polystyrene nanoplastics (PS NPs) and environmental micro- (ENV MPs) and nanoplastics (ENV NPs) at 0.008, 10 and 100  $\mu\text{g L}^{-1}$  (mean  $\pm$  SD; n=3; letters showed significant differences between conditions; red star showed significant differences between plastic condition and control,  $p < 0.05$ )

Author Statement

AC, IM, MB, AFM, JG : Funding acquisition

OL, AC, IM, MB, AFM, HPE, MM : Experimental conceptualization, methodology

MB, AFM : Macro-sized plastic debris collection

OL, AC, IM, HPE, MM, DG : Conduct the experiment

OL, CR : Data analysis and article writing

JG : Microplastics and nanoplastics synthesis

CC : Measurement of metals and metalloids concentrations in microplastics and nanoplastics :

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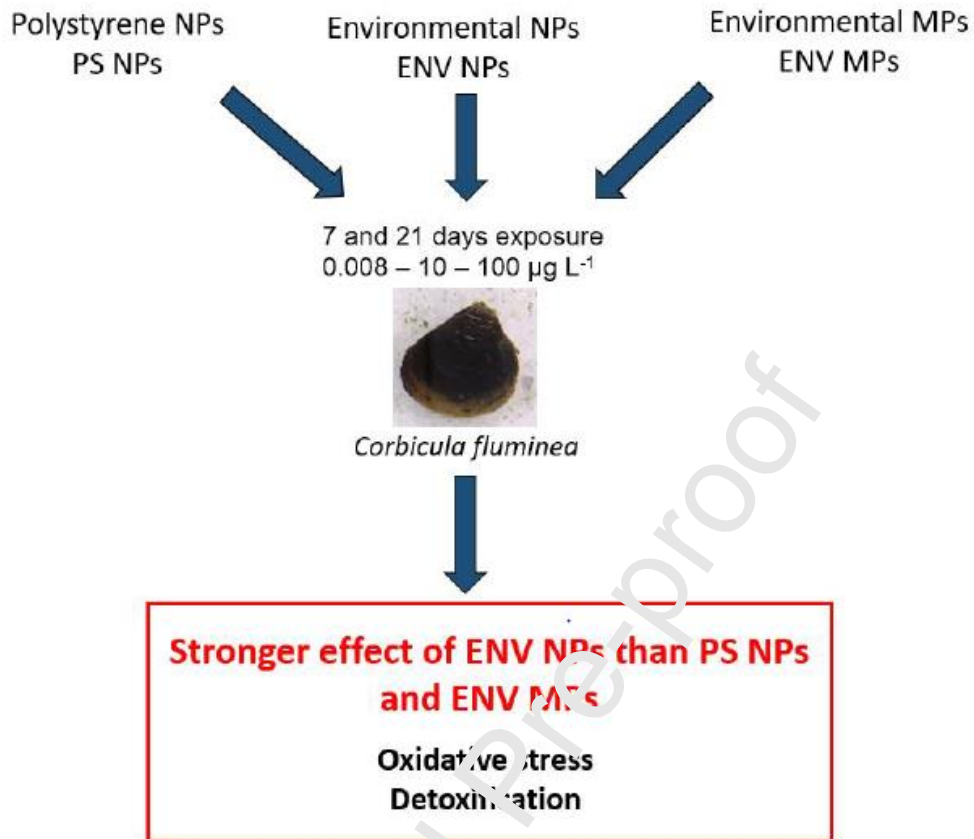
**Declaration of interests**

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Graphical abstract





## Highlights

- ENV NPs triggered more effects on detoxification and immune processes than PS NPs
- Effects of ENV MPs were less marked than ENV NPs
- Importance of using environmental MPs and NPs for realistic assessment of the risk

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