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Toxicity of environmental and polystyrene plastic particles on the bivalve *Corbicula fluminea:* focus on the molecular responses

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Abstract

Among aquatic organisms, filter feeders are particularly exposed to the ingestion of microplastics (MPs) and nanoplastics (NPs). The present study investigates the effect of environmental microplastics (ENV MPs) and nanoplastics (ENV NPs) generated from macro-sized plastic debris collected in the Garonne River (France), and polystyrene NPs (PS NPs) on the freshwater bivalve *Corbicula fluminea*. Organisms were exposed to plastic particles at three concentrations: 0.008, 10, and 100 µg L^{-1} for 21 days. Gene expression measurements were conducted in gills and visceral mass at 7 and 21 days to assess the effects of plastic particles on different functions. Our results revealed: (i) an up-regulation of genes, mainly involved in endocytosis, oxidative stress, immunity, apoptosis, and neurotoxicity, at 7 days of exposure for almost all environmental plastic particles and at 21 days of exposure for PS NPs in the gills, (ii) PS NPs at the three concentrations tested and ENV MPs at 0.008 µg L^{-1} induced strong down-regulation of genes involved in detoxication, oxidative stress, immunity, apoptosis, and neurotoxicity at 7 days of exposure in the visceral mass whereas ENV MPs at 10 and 100 µg L^{-1} and all ENV NPs induced less pronounced effects, (iii) overall, PS NPs and ENV MPs 0.008 µg L^{-1} did not trigger the same effects as ENV MPs 10 and 100 µg L^{-1} and all ENV NPs, either in the gills or the visceral mass at 7 and 21 days of exposure. This study highlighted the need to use MPs and NPs sampled in the environment for future studies as their properties induce different effects at the molecular level to living organisms.

Keywords nanoplastics · microplastics · bivalve · freshwater ecotoxicology · *Corbicula fluminea*

Introduction

Plastics are synthetic or semi-synthetic organic materials used for a wide range of applications in the industrial sector. Omnipresent in our societies, their production has continued to increase in recent decades. Thus, since the middle of the 20th century, global plastic production has increased from 2 million tons in 1950 to 380 million tons in 2015 (Geyer et al. 2017). However, only 5% of the plastics produced are recycled, mainly into secondary products which will not be further recycled and end up in landfills or the environment (Sardon and Dove 2018). The mass production of plastics, combined with high durability and low recycling rates, have led to their accumulation as wastes in the terrestrial, freshwater and marine environments (de Souza Machado et al. 2018; Dioses-Salinas et al. 2020; Horton et al. 2017).

Plastic particles ranging in size from 1 to 5 mm are defined by the term "microplastics" (MPs) (Browne et al. 2007; Fendall and Sewell 2009). MPs are considered primary or secondary depending on their sources. MPs are primary when produced during manufacture in the form of small particles. They are notably present in certain cosmetic products, skin cleansers, and production wastes from plastic

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processing plants (Wang et al. 2016). They are very stable in this form, with a lifespan of over 1000 years (Cózar et al. 2014). Secondary MPs derive from the fragmentation of larger pieces of plastic under the effect of different biotic and abiotic factors such as photodegradation, waves, wind, microorganisms, and sediment abrasion (Andrady 2011; Kale et al. 2015). Secondary MPs represent a significant part of the MPs present in the marine environment. Recently, smaller plastic particles have been identified and described as nanoplastics (NPs) (Gigault et al. 2016). These particles have colloidal properties in aqueous media (e.g., they do not sediment) and their size varies from 1 to 1000 nm in one of the three dimensions of space (Gigault et al. 2021). NPs are also introduced in significant quantities into the natural environment but their presence is difficult to estimate due to methodological challenges (da Costa et al. 2016; Hernandez et al. 2017; Koelmans et al. 2015). Owing to the massive use of plastics and to the additives they may contain, MPs and NPs pose environmental risks (Besseling et al. 2019; Koelmans et al. 2022). In addition, chemicals can be adsorbed on plastic particles due to the surface alteration of the particles and to the small size of the particles which increases their surface. When chemical conditions changed (pH for example), such pollutants can be desorbed. Moreover, due to their nanoscale properties, NPs can easily cross biological barriers and accumulate in tissues and organs (Chae and An 2017; Mattsson et al. 2018). In addition, they have a longer retention time than MPs in bivalves (Ward and Kach 2009). The ingestion of MPs and NPs by aquatic organisms is of particular concern since numerous studies have demonstrated their harmful effects (Al-Thawadi 2020; Issac and Kandasubramanian 2021). Indeed, plastic particles induce effects from the cellular to the ecosystem levels by impairing, for example, metabolic and physiological processes, morphology, food absorption, and behavior (Al-Thawadi 2020; Gardon et al. 2018; Sussarellu et al. 2016; Watts et al. 2015).

Among aquatic organisms, filter feeders are particularly exposed to the ingestion of MPs and NPs because they filter large quantities of water for food and because of their unselective feeding strategy (Wesch et al. 2016). *Corbicula fluminea* is an endobenthic bivalve used as a bioindicator for the assessment of environmental quality (Arini et al. 2019; Guo and Feng 2018; Zhou et al. 2008). These organisms assimilate small particles from both the sediment and freshwater. They can bioaccumulate chemical substances and are widely used to evaluate the toxicity of freshwater and sediment (Guo and Feng 2018). Recent studies have been conducted on this species to assess the effects of plastic particles (Fu et al. 2022; Guilhermino et al. 2018; Guo and Feng 2018; Li et al. 2021). However, the plastic particles tested in most of these studies are standard beads and are not representative of the particles in the environment. Composed of a single type of plastic, mainly PS, perfectly spherical and uniform in size, they differ from secondary MPs and NPs resulting from the degradation of plastic wastes (Gigault et al. 2018 2016; Haegerbaeumer et al. 2019). Some studies have already started to demonstrate the more deleterious effects of environmental NPs compared to reference ones at environmental levels of exposure in *C. fluminea* (Baudrimont et al. 2019) and bivalve *Scrobicularia plana* (Metais et al. 2022) underlining the relevancy of using this type of NPs for ecotoxicological studies.

Thus, we aimed to investigate how source and size (MPs and NPs) of plastic particles could impact the toxicity on the bivalve *Corbicula fluminea*. To this aim, we studied the effects of both MPs and NPs resulting from the degradation of macroplastics sampled in the field on the bivalve *C. fluminea*. We also compared the toxicity of these environmental plastic particles with standard plastic particles. We chose to focus on standard PS NPs given that there is considerably less knowledge on NPs than on MPs in the literature to date. Bivalves were exposed to plastic particles at different environmentally relevant concentrations for 21 days. Of the various analyses carried out to evaluate the effects of environmental pollutants, the measurement of gene expression levels is helpful for identifying the mechanisms involved in the toxicant-specific responses and characterizing stress-induced expression patterns (Piña et al. 2007; Snell et al. 2003). These molecular markers can also provide early-detection of environmental stress. Therefore, we chose to study the influence of different sources of plastic particles (standard and environmental) and of size scale (micro and nano) on the expression level of a panel of genes involved in the responses to environmental stressors.

Materials and methods

Collection, preparation, and characterization of environmental derived MPs and NPs

Plastic wastes were collected by hand 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). Plastic debris showing signs of weathering (damaged labels, discoloration, covered with organic matter) were sampled, rinsed in the laboratory with ultra-pure water and dried at 45 °C for 48 h before preparation for micro and nanoplastic solutions.

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 Blancho et al. (2021). Briefly, NPs and MPs were produced by coupling agitation and sonification in aquatic media. The analysis was

performed using the same method used in Blancho et al. (2021). The size range was between 235 ± 70 nm for ENV NPs and 1.2 and 300 µm 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). Plastic analysis showed that ENV NPs and ENV MPs were mainly composed of polyethylene (PE) (95%) (Supplementary Information Fig. 1). They were anisotropic, polydisperse in size (Supplementary Information Fig. 2) and possessed high levels of carboxylic groups on their surface. Zeta potential of the ENV NPs was −36.2 mV. In addition to ENV NPs, carboxylated polystyrene nanobeads (PS NPs) with 200 nm of size, 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 (12N HNO3 subgrade) using a multi-step procedure with a microwave oven (MW7000 system from Anton-Paar; increasing ramp of the temperature of 6.6 °C per minute until reaching 250 °C, then 25 min at 250 °C under 140 bar of pressure). Metal concentrations were measured by ICP-MS from Agilent Technologies (7700x Model, Agilent) (Supplementary Information Table A). The solution of three tubes was mixed, evaporated at 90 °C, and solubilized in 0.37 N HNO3 before ICP-MS measurements. The digestion and analysis process 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 and nanoplastics

For each type of plastic particle (ENV MPs, ENV NPs, and PS NPs), stock suspensions at 1 and 0.1 g L^{-1} were prepared in ultra-pure (milliQ) water at pH 7. A working solution at 0.1 mg L^{-1} was obtained for each type of plastic particle by three serial dilutions of the stock suspension at 0.1 g L^{-1} in ultra-pure water (milliQ) at pH 7 as performed in Revel et al. (2019). A specific volume of the stock suspension (1 g L^{-1} or 0.1 g L^{-1}) or the working solution (0.1 mg L^{-1}) was distributed in the aquaria to obtain the final concentrations of 0.008, 10, and 100 µg L^{-1}. Each solution was well mixed before adding it to the aquaria. No surfactant was used during MPs and NPs preparation to prevent any additional effect. A strong manual mixing of the MPs and NPs solutions was performed before adding the aliquots to the aquariums as in Revel et al. 2020a. The MPs and NPs solutions were spilled in the aquariums every 3 days just after a water change to maintain the same concentration during exposure. We also use 6 aeration systems per aquarium to enable the dispersion of MPs and NPs.

Bivalve collection and laboratory exposure assay

Individuals of *Corbicula fluminea* were collected in the lake of Parentis-Biscarrosse (France). Clams were transported to the laboratory in boxes with sediment from the collection site. Bivalves were then transferred into aquaria (30 L) containing 27 L of tap water in a temperature-controlled room at 15 °C for an acclimatization period of 7 days. Photoperiod was maintained at 12:12 h. The aquarium water was renewed entirely every 3 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 µm) and field derived nanoplastics (ENV NPs, 235 ± 70 nm) at the following concentrations: 0.008 µg L^{-1}, 10 µg L^{-1} and 100 µg L^{-1}. These concentrations were chosen in accordance with the study of Revel et al. (2020b) to expose clams to concentrations of MPs and NPs which are close to the ones measured from coastal regions (0.008 µg L^{-1}) to oceanic gyres (100 µg L^{-1}) (Goldstein et al. 2013). The experimental conditions are abbreviated in the results section: for example, ENV MPs 10 is used for environmental microplastics at 10 µg L^{-1}. One group of individuals was used as control (no added plastic particles). The use of plastic material was avoided during all the experiments.

After 7 days and at the end of the experiment at 21 days, 3 individuals per condition and per replicate were sampled for gene expression. Gills and visceral mass were dissected and immediately frozen at −80 °C for further gene expression measurements.

Analysis of gene expression by quantitative PCR

Corbicula fluminea samples were pooled by three for each condition. Triplicates were analyzed by quantitative RT-PCR for each condition. Total RNA was extracted using TRIzol reagent® (Life Technologies) from the gills and the visceral mass, according to the manufacturer's recommendations and precipitated with propan-2-ol. RNA concentration (µg . µL^{-1}) was quantified using a NanoDrop 2000 spectrophotometer (ThermoScientific®). First-strand cDNA was synthesized from 5 µg of total RNA using the Invitrogen™ SuperScript™ III kit (ThermoScientific® ; T100™ Thermal Cycler, BIORAD®) according to the manufacturer's recommendations. The expression levels of twenty genes involved in endocytosis, oxidative stress, detoxication, respiratory chain, immunity, neurotoxicity, and apoptosis and were analyzed using a set of forward and

Table 1 List of genes, protein names, functions and sequences of specific primers used in this study

Gene	Protein name	Function	Accession number	Primers (5′-3′)	Source
β-actin	Beta Actine	Reference	A4L694	GATGGATGGTCCAGACTCGT[a] GGTCTGGATCGGTGGTTCTA[b]	Arini et al. 2019
rpl7	Ribosomal protein L7		P18124	CACCATCGTTGAAGTGGTTG[a] CTTCAAACAGGCTGCCAACT[b]	Arini et al. 2019
ef1α	Elongation factor 1 alpha		J9Z6R5	CTTCGTGCCAATCTCTGGAT[a] TTCCTCTCTACAGCCCAACC[b]	Arini et al. 2019
cltl	Clathrin heavy chain 1	Endocytosis	Q00610	GCAAACATCACACAGGTTGC[a] CGTGCGTAGTTGGACACATT[b]	Arini et al. 2019
cav	Caveolin		P51636	GTCCAGCACCATACGTGGTT[a] AACTGTTGACCACCCTCTGTG[b]	Arini et al. 2019
mt	Metallothionein	Detoxication	EF185126	CGGCTATCTCCCGCGA[a] AGCTTTTACCAGAACCAAACAGT[b]	Arini et al. 2019
mdr	ATP Binding Cassette Subfamily		KJ001772	ATCCTGGTTGATGGCACTGA[a] AGGTTCCTGGCTCACAATACC[b]	Chen et al., 2015
gst	Glutathione transferase, GST class-pi		AY885667	ATGACTTCATCAAGAGTTTACCAG[a] GCATGTTCTTGAACTCTTCTGA[b]	Bigot et al., 2009
cox1	Cytochrome C oxidase	Respiratory chain	Q9G2B4	CCTGTTTGGAGAAAGGGTCA[a] CCGTGGCATTCCACTTATTC[b]	Arini et al. 2019
12 s	Mitochondrial 12 s rRNA		EF446612	AGCATTACTATGTTACGACTTACCTCA[a] AGTTCAGGTAGACGTGTAGGG[b]	Arini et al. 2019
sod2	Superoxide dismutase 2	Oxidative stress	P04179	CCAGGCTAATGGCAGACTTC[a] GTAGGCATGCTCCCAAACAT[b]	Arini et al. 2019
sod1	Superoxide dismutase 1		U3PWG5	CCAGCAGCCAGACCAGTTAT[a] AGGGAGACGCTAATGTGTCG[b]	Arini et al. 2019
cat	Catalase		A4L695	CACCAGGTGTCCTTCCTGTT[a] CTCAGCATTCACCAGCTTGA[b]	Arini et al. 2019
gpx7	Glutathione peroxidase 7		Q96SL4	AGGATGCATCTGAAGCTTGG[a] CGTTCACCACATGTCCATTC[b]	Arini et al. 2019
ache	Acetylcholinesterase	Neurotoxicity		CTTGCTCTGGATCCTGCTCC[a] TGCAAAAACCGGGACTCCAA[b]	Designed from the transcriptome
atg13	Autophagy-related protein 13	Immunity	O75143	CTCAGCTGTCCGTAACGAGAT[a] AAGTGCATTCTGAGGCGAAG[b]	Arini et al. 2019
atg12	Ubiquitin-like protein ATG12		O94817	CCTCTCAACTGCCCATTTCT[a] GTACACCAACGAGTCCTTTGC[b]	Arini et al. 2019
gal	Galectin-3		P17931	TACTGACTTCCCGCTCTTCG[a] CACTTTGATGTGCGCTTCC[b]	Arini et al. 2019
acp	Phosphatase acid			TGGCTTGTTGGCTTCCTTGA[a] AAGAGGAGGTTCTGCATGCC[b]	Designed from the transcriptome
bcl2	Apoptosis regulator Bcl-2	Apoptosis	P10415	AAGGAACAGGTCCATTCACG[a] GGGACGGATTGTTGGTGTT[b]	Arini et al. 2019
p53	Tumor protein p53		P04637	TCCTGCCACAGTCACAAATG[a] GTCGAGATTTTCCCTCCTTAGC[b]	Arini et al. 2019
bax	BCL2 associated X		Q07812	AAAGGGGAGGATGGGAGAT[a] GCTATAACTGCCCCTGCTGT[b]	Arini et al. 2019
gadd45	Growth Arrest and DNA Damage		P24522	GGAGCAGGTGATGCTGTGTA[a] CCAGCAGTGTGCCTCAATAA[b]	Arini et al. 2019

[a]Forward primer

[b]Reverse primer

reverse primers by quantitative RT-PCR. Three genes were used as housekeeping genes, including *β-actin*, elongation factor 1 α (*ef1α*) and ribosomal protein 7 (*rpl7*) (Table 1). Specific primers for *ache* and *acp* genes were designed using the software Primer 3 V 4.0. Previously, the quality of each pair of primers was checked: cDNA tests were amplified by PCR (T100™ Thermal Cycler, BIORAD®. 30 cycles: 30 s for 95 °C, 30 s for 60 °C, 30 s for 72 °C), then the amplification products were separated on 1.5% agarose electrophoresis gel. After staining with ethidium bromide, the presence and size of each amplicon were verified. Quantitative PCR (qPCR) amplifications were carried out in triplicate in 96-well microplates (CFX Connect™ Real-Time System, BIORAD®) using SYBR™ Master Mix PCR Power SYBR™ Green (Invitrogen) containing the SYBR Green dye, DNA Taq Polymerase and dNTPs. For each reaction, 1 µL of each primer (50 ng.µL^{-1}), 6.25 µL of SYBR Green mix, 3.75 µL of water treated with DEPC (DNase-free water) and 0.5 µL of cDNA were added in each well. The qPCR reactions consisted of the first step of 10 min at 95 °C (enzyme activation) followed by 40 cycles (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s) and 5 min at 72 °C. Expression levels were estimated by evaluating the fluorescence signal emitted by SYBR-Green®. This fluorescent marker binds to double-stranded DNA (dsDNA) and the fluorescence emitted is proportional to the dsDNA present in the reaction mix. Calculations are based on cycle threshold (Ct) values. The relative gene expression ratio of each target gene was calculated following the delta-delta method normalized with reference genes (Livak and Schmittgen 2001), which is defined as:

$$ratio = \frac{2 - \Delta\Delta\mathrm{Ct}\,(\mathrm{exposed})}{2 - \Delta\Delta\mathrm{Ct}\,(\mathrm{control})}$$

Statistical analysis

The statistical analyzes were performed using the software XLSTAT 2019 (version 21.4.63762). The normality of data distribution and homogeneity of variance were tested using the Shapiro-Wilk test and Bartlett test, respectively. As the assumptions for parametric tests were not met for the gene expression measurements, we used the Kruskal-Wallis test to test for differences between the treatments. As the overall test was significant, a Dunn procedure was performed to determine which means were significantly different. $p \leq 0.05$ were considered statistically significant,

Results

Gills

As shown in Table 2, at 7 days post-exposure, genes were mainly up-regulated in gills for almost all environmental plastic particles (ENV MPs 10, ENV MPs 100, ENV NPs 0.008, ENV NPs 10 and ENV NPs 100). These genes are involved in endocytosis (*cltl, cav*), detoxication (*gst*), oxidative stress (*sod2*, *sod1*), immunity (*atg12*, *acp*, *gal*), apoptosis (*bcl2*, *bax*,

Table 2 Differential gene expression observed in gills of *Corbicula fluminea* after 7 days of exposure to the different treatments

7 days exposure - Gills										
		PS NPs 0,008	PS NPs 10	PS NPs 100	ENV MPs 0,008	ENV MPs 10	ENV MPs 100	ENV NPs 0,008	ENV NPs 10	ENV NPs 100
Endocytosis	cltl	2,71					3,94	2,81		
	cav		7,27			6,44	7,57		10,1	8,43
Detoxication	mt		0,30							
	mdr		0,34		0,27		0,44	0,29		0,27
	gst			2,46		2,64	3,64		4,95	3,69
Respiratory chain	cox1		0,49	0,46					0,35	0,24
	12s				0,18		0,27	0,20	0,21	
Oxidative stress	sod2					3,87	6,61	6,03	5,00	5,93
	sod1					5,16	8,55	6,51	6,79	8,39
	cat									
	gpx7		1,52			2,04				1,56
Immunity	atg13					0,39				0,27
	atg12			6,14		6,81	13,9	8,61	5,35	
	AcP			2,54			8,33	3,32	3,32	
	gal			10,8		15,8	21,9	13,7		
Apoptosis	bcl2						8,34	7,28	7,61	5,87
	p53			4,55						
	bax					6,90	9,06	8,04	8,82	8,32
	gadd45						3,23		2,79	2,74
Neurotoxicity	AChE			7,19			5,57	3,58	5,00	

PS (polystyrene) NPs, ENV MPs, and ENV NPs from plastics collected in the Garonne River. Results are presented as fold-change factors between gene expression in controls and gene expression in exposed organisms (>1: induction, in green; <1: repression, in red). Only significant differences are represented ($p \leq 0.05$) and only factors <0.5 and >1.5 are considered as significant.

Table 3 Differential gene expression observed in gills of *Corbicula fluminea* after 21 days of exposure to the different treatments

21 days exposure - Gills										
		PS NPs 0,008	PS NPs 10	PS NPs 100	ENV MPs 0,008	ENV MPs 10	ENV MPs 100	ENV NPs 0,008	ENV NPs 10	ENV NPs 100
Endocytosis	cltl	1,43		2,27						
	cav									
Detoxication	mt									
	mdr	1,71	2,93			1,68		1,84		
	gst	8,33	12,7	7,23	10,3			8,61		
Respiratory chain	cox1				1,59					
	12s			2,71						
Oxidative stress	sod2			1,57	2,08					
	sod1									
	cat			3,55	3,50		2,19			
	gpx7							1,59		
Immunity	atg13	3,08	3,82		3,23			3,38		
	atg12	2,33		2,52	2,80					
	AcP	2,46	3,13	2,69	3,09					
	gal	2,00			2,24					
Apoptosis	bcl2	2,96	2,25	2,14	2,54					
	p53	2,83	2,48			2,89	2,66			
	bax	1,88			1,82		1,74			
	gadd45		2,82	2,29	2,64					
Neurotoxicity	AChE	2,25	2,28	2,15	2,86					

1,50
2,00
4,00
7,00
8,00
10,00
12,00

Fold change scale bar

PS (polystyrene) NPs, ENV MPs and ENV NPs from plastics collected in the Garonne River. Results are presented as fold-change factors between gene expression in controls and gene expression in exposed organisms (>1: induction, in green; <1: repression, in red). Only significant differences are represented ($p \leq 0.05$) and only factors <0.5 and >1.5 are considered as significant.

gadd45) and neurotoxicity (*ache*). Downregulations were also observed for genes involved in detoxication (*mdr*), the respiratory chain (*cox1*, *12s*), and immunity (*atg13*) for some of the environmental plastic particles. There was no clear dose-dependent effect for ENV MPs treatments and ENV NPs treatments. Results for PS NPs treatments showed different trends compared to environmental plastic particles. Only a few genes were impacted in gills with both up and downregulations. Up-regulated genes after PS NPs 100 treatment were involved in detoxication (*gst*), immunity (*atg12*, *acp*, *gal*), apoptosis (*p53*), and neurotoxicity (*ache*). Other genes were up-regulated after PS NPs 0.008 *(cltl*) and after PS NPs 10 (*cav* and *gpx7*). Down-regulated genes concerned detoxication (*mt*, *mdr*), the respiratory chain (*cox1*) after treatment with PS NP at one or two of the concentrations tested. Two genes were down-regulated for the ENV MPs 0.008 treatment (*mdr* and *12S*).

At 21 days post-exposure (Table 3), a clear difference of gene expression responses in the gills was observed between two groups: (1) the PS NPs treatment whatever the tested concentration and ENV MPs 0.008, and (2) ENV MPs 10, ENV MPs 100 and all the ENV NPs. For the first group, many genes were up-regulated, particularly concerning immunity, apoptosis, detoxication, and neurotoxicity functions. For the second group, only few genes were up-regulated for one or two treatments.

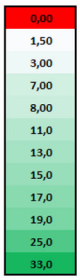
Visceral mass

After 7 days of exposure to the different plastic conditions, two trends were observed in the visceral mass (Table 4). For the first group (all PS NPs concentrations and ENV MPs 0.008), almost all the studied genes involved in immunity (*atg13*, *atg12*, *acp*, *gal*), apoptosis (*bcl2*, *p53*, *bax*, *gadd45*), neurotoxicity (*ache*) and some of the genes involved in the oxidative stress (*cat, gpx7*) and detoxication (*mdr*, *gst*) were strongly downregulated. Only a few genes were over-expressed for some of these treatments and were involved in endocytosis (*cltl*), detoxication (*mt*) and oxidative stress (*sod1*, *sod2*). For the second group (ENV MPs 10, ENV MPs 100, and all the ENV NPs concentration), the gene' responses were relatively similar to the first group for the functions related to endocytosis, detoxification, respiratory chain and oxidative stress. However, a clear difference regarding the genes involved in oxidative stress, immunity, apoptosis, and neurotoxicity was depicted, since very few of these genes were under-expressed compared to the first group.

As shown in Table 5, fewer genes were impacted after 21 days of exposure than at 7 days in the visceral mass. The PS NPs and ENV particles (MPs and NPs) did not induce the same effects. The PS NPs had little effect on the studied genes, whatever the concentration tested. Concerning the ENV MPs and NPs, some genes were under-expressed for some concentrations tested and were involved mainly in these different functions: detoxication (*mdr* and *gst*), oxidative stress (*cat*), immunity (*atg13*, *atg12* and *acp*) and apoptosis (*blc2* and *gadd45*). Some genes were up-regulated for the ENV MPs and NPs treatments such as the ones involved in endocytosis (*cav*), respiratory chain (*12 s*), oxidative stress (*gpx7*), immunity (*gal*) and neurotoxicity (*ache*) for some conditions and concentrations tested.

Table 4 Differential gene expression observed in the visceral mass of *Corbicula fluminea* after 7 days of exposure to the different treatments

7 days exposure - Visceral mass										
		PS NPs 0,008	PS NPs 10	PS NPs 100	ENV MPs 0,008	ENV MPs 10	ENV MPs 100	ENV NPs 0,008	ENV NPs 10	ENV NPs 100
Endocytosis	cltl	19,7				35,8			36,1	
	cav	0,0004					0,07	0,01		0,06
Detoxication	mt		2,78	3,03		3,96	2,90		2,41	
	mdr	0,0002		0,0001	0,0002		0,0002	0,00003		
	gst	0,001	0,001	0,001				0,001		
Respiratory chain	cox1						0,03	0,04	0,00003	0,004
	12s				0,08		0,04	0,05	0,04	0,06
Oxidative stress	sod2	2,94	10,2		7,80				9,43	11,2
	sod1			11,0		15,3	11,6	11,9	7,76	
	cat	0,005	0,003	0,002						0,003
	gpx7	0,0001	0,0005		0,0005				0,0003	0,0005
Immunity	atg13	0,0002	0,0002	0,0001	0,0003				0,0002	
	atg12	0,0003	0,0006	0,0005	0,0005					
	AcP	0,0003	0,0002	0,0001	0,0003					
	gal	0,0001	0,0006		0,0005			0,0004	0,0006	
Apoptosis	bcl2	0,0002	0,0002	0,0002	0,0003			0,0005		
	p53		0,0002	0,0001	0,0002		0,0002			
	bax	0,0003	0,0004	0,0005	0,0003					
	gadd45	0,0002	0,0003	0,0001	0,0003				0,0003	
Neurotoxicity	AChE	0,0001	0,0005	0,0004	0,0004		0,0004		0,0002	

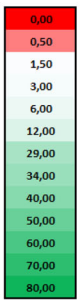


Fold change scale bar

PS (polystyrene) NPs, ENV MPs, and ENV NPs from plastics collected in the Garonne River. Results are presented as fold-change factors between gene expression in controls and gene expression in exposed organisms (>1: induction, in green; <1: repression, in red). Only significant differences are represented ($p \leq 0.05$) and only factors <0.5 and >1.5 are considered as significant.

Table 5 Differential gene expression observed in the visceral mass of *Corbicula fluminea* after 21 days of exposure to the different treatments

21 days exposure - Visceral mass										
		PS NPs 0,008	PS NPs 10	PS NPs 100	ENV MPs 0,008	ENV MPs 10	ENV MPs 100	ENV NPs 0,008	ENV NPs 10	ENV NPs 100
Endocytosis	cltl									
	cav		48,3		84,1		84,9	83,1	46,7	
Detoxication	mt							2,21		
	mdr	0,44				0,27	0,40		0,43	
	gst				0,46					
Respiratory chain	cox1			1,92						
	12s			1,63				1,57		
Oxidative stress	sod2									
	sod1									
	cat					0,45	0,47		0,43	0,35
	gpx7		1,65	2,10	1,78			1,55		
Immunity	atg13								0,24	
	atg12								0,38	
	AcP					0,43	0,49			
	gal	2,54			2,48		2,55	4,75		3,14
Apoptosis	bcl2					0,50				
	p53									
	bax									
	gadd45				0,45	0,36	0,50			
Neurotoxicity	AChE							1,70		2,31



Fold change scale bar

PS (polystyrene) NPs, ENV MPs, and ENV NPs from plastics collected in the Garonne River. Results are presented as fold-change factors between gene expression in controls and gene expression in exposed organisms (>1: induction, in green; <1: repression, in red). Only significant differences are represented ($p \leq 0.05$) and only factors <0.5 and >1.5 are considered as significant

Discussion

The present study investigated the effects of field-derived ENV MPs and NPs and standard PS NPs on the expression of genes involved in the molecular response to toxicity in gills and visceral mass, in *C. fluminea*. Our results first highlighted that the exposure led to changes in gene expression patterns at environmentally relevant concentrations whether the plastic source was manufactured plastics beads or environmental particles. Two main types of responses emerged from the analysis of gills and visceral mass : firstly, the earlier pattern of response, after 7 days of exposure, was linked to exposure to ENV NPs and MPs in gills and to PS NPs in visceral mass; secondly, after a more

prolonged exposure (21 days), the effects of PS NPs on gene expression was highlighted in gills while in the visceral mass, modifications in gene expression were instead linked to environmental plastic particles.

Endocytosis

Endocytosis is a main process involved in the uptake of nanoparticles in many species (Weng et al. 2022). In our study, caveolin (*cav*) and clathrin (*cltl*) gene expression varied significantly under NPs and MPs exposures, showing their role in the plastic particles uptake.

In the gills, endocytosis seems to be an entry pathway for MPs and NPs since the caveolin (*cav*) and clathrin (*cltl*) genes were over-expressed for specific concentrations in the three plastic conditions (both ENV MPs and NPs, and PS NPs) at 7 days of exposure. Indeed, the internalization rate of 50 nm PS NPs is lower when caveolae and clathrin endocytosis pathways were inhibited in the mussel *Mytilus galloprovincialis* (Sendra et al. 2020). These mechanisms were also already observed in oysters exposed to environmental NPs, attesting of an easy uptake of these particles in bivalves (Arini et al. 2022b). Although it has been shown that endocytosis by the caveolin or clathrin pathways is limited to particle sizes less than 500 nm or 200 nm respectively (Rejman et al. 2004), we hypothesize that small particles of MPs present in our solution can pass through these entry routes into organisms and/or we suggest the presence of NPs in the solution of MPs.

Oxidative stress and detoxication

In the gills, the genes related to oxidative stress (*gpx*, *sod1, and sod2*) were overexpressed for ENV MPs and NPs at 7 days of exposure. The *cat*, *sod2* and *gpx* genes were overexpressed after 21 days of exposure to PS NPs for some concentrations. Conversely, *cat* and *gst* genes were under-expressed in the visceral mass after exposure to PS NPs for 7 days. Catalase is an enzyme that acts as a defense mechanism against reactive oxygen species, allowing the disproportionation of hydrogen peroxide into water and dioxygen. The GST enzyme protects cells against toxicants by conjugating the glutathione as substrate to xenobiotics. The increased expression of both *cat* and *gst* genes observed in the gills of *C. fluminea* is a sign of cellular oxidative stress. A previous study also showed an increase in the activity of the catalase in the gills of *C.fluminea* after an exposure to PS MPs (200 µm) at a concentration of 2 mg L^{-1} for 7 days (Parra et al. 2021), while in our study, this is observed for PS NPs and ENV MPs at considerably lower concentrations. The gene relating to the detoxification system *mdr* was overexpressed in the gills after 21 days for the different plastic conditions and specific concentrations. This may be related to the increased expression of the *gst* gene. Indeed, GSTs are enzymes that catalyze the conjugation of reduced glutathione (GSH) with metabolites and reactive electrophiles, representing an essential chemical detoxification route. This suggests the presence of additives and/or some chemical compounds adsorbed on the surface of the plastic particles. This is consistent with the high metal concentrations measured in the ENV MPs and NPs used in this study (SI Table A).

Respiratory chain

12S ribosomal RNA refers to the mitochondrial metabolism. Thus, an overexpression of the 12S gene represents an increasing number of mitochondria necessary to respond to oxidative stress in the bivalves. In our study, an under-expression was observed in both gills and visceral mass after 7 days of exposure. The same observation was followed by a decrease in the activity of isocitrate dehydrogenase, involved in the Krebs cycle and therefore mitochondrial activity, in the fish *Pomatoschistus microps* after exposure to 0, 18.4, and 184 µg L^{-1} of PE MPs (1–5 µm) for 96 h (Oliveira et al. 2013). At the opposite, an over-expression of the 12S gene was demonstrated in the visceral mass of oysters *Isognomon alatus* after 7 days of exposure to PS NPs and derived-field NPs at 7.5 µg L^{-1} (Arini et al. 2022a). In their study, the authors suggest that the overexpression of the *12S* gene was linked to the repression of the *cox1* gene and would be involved in a compensatory mechanism aimed at maintaining mitochondrial metabolism (Arini et al. 2022a). In our study, we observed both the *cox1* and *12S* genes repression after 7 days of exposure to ENV MPs 100 and all ENV NPs in the visceral mass, suggesting an excessive oxidative stress which the mitochondria cannot support.

Immunity

The responses of the organisms to the environmental and polystyrene particles exposure were different in the gills and the visceral mass. In the gills, ENV MPs and NPs induced an over-expression of 3 of the 4 genes involved in immunity after 7 days of exposure (*atg12*, *AcP*, *gal*). After 21 days of exposure, the organisms exposed to the PS NPs showed an over-expression of the 4 genes studied (*atg13*, *atg12*, *AcP,* and *gal*). This indicates an important immune system activity even at low concentrations of plastic particles. Such a shift in the immune response has already been reported for bivalves exposed to MPs and NPs (Auguste et al. 2020; Mkuye et al. 2022).

In the visceral mass, we observed an opposite trend. An intense repression was depicted after 7 days of exposure to PS NPs and ENV MPs 0.008 whereas little effect was

observed for ENV MP 10 and 100 and ENV NPs. After 21 days of exposure, almost no effect of PS NPs and a down-regulation of some genes were observed for the ENV MPs and NPs. Our results suggested that PS NPs induced a stronger response in the short term than ENV MPs and NPs. Due to their small size (200 nm) and potentially their carboxyl groups, PS NPs may reach the visceral mass faster while ENV MPs and NPs may tend to be retained in the gills explaining the responses observed at 7 days of exposure. These results are in agreement with two studies which demonstrated a more significant accumulation of PS plastic particles in the digestive gland tissues than in the gills of the mussel *Mytilus galloprovincialis* (Fabbri et al. 2020; Wei et al. 2021).

Apoptosis

Apoptosis is the process of programmed cell death which plays a significant role in the immune response triggered by various factors including virus, diseases and toxic agents (Ekert and Vaux 1997; Romero et al. 2015). Our results on apoptosis were consistent with those obtained for immunity genes and demonstrated significant differences in the response to the two types of plastics (PS NPs vs ENV MPs and NPs), both in the gills and in visceral mass. At 7 days of exposure, the ENV MPs and NPs induced an up-regulation of 3 genes involved in apoptosis processes (*bcl2*, *bax* and *gadd45*) in gills. In contrast, only one gene (*p53*) was up-regulated for the highest concentration of PS NPs. The apoptosis response induced by environmental plastic particles can be related to eliminating damaged cells to maintain the tissue's integrity and to preserve the physiological activity of gill filaments (Romero et al. 2015). In the visceral mass, after 7 days of exposure, organisms exposed to PS NPs and ENV MPs 0.008 µg L^{-1} showed an intense repression of the 4 genes involved in apoptosis. These plastic particles could cause impairment of promoting factors that have an influence on the expression of genes implicated in apoptosis. Qi et al. (2023) also studied the impact of NPs in mussels. They explained that the down-regulation of gene expression also observed after NPs exposure may suggest different stages of an inflammatory response and hence demonstrate that NPs exposure may exert more toxicity than MPs exposure.

In contrast, in our study, organisms exposed to environmental particles showed little or no effect for MPs 10 and 100 µg L^{-1} and NPs. MPs have been shown to induce apoptosis in bivalves, particularly via caspase-related genes (Mkuye et al. 2022; Shi et al. 2020; Sun et al. 2021). However, very few studies described the effects of NPs on apoptosis processes in bivalves. One study related to direct exposure to environmental NPs derived from plastic macro-wastes reported effect on apoptotic genes in gills and visceral mass in the oyster *I. alatus* (Arini et al. 2022b). Our divergent results from those on the oyster *I. alatus* could be partly explained by the different environmental plastics tested, specifically by differences in plastic characteristics (i.e., composition, surface charge, size, shape, additives and adsorbed chemicals).

Neurotoxicity

Acetylcholinesterase (AChE) is the primary enzyme responsible for the hydrolytic metabolism of the neurotransmitter acetylcholine (ACh) into choline and acetate to remove the neurotoxic effects of pollutants. The exposure to plastic particles induced an inhibition of the AChE activity in different bivalves (Avio et al. 2015; Oliveira et al. 2013; Ribeiro et al. 2017). This is consistent with the inhibition of *ache* gene expression we observed in the visceral mass after an exposure of 7 days to PS NPs (for all tested concentrations) and ENV MPs (0.008 µg L^{-1}). This inhibition may reflect a possible disturbance of nerve impulse transmission and could be due to the toxicity of plastic particles and the chemical compounds they carry.

In opposition, our results indicated an induction of the *ache* gene in the gills after a 7 days exposure to ENV MPs (100 µg L^{-1}), ENV NPs (0.008 and 10 µg L^{-1}) and PS NPs (100 µg L^{-1}), and after a 21 days exposure to PS NPs and ENV MPs (0.008 µg L^{-1}). In the same way, an increase of the AChE activity has been reported in barnacle nauplii *Amphibalanus amphitrite* exposed to PS MPs for 48 h at 0.001, 0.01 and 1 mg L^{-1} (Gambardella et al. 2017) and in the freshwater insect larvae *Culex quinquefasciatus* exposed to PE at 4.24×10^6 particles m^{-3} for 5 days (Malafaia et al. 2020). This increase in *ache* gene expression in our study may be related to the inflammation of the visceral mass since it has been reported that inflammatory conditions can trigger the up-regulation of *ache* gene expression (Oliveira et al. 2012). It would be interesting to compare the *ache* gene expression levels with animal behavioral responses such as valve movement activity or filtration capacity. Indeed, inhibition of AChE activity was reported combined with a decrease of the filtration capacity of *C. fluminea* exposed to 10 mg mL^{-1} of PS NPs (Guo et al. 2021). Moreover, disturbances in the behavior of zebrafish at the larval stage were measured together with an inhibition of AChE activity after an exposition to 2 mg L^{-1} of MPs (Santos et al. 2021).

Testing environmental particles for a realistic assessment of environmental risk

In this study, we tested the effects of manufactured PS NPs and environmental MPs and NPs derived from macroplastics sampled in the environment on *C. fluminea*. These

particles differ in plastic composition, size, shape, additives, adsorbed pollutants than the commercial ones. We showed that the two types of particles (manufactured or environmental) induced differential responses in the gills and visceral mass regardless of the sampling time. Therefore, in the gills, the genes involved in immunity, apoptosis, and neurotoxicity are overexpressed after 7 days of exposure to the ENV MPs and NPs. In contrast, it was not the case for the PS NPs. At 21 days, these results are reversed with an overexpression of these genes for the PS NPs and only the ENV MPs 0.008. In the visceral mass, the organisms exposed to the PS NPs and ENV MPs 0.008 showed an intense repression of the genes involved in immunity, apoptosis, and neurotoxicity after 7 days of exposure whereas little effect was observed for the organisms exposed to the ENV MPs 10 and 100 and all the ENV NPs. Such differences between the effects of manufactured and environmental particles have already been demonstrated in oysters *Isognomon alatus* (Arini et al. 2022a, b; Lebordais et al. 2021). In these studies, the authors showed notably that nanoplastic particles derived from macroplastics sampled in the environment triggered more effects on gene expression than PS NPs. There is no clear hypothesis that PS NPs and the lower concentration of ENV MP would induce similar toxicity profiles in the visceral mass. However, low MP concentrations contained potentially smaller particles than higher concentrations, where aggregation/agglomeration of MPs particles could occur (Revel et al. 2019). This could potentially explain the response similarities between these two exposure conditions.

As part of this study, the effects of environmental particles of different sizes: ENV MPs (1.2–300 µm) and ENV NPs (235 ± 70 nm) were tested. The results did not demonstrate differences in molecular responses between the organisms exposed to the two particle sizes. In contrast, it has been found that the small size of NPs and their high surface area make them more toxic to the organisms than MPs (Zhang et al. 2021). However, studies investigating the effects of NPs on aquatic organisms have emerged in recent years and there is still a knowledge gap on this topic (Ferreira et al. 2019). Furthermore, it would be relevant to study the comparison of the toxicity of environmental and reference MPs in future studies. In addition to this, although we changed the water every 3 days and used an aeration system including 6 inlets per aquarium in order to maintain the concentrations of plastic particles tested, we cannot rule out the hypothesis that particles plastics have adhered to bivalves and aquariums. In order to test this hypothesis, methodological tools should be developed in the future in order to be able to measure low concentrations of MPs and NPs in water but also in different matrices such as organism tissues.

Indirect toxicity of MPs and NPs can also be due to the additives they contain and/or the pollutants adsorbed on their surface. These chemical compounds can be transferred to the organisms (Avio et al. 2015; Gomiero et al. 2018) and can lead to joint toxicity (synergistic, additive, antagonistic, independent) (Ding et al. 2022). But studies on the effects of MPs and NPs from the environment and whose pollutants have been characterized are currently very scarce. In our study, the concentrations of different metals and metalloids in the environmental MPs and NPs were measured. They were found to be very high and could be related to the immune responses, apoptosis, and neurotoxicity observed in the gills and visceral mass in *Corbicula fluminea*. Another study revealed a higher growth inhibition of a freshwater algae with environmental NPs compared to manufactured NPs (Baudrimont et al. 2019). The concentration of different trace metals was shown to be higher in the environmental NPs than in the manufactured ones which could explain the toxicity differences between the two types of plastic particles (Baudrimont et al. 2019). Moreover, a mixture of MPs and mercury has been shown to cause oxidative stress and lipid peroxidation damage in *C. fluminea* (Oliveira et al. 2018). However, the authors also pointed out antagonistic effects between MPs and mercury on filtration rate and the enzymatic activities of ChE and GST (Oliveira et al. 2018). The interactions between the MPs, NPs, and the chemicals are complex and far from understood. Additional research is needed to better understand the mechanisms of toxicity of MPs and NPs from the environment.

Finally, other parameters related to freshwater environments must be evaluated in order to better understand the toxicity of MPs and NPs. The water chemistry in the environment can impact the long-term fate of plastic particles and their toxic effects (Ding et al. 2021). In addition, the characteristics of plastic particles in the environment (size, shape, and density for example) can also influence their horizontal or vertical distribution and their bioavailability for living organisms (Ding et al. 2021). Testing the effects of plastic particles derived from macrodebris collected in various freshwater environments could also help to better assess their environmental risk.

Transcriptomic tools, including the measurement of gene expression levels, represent sensitive tools for detecting early responses and establishing toxicity profiles (Piña et al. 2007). They are suitable for studying the toxicity of MPs and NPs since they can provide information on a large number of parameters in a short time (Barrick et al. 2019). Given the diversity of MPs and NPs, it appears advantageous to use high-throughput screening techniques such as real-time quantitative PCR before performing other analysis. It would therefore be interesting to complete the transcriptomic data with a multimarker approach. Parameters including sub-individual markers (eg biochemical biomarkers involved in responses to stress factors) and individual (eg burial behavior, filtration capacity) could provide

valuable information for the assessment of the toxicity of MPs and NPs.

Conclusion

The present study evaluated the effects of PS NPs and environmental MPs and NPs in the bivalve *C. fluminea* at environmentally relevant concentrations and under the same laboratory conditions. We have evidenced major differences in the bivalve molecular responses between manufactured NPs composed of polystyrene and field-derived MPs and NPs especially in the oxidative stress, immunity, apoptosis, and neurotoxicity. These results highlight the importance of conducting further investigations including plastic particles from the environment, from nano to micro size and to fully characterize these particles (composition, shape, size, chemicals, etc.) for a realistic assessment of environmental risk.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s10646-024-02769-5.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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