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► **To cite this version:**

Clémentine Labbé, Isabelle Métais, Hanane Perrein-Ettajani, Mohammed Mouloud, Antoine Le Guernic, et al.. Effect of aging on the toxicity of polyethylene microplastics on the estuarine bivalve *Scrobicularia plana*. *Environmental Pollution*, 2024, 361, pp.124805. <10.1016/j.envpol.2024.124805>. <hal-04765702>

HAL Id: hal-04765702

<https://hal.science/hal-04765702v1>

Submitted on 4 Nov 2024

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Effect of aging on the toxicity of polyethylene microplastics on the estuarine bivalve *Scrobicularia plana*[☆]

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ARTICLE INFO

Keywords:

Microplastic
Mollusk
Comet assay
Catalase activity
Burial

ABSTRACT

Microplastics (MP) are now present in all ecosystems and undergo weathering processes, including physical or chemical degradation. Although most studies have been carried out on MP toxicity in the marine ecosystem, interest is growing for the terrestrial and entire aquatic compartments. However, the interface between both environments, also known as the soil/water continuum, is given little consideration in MP toxicity studies. Only a few studies considered the toxicity of artificially aged or soil field-collected MP on species living at this interface. The present study evaluates the impact of artificial and field aging polyethylene (PE) MP on the bivalve *Scrobicularia plana*, a key organism of the estuarine compartment, living at the soil/water interface. Clams were exposed for 21 days to environmental concentrations (0.008, 10 and 100 $\mu\text{g L}^{-1}$) of unaged as well as artificially and field aged PE MP. Toxicity was assessed from individual to molecular levels including condition index, clearance rate, burrowing behavior, energy reserves, enzyme activities and DNA damage. Results showed differential effects at all biological levels depending on the type and the concentration of the MP tested. Indeed, a decrease in burrowing behavior was observed in *S. plana* exposed to aged and field PE at low concentration (0.008 $\mu\text{g L}^{-1}$). In the gills of clams, exposures to aged PE (0.008 and 100 $\mu\text{g L}^{-1}$), virgin PE (10 $\mu\text{g L}^{-1}$) and field PE (all tested concentrations) decreased CAT activity while DNA damage increased after exposure to virgin PE (0.008 $\mu\text{g L}^{-1}$ and 10 $\mu\text{g L}^{-1}$) and field PE (0.008 $\mu\text{g L}^{-1}$). Our findings suggest that aging modifies the toxicity profile of PE polymer on *S. plana* and considering plastic from field at environmental concentrations is important when performing ecotoxicological studies.

1. Introduction

Due to its numerous properties and societal benefits (Andrady & Neal, 2009), plastic has rapidly become a dominant material in our daily lives and is sometimes considered one of the markers of the Anthropocene (Zalasiewicz et al., 2016). Its production has been constantly increasing since the 1950s, reaching 390 Mt per year in 2021 (Plasticseurope, 2022). While more than half of the total plastic production is dedicated to the packaging and construction sectors, many other areas consume plastics (e.g. agriculture, sport, automotive) (Plasticseurope, 2022). Jointly, this massive production and poor management of plastic waste led to an accumulation of plastics in the environment (Borrelle et al., 2020), making plastic pollution a major threat to wildlife and to ecosystems health (MacLeod et al., 2021). Once

in the environment, large plastic debris are broken down by solar UV, water, wind (Andrady, 2017) and microorganisms' action (Kale et al., 2015) into smaller fragments called microplastics (MP, plastic particles between 1 μm and 5 mm in size) (Frias & Nash, 2019)). This fragmentation is accompanied by changes in the physical and chemical nature of plastics, as well as in their properties (e.g. shape, color, roughness), which can impact their toxicity (Tirkey & Upadhyay, 2021). While some of these MP accumulate in the soil where they are discharged directly (e.g. sewage sludge, landfill), others are transported into rivers by wind or by surface runoff, to end up in the sea (Horton & Dixon, 2018). Although direct inputs into aquatic compartments exist (e.g. inadequate waste disposal, fishing activities), most MP found in freshwater and marine environment have a land origin (Koutnik et al., 2021; Lwanga et al., 2022). Lebreton & Andrady (2019) suggest that rivers are a major

[☆] This paper has been recommended for acceptance by Maria Cristina Fossi.

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transport route for plastics to the ocean. However, some recent studies also present the atmosphere as an important way in the long-distance transport of plastic particles, in particular fibers (Xiao et al., 2023), and suggest the ocean as a potential source of MP for atmosphere and then to remote terrestrial regions (Ryan et al., 2023). Despite the presence of MP in all ecosystems, numerous studies have been carried out on the toxicity of MP on the marine compartment, considering the terrestrial and aquatic compartments disconnected from each other (C. Wang et al., 2021). However, these compartments are interconnected and essential ecological processes such as microbiota and nutrient transfer occur at their interface (Lamy et al., 2022). Some studies focused on characterizing MP contamination and modelling particle transport in catchment areas or in environments at the soil/water interface, such as estuaries (Roweczyk et al., 2022), riverbanks (Kundu et al., 2022), groundwater (Shu et al., 2023), wetlands (Su et al., 2023) or sandy beaches (Ronda et al., 2023). MP accumulation has been suggested in these interface environments, particularly in sediment matrix (Jones et al., 2020) acting as a sink for MP contamination (Defontaine et al., 2020). This soil/water continuum should therefore be further considered in MP toxicity studies (Lamy et al., 2022) assessing effects of MP from the land on organisms living in these environments.

The use of aged MP in ecotoxicological studies on aquatic organisms is currently increasing. Generally, the MP tested were artificially aged using a UV chamber (Fu et al., 2019) or in water under laboratory conditions (Jemec Kokalj et al., 2019). Vroom et al. (2017) aged, in laboratory, polystyrene beads and fragments in filtered seawater (3 weeks, in the dark) comparing the ingestion of aged and pristine microparticles by marine zooplankton. The preferential ingestion of aged MP was suggested to be linked to the development of biofilm on the MP surface, which pointed out the importance of aging process in the potential exposure or interaction with organisms. Recently, more environmentally realistic studies, using naturally aged microplastics derived from environmental macro-waste were published to better understand their effect on aquatic organisms (Guimarães et al., 2021; Métais et al., 2023b). In their study, Kanimozhi et al. (2024) compared the impact of MP (PP; <125 µm) generated from plastic waste with that of MP from new commercial products at different concentrations (0.25, 0.5, and 1 mg mL⁻¹) on the physiological responses of *Artemia salina* after a 48 h exposure. A more pronounced oxidative stress response was observed in organisms exposed to weathered MP.

Bivalves have an ecological significance for the health of aquatic ecosystem. They are particularly vulnerable to MP (Khanjani et al., 2023), in particular because of their filtering behavior, which brings them in direct contact with MP particles with characteristics similar to those of their usual food (Walkinshaw et al., 2020). Most toxicity studies on bivalves used commercial MP or MP produced from commercial products. Sussarellu et al. (2016) demonstrated that a 2-month chronic exposure of adult Pacific oysters (*Crassostrea gigas*) to virgin commercial polystyrene MP (2 and 6 µm, 0.023 mg L⁻¹) impacted their energy metabolism and reproductive function. The immune function of the Mediterranean mussel (*Mytilus galloprovincialis*) was also impacted by a 72 h exposure to commercial polymethylmethacrylate MP (10 and 50 µm, 0.1–10 mg L⁻¹) (Pavičić-Hamer et al., 2022). However, the use of MP representative of the environment in terms of size, concentration or aging should now be a priority in order to better understand the toxic effects of MP (Paul-Pont et al., 2018). Only a few studies have been carried out on the toxicity of aged MP as artificially aged polyethylene (PE) MP on the blue mussel, *Mytilus edulis*, at the physiological (Moncrieffe et al., 2023) or molecular level (Jaouani et al., 2023). Another study used a mixture of MP generated from environmental waste to evaluate the impact of these aged MP on the freshwater bivalve, *Corbicula fluminea* (Vidal et al., 2023). A long-term exposure to a mixture of naturally aged MP (PE, PP and PVC, 0.1 and 10 mg L⁻¹, 138 µm) altered significantly the mortality, oxidative stress and lipid peroxidation in adult oysters *Crassostrea gigas* (Bringer et al., 2022). *Scrobicularia plana* is an endo-benthic bivalve that lives in the sediments of estuarine

and coastal areas. Widely distributed, with a sedentary lifestyle, easy to collect, and robust under laboratory-controlled conditions, this species meets all the criteria which make it a relevant bioindicator of the health status of the intertidal areas of estuaries (Guerreiro, 1998; Santos et al., 2011). *S. plana* is a filter-feeder and a facultative deposit-feeder bivalve, living partly buried in sediment. This bivalve is therefore particularly vulnerable to contaminants, including MP, as it can be exposed to both particles suspended in the water and those present in sediments. For all these reasons, *S. plana* was previously widely used in ecophysiological and ecotoxicological studies (Amorim et al., 2020; Mouneyrac et al., 2014; Trombini et al., 2022). However, only several studies examined the impact of MP on *S. plana*. These few studies mainly explored the effect of co-exposure to commercial LDPE MP and other adsorbed contaminants such as benzo [a]pyrene (Bebiano et al., 2022; Rodrigues et al., 2022), perfluorooctane sulfonic acid (Islam et al., 2021; O'Donovan et al., 2018) or oxybenzone (O'Donovan et al., 2020). A 14-day exposure to commercial PS MP (1 mg L⁻¹, 20 µm diameter) showed the presence of MP in clam's tissues (Ribeiro et al., 2017), confirming the capacity of *S. plana* to accumulate MP particles. In addition, environmental aged MP (0.008, 10, and 100 µg L⁻¹, <300 µm) were demonstrated to alter behavior, oxidative stress and detoxication process in *S. plana* after a 21-day exposure (Métais et al., 2023a).

Only a few studies considered the toxicity of artificially aged or soil field-collected MP on species living at the soil/water interface. The aim of this study was to evaluate the impact of artificial and field aging polyethylene (PE) MP on the bivalve *S. plana*, a key organism of the estuarine compartment living at the soil/water interface. PE is the most produced type of polymer (Plasticseurope, 2022) and one of the most frequently found polymers in terrestrial and aquatic environments. Its potential toxicity was reported in numerous studies at all trophic levels (El-Sherif et al., 2022). In this study, clams were exposed in the laboratory to environmental concentrations (0.008 µg L⁻¹, 10 µg L⁻¹ and 100 µg L⁻¹) of artificially aged and unaged PE MP as well as PE MP from field collected macroplastics. Toxicity was assessed at different levels of the biological organization (from molecular to individual levels). For this purpose, the physiological status, as well as burrowing behavior were explored at the individual level. Oxidative stress, neurotoxicity, immunity, detoxication and genotoxicity were also evaluated. To our knowledge, this study is the first one to compare toxicity of artificially aged MP and environmental field aged MP on the toxicity of an estuarine organism.

2. Materials and methods

2.1. Preparation and characterization of microplastics

In this study, three types of MP were tested: virgin LDPE (vPE), aged LDPE (aPE) and PE from the field (fPE). Virgin and aged LDPE MP were those used in Jaouani et al. (2023). Briefly, aged MP were obtained from virgin LDPE artificially irradiated for 30 days in a UV-light chamber (Jaouani et al., 2023) at 70 °C (Julienne et al., 2019). Virgin and aged LDPE were then ground using a cryogenic mechanical miller (SPEX, 6770 Freezer-mill) and sieved on a stainless-steel sieve of 400 µm to retain the entire fraction of particles less than 400 µm. Field LDPE MP were those used by Métais et al. (2023b) issued from plastic litter collected in the Seine estuary. Each macroplastic was cryogrounded (Freezer/mill 6770, prex Sampleprep). The powder obtained was then sieved to retain only the fraction <100 µm (10 min, Retsch AS 200). MP from two previous studies done in our laboratory (Jaouani et al., 2023; Métais et al., 2023b) were used to allow comparison of the effects observed in this study with those of both previous studies.

In our experiment, for all MP types, additional morphometric analysis (Morphologi 4-ID, Malvern) was performed on MP powders to characterize particles size and shape (>1 µm). Around seven mm³ of MP powder were dispersed by injecting compressed air onto a glass plate. The particles thus deposited were photographed individually under a

microscope (x 10) and then analyzed using Morphologi software. The maximum particle size (Max. Distance) was defined as the maximum distance between any two points on a particle and was given in μm . The shape of a particle was characterized by the value of the Aspect Ratio corresponding to the ratio between its width and length. This value ranged from 0 (fiber-like particles) to 1 (spherical particles).

2.2. Suspensions of microplastics

For each type of microplastic particles (vPE, aPE, and fPE), suspensions at concentrations of 1 g L^{-1} , 0.1 g L^{-1} and 0.1 mg L^{-1} were prepared in ultrapure water (Milli-Q), pH 7 (Revel et al., 2019). A specific volume of the suspensions (1 g L^{-1} , 0.1 g L^{-1} or 0.1 mg L^{-1}) was distributed into the aquaria to obtain the final concentrations of 0.008, 10, and $100 \mu\text{g L}^{-1}$. No surfactants were used during the MP preparation to avoid any additional effects. Each solution was hand mixed before adding to the aquaria. All MP solutions were added in the aquaria every 3 days, immediately after the water change to maintain the same concentration throughout the exposure.

In addition, a Transmission electron microscopy (TEM) analysis was performed on 1 g L^{-1} stock solutions to detect the presence of particles smaller than $1 \mu\text{m}$. Briefly, solutions were filtered on $5 \mu\text{m}$ filter units (Sartorius Minisart®) to eliminate the larger particles. Three microliters of each solution were deposited on Formvar Carbon coated 200 mesh copper grids (Agar Scientific AGS162) pre-treated by glow discharge and then let to evaporate for 2 h at room temperature. Samples were imaged with a JEOL JEM-1400 PLUS transmission electron microscope at 120 kV (JEOL) and recorded with a JEOL CCD Camera Ruby (8 M pixel) using JEM1400 PLUS software.

2.3. Organism sampling and laboratory exposure assay

Individuals of *S. plana* were hand-collected at low tide in sediment on an estuary of the French Atlantic coast ($47^{\circ}26'36'' \text{ N}$, $2^{\circ}27'4'' \text{ W}$; Pont-Mahé). Clams were brought back to the laboratory in metal buckets filled with water and sediment from the sampling site and introduced into aquaria (30 L) containing 27 L of aerated artificial seawater (Tropic Marine) at 25 psu in a temperature-controlled room at $16 \pm 1^{\circ} \text{ C}$ for an acclimatization period of 9 days. A photoperiod of 12:12 h was set up. A total of 610 clams were distributed in ten glass aquaria (61 clams per aquaria). The artificial seawater was fully renewed every 3 days before addition of the MP solutions. The clams were fed once a week with the marine microalgae *Tetraselmis suecica* (Teramer).

Clams were then exposed for 21 days to three MP conditions: virgin polyethylene microplastics (vPE MP, $<400 \mu\text{m}$), aged polyethylene microplastics (aPE MP, $<400 \mu\text{m}$), and field derived microplastics (fPE MP, $<100 \mu\text{m}$) at three different concentrations: $0.008 \mu\text{g L}^{-1}$, $10 \mu\text{g L}^{-1}$, and $100 \mu\text{g L}^{-1}$; and to a negative control without any added plastic. The three tested concentrations were chosen according to environmentally relevant MP concentrations used in Revel et al. (2020), representative of coastal regions and gyres.

2.4. Ecophysiology measurement

The physiological status of exposed clams was monitored through several parameters including the condition index (CI), the clearance rate (CR) and energetic reserves status.

2.4.1. Condition index

At the end of the acclimatization period, the physiological status of the clams at T0 was determined on 10 sampled individuals. After 21 days exposure (T21), 10 clams per condition were sampled. The total and the flesh weights of clams were determined individually. The condition index (CI) was calculated as follows:

$$\text{CI} = [(\text{wet weight of soft tissues}/\text{total wet weight}) * 100] \text{ (AFNOR, 1985.)}$$

Although dry weight is generally used to calculate the condition index, the wet weight condition index was chosen in our study to limit the number of individuals required, as it allows measurements of IC and energy reserves on the same specimens. A positive correlation between dry weight and wet weight was reported in *S. plana* allowing the integration of the wet weight in the calculation of the condition index (Mouneyrac et al., 2008).

2.4.2. Clearance rate

Clearance rate (CR), defined as the rate at which clams can remove particles from solution (Salerno et al., 2018), was estimated for clams previously exposed for 21 days to vPE, aPE, fPE MP (0.008 , 10 , and $100 \mu\text{g L}^{-1}$) or to artificial seawater without added MP (control condition) using a system adapted from Revel et al. (2019). Clams ($n = 3$) were placed individually on metal grids immersed in 600 mL beakers filled with 400 mL of water from the aquaria for 1 h before starting measurements. Clams were then fed with *Tetraselmis suecica* (Teramer) at an initial concentration of $300,000 \text{ cells mL}^{-1}$. A fine bubble aeration was installed on the beaker wall to promote mixing without resuspending feces. The clearance rate was measured by assessing the concentration of the algal solution every 15 min for 45 min according to the classical algal cell counting technique on Malassez counting chamber (Utermöhl, 1958) as previously described in Vidal et al. (2023). The clearance rate was expressed as the number of cells of *T. suecica* per clam per hour.

2.4.3. Energy reserves

The status of energy reserves was evaluated by measuring glycogen, lipid and protein concentrations in the total homogenate of 10 clams at the beginning of experiment and after a 21-day exposure to aPE, vPE, fPE or no MP (control) as in Vidal et al. (2023) with a minor modification of the grinding buffer (Citrate buffer (0.1 M citric acid monohydrate, 0.1 M sodium citrate tribasic dihydrate)). Lipid, glycogen and protein concentrations were expressed in mg g^{-1} of wet weight (ww).

2.4.4. Burrowing test

Burrowing tests were performed at T21 as described in Métais et al. (2023a). The burial behavior was evaluated by placing 10 individuals per condition on the sediment surface in the dark and observing how many of them burrowed at frequent intervals: every 5 min for the first hour, 10 min for the second hour, and 20 min for the third to the sixth hours. The percentages of unburied individuals over time were determined for each condition.

2.5. Biomarkers

2.5.1. Enzymatic activities

Enzyme activities (catalase (CAT), glutathione-S-transferases (GST), acetylcholinesterase (AChE) and acid phosphatase (AcP) were quantified in gills and digestive gland tissues of 9 clams per condition according to the protocols used in Métais et al. (2023a). The same individual homogenate was used to evaluate all enzyme activities. All specific activities were expressed either in $\mu\text{moles min}^{-1} \text{ mg protein}^{-1}$ (CAT) or in $\text{nmoles min}^{-1} \text{ mg protein}^{-1}$ (GST, AChE, AcP).

2.5.2. Genotoxicity (comet assay)

The comet assay was carried out as previously described by Métais et al. (2023a) on gills and digestive glands of three clams per condition. One hundred nuclei were analyzed per slide and observed using an optical fluorescence microscope (Olympus BX60, $\times 40$) equipped with a CDD camera (Lucas-S, Andor Technology) and a Komet 6 image analysis system (Kinetic Imaging Ltd). DNA damage was expressed as percentage of the DNA present in the comet tail (% Tail DNA) for each nucleus.

2.6. Quality control

To prevent cross-contamination, all equipment used in the laboratory was previously rinsed with milliQ water and 70 % ethanol, dried at room temperature in a hood and stored under aluminum foil to avoid contact with ambient air. During the experiments, cotton laboratory coats and nitrile gloves were worn.

2.7. Statistical analysis

Statistical analysis was carried out using XLSTAT 2019 (version 21.4.63762) and R software (version 4.0.3). Normality of data distribution and variances homogeneity among groups were tested using the Shapiro–Wilk test and Bartlett test, respectively. If the data followed the conditions for applying parametric tests, differences between the treatments were tested using an ANOVA. A significative ANOVA was followed by a Tukey post hoc test to determine which means were significantly different. If the data did not comply with the parametric tests' conditions, Kruskal–Wallis test was used to test differences between the treatments. If the overall test was significant, a Dunn procedure was performed to determine which means were significantly different. For the burrowing test, the percentages of unburied individuals were first ln-transformed to linearize the data and then the regression coefficients of the least-square regression lines were compared using a covariance analysis (ANCOVA). In all cases, p-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Characterization of tested microplastics and exposure solutions

According to the morphometric analysis, the particles size ranged from 1.2 to 607 μm for aPE MP (mean size, 32 μm), between 1.2 and 571 μm for vPE MP (mean size, 34 μm) and from 1.2 to 446 μm for fPE (mean size, 28 μm) (Table S1). The three types of MP powders exhibited a diversity of particle shapes with a mean aspect ratio of 0.623, 0.568 and 0.627 for aPE, vPE and fPE, respectively (Table S1). Concerning the nanoparticle fraction, the TEM analysis revealed the presence of nanoparticles ($<1 \mu\text{m}$) in all the tested stock solutions (Fig. 1) as in Jaouani et al. (2023) and Métais et al. (2023b).

3.2. Physiological status

No significant differences in the mean of CI were observed in clams between the beginning of the experiment at T0 (15.49 ± 2.86) and the control after 21 days of exposure (13.15 ± 2.05) and between the control and all MP conditions after 21 days of exposure, with a mean CI of the organisms comprised between 12.22 ± 2.09 and 15.30 ± 2.93 (Table S2).

After 21 days of exposure, MP did not induce any significant impact on the CR of *S. plana*, independently of MP types, concentrations and measurement time (Fig. S1).

Concerning the status of energy reserves, as shown in Fig. 2A and B, a 21-day exposure to aPE, vPE and fPE did not significantly modify the concentration of glycogen and lipids in *S. plana*, regardless of the exposure concentration condition. However, significantly higher proteins levels were observed in clams exposed to 100 $\mu\text{g L}^{-1}$ of aPE and vPE and to 10 $\mu\text{g L}^{-1}$ of fPE compared to control group (Fig. 2C).

3.3. Burrowing test

Percentage of unburied *S. plana* in sediment as a function of time after 21 days of exposure to different sources of microplastics (aPE, vPE, fPE) at different concentrations (0.008, 10, and 100 $\mu\text{g L}^{-1}$) are presented in Fig. 3. Slopes of the least-square regression lines after ln transformation of the raw data are reported in Table 1: the lower the

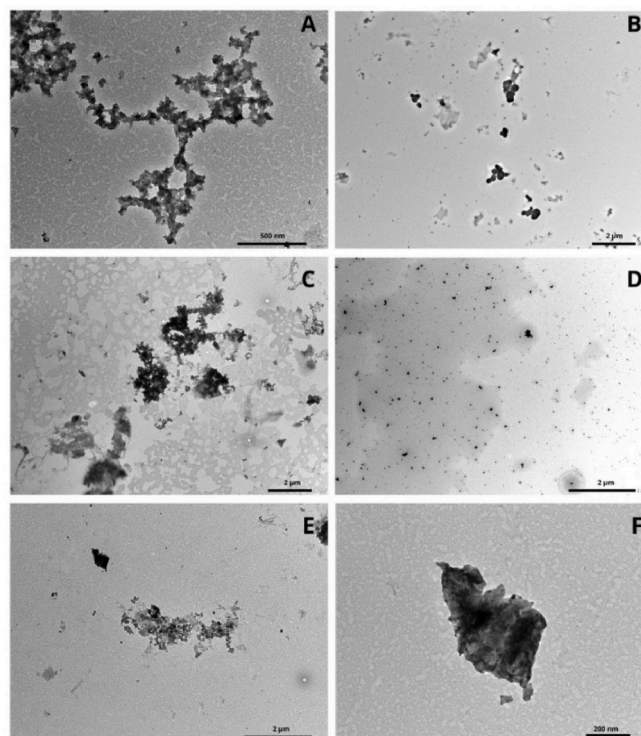


Fig. 1. – TEM images of stock solutions (1 g L^{-1} , 5 μm filtered) of aged PE (A and B), field PE (C and D) and virgin PE (E and F).

slope value, the higher the burial speed.

The burrowing kinetics were significantly impaired in *S. plana* exposed to 0.008 $\mu\text{g L}^{-1}$ of aPE and fPE (slopes = - 0.0018 and - 0.0013 respectively) compared to control (slopes = - 0.0037). No significant effects on burrowing behavior were observed following exposure to vPE compared to control. Regarding the concentration effect within a condition, there was no significant difference in burrowing behavior of bivalves when the concentration of vPE MP increased. However, the burial rate was increased for bivalves exposed to 10 $\mu\text{g L}^{-1}$ of aPE and fPE MP (slopes = - 0.0035 and - 0.0044 respectively) compared to the burial rates observed at 0.008 and 100 $\mu\text{g L}^{-1}$. Comparison between exposure to aPE, vPE and fPE MP at the same concentrations showed no difference at 0.008 and 100 $\mu\text{g L}^{-1}$. Exposure to 10 $\mu\text{g L}^{-1}$ of vPE MP led to a significant increase of burrowing kinetics (slope = - 0.006) compared to exposure to aPE and fPE MP at the same concentration (slopes = - 0.0035 and - 0.0044 respectively).

3.4. Enzymatic biomarkers

Oxidative stress in clams was monitored through measurement of CAT activity. After 21 days of exposure, CAT enzymatic activity decreased in gills of organisms exposed to aPE (0.008 $\mu\text{g L}^{-1}$ and 100 $\mu\text{g L}^{-1}$), vPE (10 $\mu\text{g L}^{-1}$) and fPE (all exposure concentrations) compared to control organisms (Fig. 4). In digestive mass, no significant difference in CAT activity was observed between all tested conditions.

Regarding the immunity function, the neurotoxicity and detoxication system, no significant difference in ACP, AChE and GST activities, respectively, were observed in gills and digestive glands of clams exposed to MP compared to control (Fig. 5).

3.5. Genotoxicity

In gills, after 21 days of exposure, DNA damage were significantly increased in organisms exposed to vPE (0.008 $\mu\text{g L}^{-1}$ and 10 $\mu\text{g L}^{-1}$) and fPE (0.008 $\mu\text{g L}^{-1}$) but decreased in organisms exposed to high

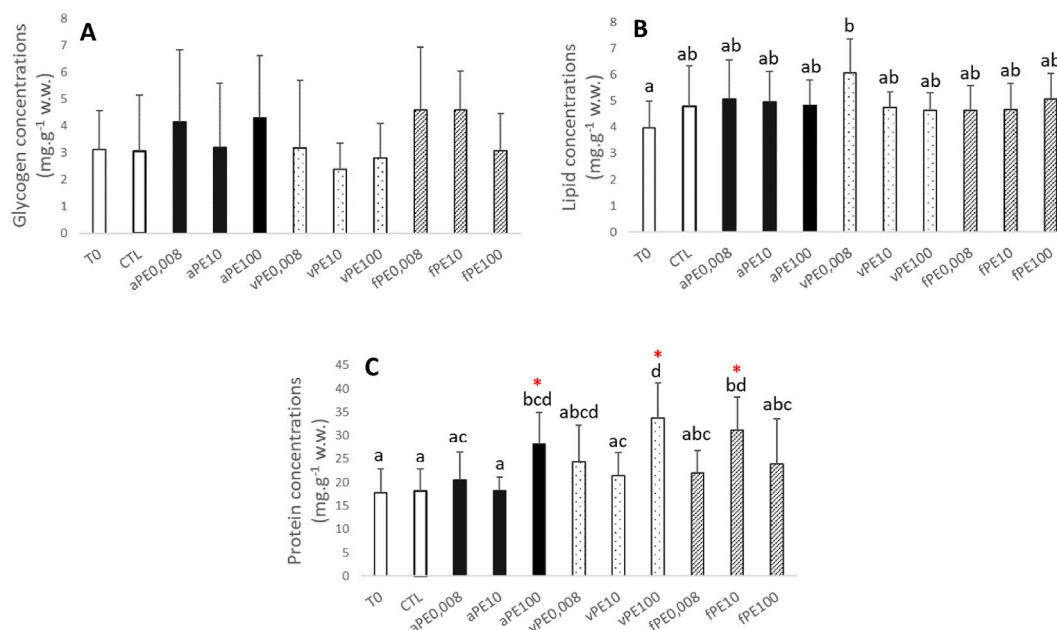


Fig. 2. Glycogen (A), lipids (B) and proteins (C) content in whole organism of *S. plana* at the beginning of exposure (T0) and after a 21-day exposure to a control condition (CTL) and to aged PE (aPE), virgin PE (vPE) or field PE (fPE) at 0.008, 10, and 100 $\mu\text{g L}^{-1}$ (mean \pm SD, letters a, b, c, d for significant differences between conditions $p < 0.05$ and the red star for significant differences between conditions and control $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

concentrations of fPE (100 $\mu\text{g L}^{-1}$) compared to control organisms (Fig. 6A). No significant effect was observed following exposure to aPE, regardless of the exposure concentration. For all tested plastic conditions, the highest concentrations induced significantly lower DNA strand breaks as compared to lowest doses. In digestive glands, significant decreases of DNA damage were observed after clam exposures to aPE (10 $\mu\text{g L}^{-1}$ and 100 $\mu\text{g L}^{-1}$) and fPE (100 $\mu\text{g L}^{-1}$) compared to control organisms whereas exposure to vPE (all tested concentrations) did not significantly modify the levels of DNA damage (Fig. 6B).

4. Discussion

Once in the environment, plastics undergo weathering processes, including physical or chemical degradation, which involve various mechanisms such as photolysis, thermolysis, oxidation, ozone-induced reactions, hydrolysis, and biological breakdown (Singh & Sharma, 2008; Webb et al., 2013). However, degradation patterns differ among plastic polymers when exposed to environmental conditions. This variability in plastic aging poses a challenge for ecotoxicity assessment, as it can lead to the release of chemical additives and degradation by-products, creating a complex mixture of stressors (Fred-Ahmadu et al., 2020; Gaur et al., 2022; Webb et al., 2013). Evaluating ecotoxicity requires therefore distinguishing the physical effects of microplastics from those stemming from additives (Barrick et al., 2021).

As majority of previous studies focused on the effects of commercial MP (Leistenschneider et al., 2023), not representative of those found in natural environments (Paul-Pont et al., 2018), the originality of the present study was to assess the impact of artificial and field aging polyethylene microplastics (PE MP) on the bivalve *S. plana*, a key organism of the estuarine compartment living at the soil/water interface. This bivalve is a filter-deposit-feeder able to ingest MP via its gills, the first organ in contact with particles in suspension in water, and through its inhalant siphons (Ribeiro et al., 2019), demonstrating its relevance for MP risk assessment.

4.1. Environmental experiment conditions

PE was chosen as a model plastic polymer for this study as it is the most produced type of polymer (Plasticseurope, 2022), especially in packaging and single-use products (Plasticseurope, 2022). PE is also one of the most frequently found polymers in terrestrial and aquatic environments, notably in estuaries (Jones et al., 2020). Furthermore, effects of PE MP were previously reported on bivalves (Abidli et al., 2021; Moncrieffe et al., 2023; Teng et al., 2024). In the present study, the average size of PE MP was comprised between 28 μm (fPE) and 34 μm (vPE) with the presence of particles $< 10 \mu\text{m}$. These sizes were in the same range as those tested in previous studies and are close to the size of the phytoplankton and particles on which *S. plana* usually feed (Hughes, 1969). This allows us to consider that the exposed clams may have filtered or ingested the PE MP tested in our study.

Another point to consider was the MP concentrations chosen for the study. Environmental MP concentrations were selected to be consistent and relevant with environmental conditions. The use of environmental concentrations in ecotoxicological studies is needed to support political decisions but is confronted to the lack of available data concerning small sizes MP ($< 10 \mu\text{m}$). Most of field quantification studies focused on particles larger than 300 μm (Paul-Pont et al., 2018) while laboratory studies tend to use particles smaller than 100 μm (Bucci et al., 2020). In this study, three concentrations were tested, representative of coastal regions (0.008 and 10 $\mu\text{g L}^{-1}$) (Desforgues et al., 2014; Ivar do Sul et al., 2014) and gyres such as the North Pacific gyre (100 $\mu\text{g L}^{-1}$) (Goldstein et al., 2012). However, *S. plana* may encounter even higher concentrations of MP by living at the sediment-water interface, where MP concentrations are estimated to be stronger (Martin et al., 2017) and because estuaries may accumulate microplastics particles, trapped on the estuary bed (Defontaine et al., 2020).

4.2. Individual responses

Previous studies reported the ability of *S. plana* to accumulate PS MP (1 mg L^{-1} , 20 μm) in gills and digestive gland after a 14-day exposure and a 7-day period of depuration (Ribeiro et al., 2017). The ingestion of

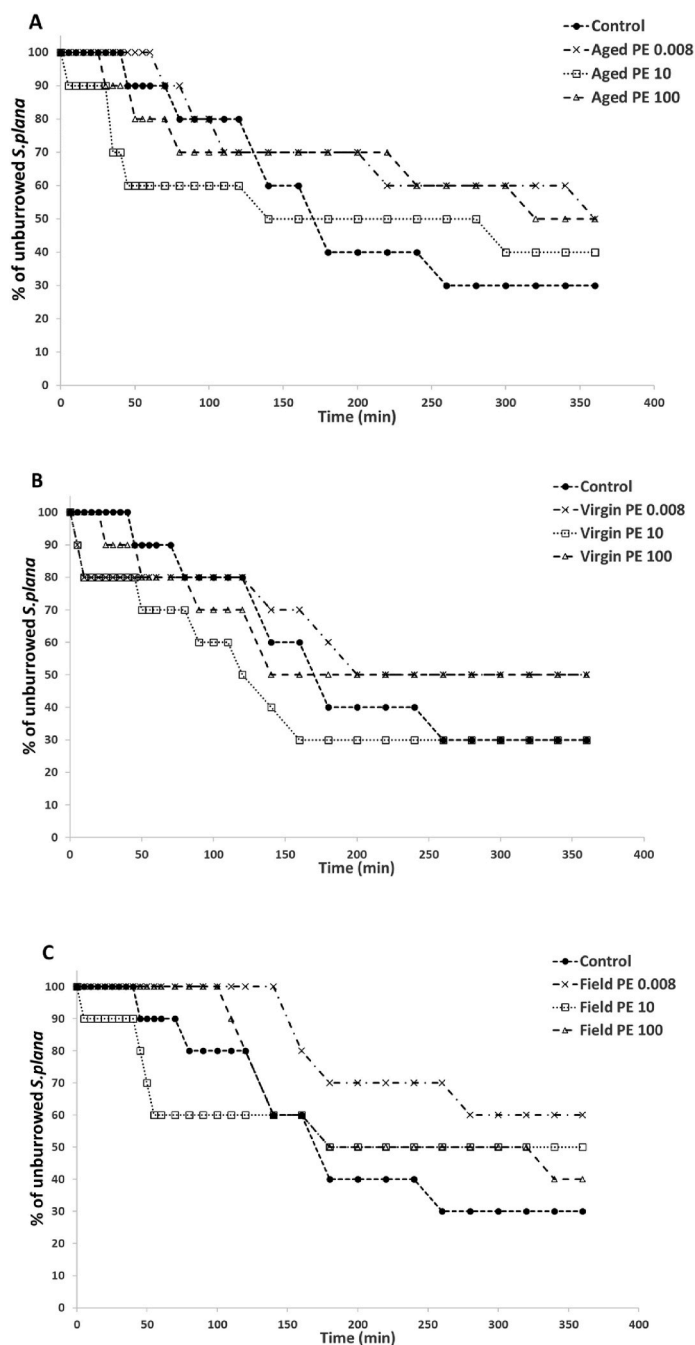


Fig. 3. Percentage of unburied clams *S. plana* ($n = 10$) at different times (min). Animals were previously exposed for 21 days to a control condition and to aged PE (A), virgin PE (B) or field PE (C) at 0.008, 10, and 100 $\mu\text{g L}^{-1}$.

PE MP (1 mg L^{-1} , 4–6 and 20–25 μm , 14 days) was also previously reported (Rodrigues et al., 2022) and demonstrated to be size dependent with a lower ingestion level for smallest MP related to a reduction of the clearance rate (Islam et al., 2021). In the present study, condition index and clearance rate of *S. plana* were not affected by a 21-day exposure to PE MP, regardless of MP type and concentrations. The reported values for the condition index (mean 13.96 ± 2.33) were consistent with good physiological conditions of *S. plana* (Mouneyrac et al., 2008). The capacity of bivalves to ingest MP can be influenced by numerous parameters (e.g. exposure time, seasonal variations). Particle size and morphology are factors influencing MP accumulation in bivalve tissues and are therefore important to consider when conducting ecotoxicological studies (Khanjani et al., 2023). The morphometric diversity of the MP used in this study is consistent with the plurality of MP shapes

that can be found in the environment. Moreover, the TEM analysis revealed the presence of particles in the nanometer size range ($<1 \mu\text{m}$) in the exposure solutions. It is consistent with other studies using lab-produced MP (Vidal et al., 2023). In environment, previous works demonstrated the generation of nanoplastics (NP) from the degradation of microplastics (Lambert & Wagner, 2016) and their occurrence in environmental matrices (Ter Halle et al., 2017). NP are expected to be more toxic for bivalves than MP (Métais et al., 2023a) and exclusion of this size category when performing MP exposure could minimize effects on organisms. In this study, the analysis focused on the impact of MP. However, it is important not to minimize the potential role of NP in the observed toxicity, even present in small quantities.

Behavioral biomarkers are useful toxicity indicators in bivalves due to their sensitivity to contaminants exposures even at low-

Table 1

Slopes of burrowing tests for *S. plana* in the control condition and after a 21-day exposure to aged PE (aPE), virgin PE (vPE) or field PE (fPE) microplastics at 0.008, 10, and 100 $\mu\text{g L}^{-1}$ ($N = 10$).

	Concentrations ($\mu\text{g L}^{-1}$)	Slopes
Control	–	–0.0037
Aged PE	0.008	–0.0018
	10	–0.0035 ^a
	100	–0.0021
Virgin PE	0.008	–0.0029
	10	–0.006 [#]
	100	–0.0036
Field PE	0.008	–0.0013
	10	–0.0044 ^a
	100	–0.0025

^a For significant differences among concentrations within the same MP, # for significant differences among treatments within the same concentration and in bold for significant differences between control and conditions $p < 0.05$.

concentrations (Boldina-Cosqueric et al., 2010). In our study, a decrease in burrowing behavior was observed in *S. plana* exposed to aged and field PE at low concentration (0.008 $\mu\text{g L}^{-1}$). These results are in accordance with Métais et al. (2023a) that demonstrated an impairment of burrowing kinetics on the same species exposed to the lower concentration of an environmental mixture of MP (0.008 $\mu\text{g L}^{-1}$, 21 days, $<300 \mu\text{m}$). Decreased burrowing rates were also shown in clams (*S. plana*) exposed to tire wear particles ($<250 \mu\text{m}$) at all tested concentrations (0.2, 1, and 5% dry weight sediment; 21 days) (Garrard et al., 2022). Our results suggest that aging processes may modulate the toxicity of PE MP on burial behavior, since only exposures to artificially or field aged MP led to a decrease of *S. plana* burrowing kinetics. In the same way, non-aged PE MP exposure (1, 10 and 25 mg kg^{-1} of sediment, 4–6; 20–25 and 125–500 μm , 4 weeks) did not modify burrowing behavior in sediment-dwelling bivalves (*Ennucula tenuis* and *Abra nitida*) in comparison to control group (Bour et al., 2018). Burrowing behavior can be impacted by biotic and abiotic parameters (Boldina-Cosqueric et al., 2010), such as salinity (Verdelhos et al., 2015), temperature (Przeslawski et al., 2009) or the presence of metal (Shin et al., 2002). This supports the hypothesis that even small changes in physical and chemical characteristics of MP due to aging process could induce a specific response of burrowing behavior. Impaired burrowing behavior has been previously linked to inhibition of the neurotransmitter system, and in particular to the inhibition of AChE activity (Amiard-Triquet, 2009). The decrease in AChE activity was previously associated with reduction of burrowing kinetics in *Hediste diversicolor* exposed to PS NP (0.005–0.5 mg L^{-1} ; 28 days; 100 nm) (Silva et al., 2020). However, in the present study, no significant difference in AChE activity was observed in the gills and digestive gland of clams exposed to MP, regardless of MP type or concentration. Similar results were observed in *S. plana* exposed to CuO nanoparticles with a burrowing decrease without changes in AChE activity (Buffet et al., 2011). Bonnard et al.

(2009) also reported behavioral impairments that were not linked to an inhibition of AChE activity in *S. plana* exposed to copper and suggested an involvement of metabolic or physiological mechanisms. An analysis of AChE activity in tissues directly involved in burrowing behavior, i.e. foot and adductor muscles, could also provide further insight into toxicity mechanisms. A possible link between feeding rate and burrowing behavior was previously mentioned (Garrard et al., 2022), unlike the present study where the clearance rate of exposed *S. plana* was not impacted. Thus, toxicity mechanisms involved in burrowing impairments following MP exposure still need to be clarified. Burrowing ability is a key function for sediment-dwelling organisms such as *S. plana*, protecting clams from predation (Griffiths & Richardson, 2006) and allowing them to feed at low tide. A decrease in burial kinetics in presence of MP could therefore impact population maintenance (Bertrand et al., 2017).

Related to feeding behavior and as MP can be captured and entered the digestive system, the impact of MP exposure on clam's energy reserves must be explored (Urban-Malinga et al., 2021). In our study, lipid and glycogen contents were not impacted by MP exposure whereas higher protein contents were observed in clams exposed to the highest concentration (100 $\mu\text{g L}^{-1}$) of aPE and vPE and to 10 $\mu\text{g L}^{-1}$ of fPE. Previous studies reported reductions in protein content of burrowing bivalves exposed to tire particles (Garrard et al., 2022) or PE MP (Bour et al., 2018), suggesting a decrease in energy reserves, as proteins may be a metabolic reserve alternative in bivalves facing a stress. Regulation of protein expression was previously reported in digestive glands of *S. plana* as a response to pollutant stressors (González-Domínguez et al., 2016). In our study, the increase of protein content in response to MP exposure could suggest an up-regulation of proteins involved in protective functions of organisms such as heat shock proteins. Additional proteomic analyses could provide further information.

4.3. Molecular and cellular responses

Catalase (CAT) is one of the first line protective enzymes in the antioxidant defense system, catalyzing dismutation of H_2O_2 into H_2O and O_2 . In our study, exposures to aPE (0.008 and 100 $\mu\text{g L}^{-1}$), vPE (10 $\mu\text{g L}^{-1}$) and fPE (all tested concentrations) significantly decreased CAT activity in the gills of clams. This decrease could suggest a disruption of the detoxification system in exposed organisms. Similar results were observed in gills of scallops (*Chlamys farreri*) exposed to PE MP for 28 days (10 and 1000 $\mu\text{g L}^{-1}$, $36.72 \pm 24.37 \mu\text{m}$) (Teng et al., 2024) and in digestive gland of *Mytilus* spp. Exposed to PS MPs (32 $\mu\text{g L}^{-1}$, 2 and 6 μm) for 7 days (Paul-Pont et al., 2016). An impairment of the antioxidant response (CAT and GST) was also reported in the Mediterranean mussel *Mytilus galloprovincialis* after a 14-days exposure to PE MP (100 and 1000 $\mu\text{g L}^{-1}$, 40–48 μm) (Abidli et al., 2021). However, Métais et al. (2023a) reported higher levels of CAT activity in gills of *S. plana* exposed to environmental MP (21 days, 100 $\mu\text{g L}^{-1}$, $<300 \mu\text{m}$) and this result was in accordance with most experiments reporting CAT activity in bivalves

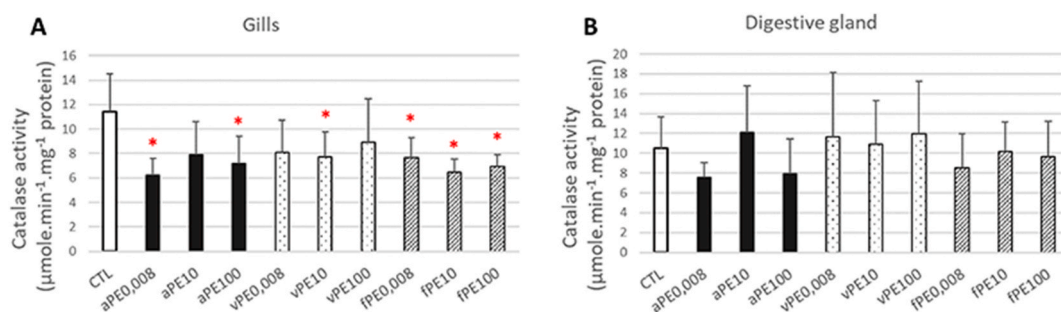


Fig. 4. – Catalase activity in gills (A) and digestive glands (B) of *S. plana* after a 21-day exposure to a control condition (CTL) and to aged PE (aPE), virgin PE (vPE) or field PE (fPE) at 0.008, 10, and 100 $\mu\text{g L}^{-1}$ (mean \pm SD, red star for significant differences between conditions and control $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

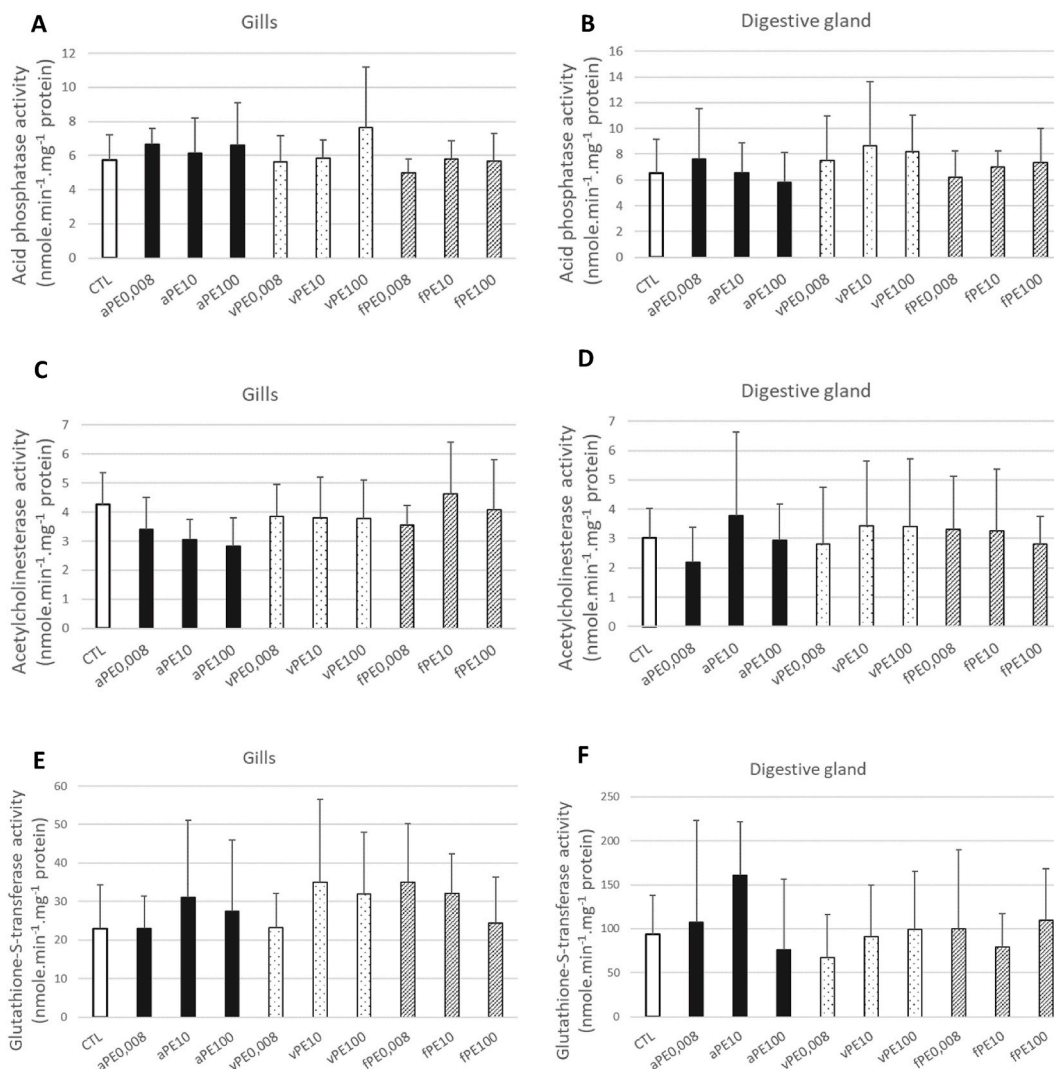


Fig. 5. – Acid phosphatase, acetylcholinesterase and glutathione-S-transferase activity in gills (A, C, E respectively) and digestive glands (B, D, F respectively) of *S. plana* after 21 days of exposure to a control condition (CTL) and to aged PE (aPE), virgin PE (vPE) or field PE (fPE) at 0.008, 10, and 100 $\mu\text{g L}^{-1}$ (mean \pm SD).

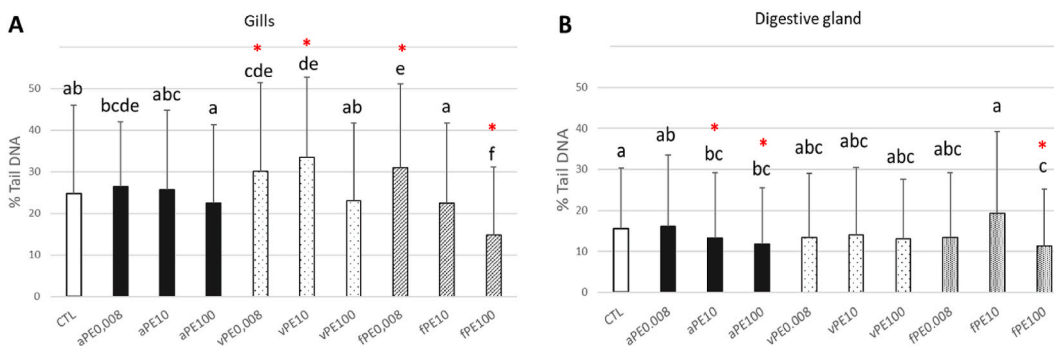


Fig. 6. DNA integrity in gills (A) and digestive glands (B) of *S. plana* after a 21-day exposure to a control condition (CTL) and to aged PE (aPE), virgin PE (vPE) or field PE (fPE) at 0.008, 10, and 100 $\mu\text{g L}^{-1}$ (mean \pm SD, letters a, b, c, d, e, f for significant differences between conditions $p < 0.005$ and the red star for significant differences between conditions and control $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

exposed to MP (Revel et al., 2019; Ribeiro et al., 2017). The meta-analysis of Li et al. (2022) on bivalves concluded that microplastics are oxidative stressors and that impacts on the antioxidant system are increasing with the duration of exposure. However, oxidative stress responses to MP are variable (Li et al., 2022); differential effects could be

explained by the characteristics or concentrations of tested MP. Indeed, most of concentrations reported in this meta-analysis were higher than those tested in our study. The presence of plastic additives could also impact the antioxidant system. Mejdoub et al. (2017) measured a decrease in CAT activity in *Mytilus galloprovincialis* exposed to chemicals

such as heavy metals and CAT is described as a lead target (Marques et al., 2016). The impact of diet should also be considered when investigating oxidative stress, as physiological effects can be modulated by the presence of food (Wang et al., 2021). In our study, reduced CAT activity could unbalance the antioxidant response, leading to a delayed increase in ROS (Reactive Oxygen Species) and oxidative stress in exposed organisms (Li et al., 2022).

DNA damage was monitored using the comet assay, a method previously developed to assess genotoxicity of contaminants in numerous ecotoxicological studies (Fairbairn et al., 1995). DNA damage was previously linked to ROS accumulation that can interact with DNA and modify its integrity (Parolini & Binelli, 2012). The disruption of the antioxidant system, caused here by the decrease of CAT activity in gills, could thus be responsible for increasing levels of ROS and consequently DNA damage. An assessment of ROS production in tissues would be necessary to confirm this hypothesis. In this study, DNA damage increased in gills of clams exposed for 21 days to vPE ($0.008 \mu\text{g L}^{-1}$ and $10 \mu\text{g L}^{-1}$) and fPE ($0.008 \mu\text{g L}^{-1}$) compared to controls, which wasn't consistent with the previous hypothesis. Furthermore, lower DNA damage was measured in gills of clams exposed to the higher concentration of fPE ($100 \mu\text{g L}^{-1}$) and in digestive glands of clams exposed to aPE ($10 \mu\text{g L}^{-1}$ and $100 \mu\text{g L}^{-1}$) and field PE ($100 \mu\text{g L}^{-1}$) compared to control organisms. Globally, in gills, for all tested plastic conditions, the highest concentration exposures induced significantly lower DNA damage compared to lowest doses suggesting a dose-dependent effect. Other studies testing higher (1 mg L^{-1}) concentrations of PS MP (Ribeiro et al., 2017) or LDPE (Low Density PolyEthylene) MP (O'Donovan et al., 2020) reported no significant differences in DNA integrity in *S. plana* hemocytes after a 14 day exposure. Métais et al. (2023a) observed that a 7-day exposure to environmental MP increased DNA damage in *S. plana* gills but these damages disappeared after 21 days of exposure. Absence or low levels of DNA damage at high toxicant concentrations or after a long exposure time was previously attributed to the activation of DNA repair system (Parolini & Binelli, 2012): a threshold concentration of toxic substances accumulated in exposed organisms may be required to activate the DNA repair system. In our study, the lowest exposure concentrations of PE MP might not have activated this repair system and were therefore more susceptible to cause an increase in DNA damage in exposed clams. On the contrary, exposure of *S. plana* to the highest concentrations of MP might have activated the repair system and thus reduced DNA damage. Furthermore, at high MP exposure concentration, apoptosis could likely occur and dead cells could be removed when performing the comet assay. Indeed, a possible interaction between apparition of apoptotic cells and measurement of DNA damage using comet assay was reported by Choucroun et al. (2001). It would be thus necessary to monitor apoptotic biomarkers such as caspase-3-activity to confirm the apoptosis hypothesis (Châtel et al., 2017).

4.4. Perspectives for understanding effect of aging on profile toxicity of PE

All types of PE MP tested in the present study (aged PE, virgin PE and field PE) impacted clams' physiological functions at individual, cellular or molecular level. However, depending of the type of PE MP aging, the toxic concentrations and the impacted biomarkers were different. The MP produced from macrowaste collected on the Seine estuary (field PE; fPE) presented the most marked toxicity profile, whereas non-aged PE MP (virgin PE; vPE) had the least impact on studied biomarkers. Aging processes of MP under the effect of light, wind, water and microorganisms lead to changes in their physical and chemical properties and consequently in their toxicity mechanisms. Chen et al. (2024) highlighted the complexity of the degradation process under environmental conditions compared to laboratory simulated ones. Complexity and stability of MP biofilm (Deng et al., 2024) and metal accumulation on MP surface (Xie et al., 2021) were studied under various MP aging conditions and demonstrated different MP properties that could explain the observed variations in the toxicity profiles. On the other hand, no

toxicity difference was reported by Jemec Kokalj et al. (2019) when exposing daphnia, duckweed and zebrafish to PE MP aged in natural waters and wastewaters or to virgin PE MP. Due to a modification of the physical MP properties, aging processes may be associated with the release of chemical additives and an increased adsorption of pollutants (He et al., 2023) that could lead to a higher level of ecotoxicity impacts in organisms (Moncrieffe et al., 2023). The observation of non-linear dose-response on the biomarkers studied prompts to characterize more precisely the possible presence of endocrine-disrupting compounds associated with MP (Gu et al., 2021; Métais et al., 2023b). The characterization of chemical adsorbed on the MP surface could thus provide deeper information on the toxicity mechanisms of tested PE MP.

5. Conclusion

This study provided a first insight into the toxic effects of artificial and natural aged PE versus non-aged MP in the estuarine clam *S. plana* exposed through environmental conditions. The differences observed in the toxicity profiles following clam exposures to artificial and natural conditions underline the difficulty of studying and comparing the toxicity of environmental MP samples, particularly in terms of size and shape heterogeneity within a sample, of chemical additives or pollutants adsorbed on particles, and of degradation mechanisms. Furthermore, the use of wild animals in MP toxicity studies highlights another challenge, namely the possible acclimatization of these organisms to MP at the site where they live. Measurement of contamination levels at organism sampling sites should be completed and future work carried out in this direction to better understand how the life history of organisms could modulate their response to MP exposure in the laboratory.

This work brings new results on the effects of aging on toxicity of MP and invites further investigations in this area, using MP mimicking environmental MP with limited parameter variations, to fully understand the major mechanisms involved in toxicity phenomena. Additional studies must be conducted considering MP physicochemical characteristics throughout their lifecycle by assessing their behavior and fate along the soil-water continuum for future regulatory needs.

CRedit authorship contribution statement

Clémentine Labbé: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Isabelle Métais:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Hanane Perrein-Ettajani:** Methodology, Formal analysis. **Mohammed Mouloud:** Methodology. **Antoine Le Guernic:** Methodology. **Oihana Latchere:** Methodology. **Nicolas Manier:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Amélie Châtel:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by Région Pays de la Loire and Institut National de l'Environnement Industriel et des RISques (INERIS). The authors would like to thank Fabienne Lagarde and Margaux Glais from Institut des Molécules et Matériaux du Mans (IMMM) for the access and

support with the morphometric platform. They are also grateful to Farid Aït ben ahmad and Yohann Baillon from INERIS (DRC/CARA/ANAE unit) for the TEM analysis. The authors thank Mélanie Bruneau and Didier Georges for her technical assistance as well as Mathilde Madre and Lalie Supiot for the help during their internship in UCO.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2024.124805>.

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