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Sublethal Impacts of Polyethylene Microplastics on Freshwater Amphipods: Genotoxic and Metabolic Responses in *Echinogammarus veneris* and *Cryptorchestia garbinii* (Crustacea, Amphipoda)

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Abstract

Microplastic (MP) contamination represents a global threat to aquatic ecosystems, yet its biological effects remain poorly understood. This study investigates the short-term impacts of polyethylene (PE) microparticles on two amphipod species: the semi-terrestrial *Cryptorchestia garbinii* and the aquatic *Echinogammarus veneris*. Amphipods are exposed to MPs both in water and through dietary intake. After 24 h, *C. garbinii* ingested an average of 9.6 ± 1.2 particles per individual, while *E. veneris* ingested 12.5 ± 2.8 particles, confirming an active uptake of microplastics. The mean particle size decreased from ≦50 µm in the food tablets to 18–25 µm in the digestive tract, suggesting fragmentation during digestion and highlighting the ecological role of amphipods in generating smaller, potentially more bioavailable particles. Both species exhibited a marked increase in DNA damage, together with variations in energy-reserve allocation (glucose, glycogen, and lipids) consistent with acute metabolic stress. To our knowledge, this represents the first evidence of genotoxicity ever reported in *C. garbinii*, expanding current understanding of the biological responses of amphipods to plastic pollution. These findings highlight the vulnerability of detritivore species to MPs exposure and, given their role in nutrient cycling, emphasize the need for further research on the ecological implications of MPs contamination.

Keywords: comet assay; energy reserves; trophic exposure; Talitrids; Gammarids

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1. Introduction

Microplastic (MP) contamination represents a major global concern with growing evidence of its widespread occurrence across aquatic environments. Despite extensive research, the effects of MPs on organisms remain poorly understood. Global plastic production reached 430 million tonnes in 2024, and [1] it is estimated that between 10 and 40 million tonnes of microplastics are released into the environment each year [2]. Microplastics and nanoplastics (NPs) are increasingly recognized as emerging environmental contaminants of global concern. Once introduced, these particles can remain for hundreds of years, accumulating across all environmental compartments [3] and the trophic chain. They have contaminated all aquatic and terrestrial environments, from the deep sea to polar regions, posing a threat to organisms that may mistake them for food and ingest them [4–6]. Since the term microplastics was first introduced by Thompson et al. [7], several studies have documented their uptake and biological effects in a wide range of marine

and freshwater organisms. Several studies have already reported biological impacts of microplastics on aquatic organisms, highlighting their toxic potential [8]. Microplastics have been detected throughout the biosphere, affecting more than 1300 species across aquatic and terrestrial ecosystems, from small invertebrates to top predators, with evidence of impacts at multiple biological levels. Their pervasive presence extends to human environments as well, being found in food, water, air, and even in human organs, where early studies suggest possible adverse effects [2,9]. Documented effects include physical damage to tissues, reduced feeding and growth, induction of oxidative stress, inflammation, and impairment of reproduction [10–12]. Moreover, microplastics can act as vectors for other contaminants, enhancing their bioavailability and toxicity [13]. Such effects have been observed in fish, bivalves, and crustaceans, indicating that MPs represent a risk at both individual and population levels [14,15]. Together, these findings suggest that MPs pose a significant toxicological risk at both individual and population levels, although the magnitude and mechanisms of their impacts vary depending on the species, polymer type, particle size, and exposure conditions.

Their environmental presence is expected to double every 10 to 15 years [16], underscoring the principle that "dose makes the poison". To address the situation, it is necessary to develop effective monitoring methodologies and study the biological effects caused. One promising approach involves the use of bioindicator organisms whose physiological responses or population changes can reflect environmental modifications [17]. Observing the exposure to MPs and their uptake in these species allows us to gather crucial information about the resulting biological effects. Choosing suitable bioindicator species requires careful assessment of their ecological significance, capacity to accumulate MPs, and feasibility for monitoring, but in recent years, several species have been suggested and tested. Among aquatic invertebrates, crustaceans are widely used as model organisms to study the effects of microplastic exposure. Laboratory and field studies have reported ingestion, reduced feeding activity, oxidative stress, and reproductive impairment in copepods, amphipods, and decapods [18–21]. Their ecological importance, wide distribution, and sensitivity make them valuable indicators for assessing the biological impacts of microplastics in aquatic ecosystems [22]. Despite this growing body of evidence, several aspects of MP toxicity remain unclear, particularly concerning species that play a key ecological role in nutrient cycling and energy transfer within aquatic food webs.

Gammarid amphipods serve as valuable bioindicators for environmental contamination studies [23,24]. They are common in aquatic ecosystems and play an essential role in nutrient cycling. Recent research has highlighted their ingestion of microplastics and their potential as bioindicators of this pollutant [5,25,26]. As key detritivores, they can also ingest microplastics, aiding their spread through the food chain [27]. Their role in ecotoxicology studies underscores their value as model organisms [28] and the Circum-Mediterranean species *Echinogammarus veneris* (Heller, 1865) (Figure 1), is a promising choice for ecotoxicological assessments within its natural range [27,29,30].

Talitrid amphipods are dominant crustaceans on sandy beaches, serving as both detritivores and a food source for other organisms. Their vulnerability to pollution and sensitivity to environmental changes make them valuable bioindicators [31]. *Cryptorchestia garbinii* Ruffo et al. 2014 (Figure 1) is a talitrid amphipod found in coastal areas of brackish and fresh waters across the Mediterranean, Central Europe, and England. It has been used as a model organism for studying cellular processes and has been shown to ingest microplastics [5,25,32]. The ingestion of microplastics poses a significant threat to individual organisms, because MPs can cause physical damage, facilitate the uptake of other contaminants, and disrupt physiological processes, having implications for population level, community and, in the end, entire ecosystems. By studying the effects of microplastics on

amphipods, we can gain valuable insights into the impacts of plastic pollution on aquatic ecosystems and human health.

Figure 1. Amphipod species used in this study: (**left**) *Echinogammarus veneris* (Heller, 1865) and (**right**) *Cryptorchestia garbinii* Ruffo et al., 2014.

In this study, we assess the impacts of microplastics on the two amphipod species previously described, *C. garbinii* and *E. veneris*, representative of freshwater and brackish habitats, where they play a key role in organic matter decomposition and trophic transfer Among the various polymers occurring in the aquatic environment, polyethylene (PE) was selected for its high environmental relevance and frequent detection in Italian freshwater ecosystems [33]. For dietary exposure, we employed DECOTABs (DEcomposition and COnsumption TABlets, DTs), previously validated for aquatic toxicity assessments [34,35] and for evaluating MPs' effects [32]. Their stability in water makes them particularly suitable for ensuring that MPs exposure occurs through the supplied food (Table 1). This combined approach provides a more complete view of the potential impacts of MPs on amphipods.

Table 1. Overview of the experimental design used to assess the effects of polyethylene microplastics in two amphipod species. The table summarizes the habitat, type of exposure, and endpoints measured for *Cryptorchestia garbinii* and *Echinogammarus veneris*. Endpoints include genotoxicity (DNA damage), metabolic biomarkers (glucose, glycogen, lipids), ingestion, and fragmentation of microplastic particles. We analised 80 individuals per treatment, obtained after two independent experimental runs. Additional specimens were used for gut MP quantification as described in Section 2.5.

	Habitat	Experimental Treatment	Endpoints	
Cryptorchestia garbinii	Supralittoral	-Trophic exposure (DTs)	DNA damage; Glucose, Glycogen, Lipid concentrations; Ingestion and fragmentation	
Echinogammarus veneris	Aquatic	-Water exposure -Trophic exposure (DTs)	DNA damage; Glucose, Glycogen, Lipid concentrations; Ingestion and fragmentation	

Some critical toxicological endpoints were assessed to estimate the impact of microplastic exposure. A 24 h exposure period was selected to assess the acute effects of microplastics, enabling the detection of early biochemical and cellular responses before potential recovery mechanisms could occur. Specifically, after 24 h of exposure, we evaluated changes in the allocation of energy reserves, including glucose, glycogen, and lipids, along with a

genotoxicity assessment, using the comet assay on circulating haemocytes. Moreover, we detected and quantified the MPs in the dissected digestive systems, and compared the average sizes of the microplastics provided as food with those detected.

This study aims to enhance the understanding of microplastic toxicity by providing new evidence of their short-term biological effects in amphipods.

2. Methods

2.1. Echinogammarus veneris Sampling and Lab Maintaining

Individuals were collected at the Fontana di Muro spring (Pontinia, Latium, Italy) using a hand net and transferred to the laboratory. There, they were maintained in 10 L aerated glass aquaria filled with water from the sampling site to minimize acclimation stress and kept under controlled environmental conditions (15 °C, 12:12 h light/dark cycle) in a thermostatic cabinet. The amphipods were supplied with commercial dry fish food *ad libitum* [30]. After a three-week acclimation period, adult individuals (praecopula pairs) were selected and subsequently used for exposures.

2.2. Cryptorchestia garbinii Sampling and Lab Maintaining

Samples of *C. garbinii* were collected by hand or using an aspirator along the shores of Lake Albano (Castel Gandolfo, Latium, Italy) and promptly transferred to the laboratory under temperature-controlled conditions. In the lab, individuals were housed in glass containers placed inside a thermostatic cabinet set at 20 °C with a 12:12 h light/dark cycle. Moisture was maintained by periodically adding water, and the animals were fed fish food and small pieces of blotting paper, reflecting their preference for cellulose-based materials. Soil from the collection site was used as substrate. The specimens were acclimated for three weeks before exposures.

2.3. Water Exposures

To evaluate the effects of PE (Polyethylene) microparticles on *E. veneris*, individuals were exposed to a suspension of PE at a concentration of 0.04 g/L (approx. 5000 MP particles/mL) in 150 mL of dechlorinated tap water, to ensure standardized and reproducible experimental conditions and to avoid variability in water chemistry among trials. This concentration was chosen to induce measurable biological responses during a 24 h (acute) exposure, in line with previous ecotoxicological studies on aquatic invertebrates [36,37] and in our prior work on amphipods [27]. Moreover, it is within the ranges recorded in freshwater environments [38,39], so the concentration tested in our study can be considered environmentally realistic.

Small glass beads were also added to each beaker to mimic the substrate. We used commercial PE MPs (Sigma-Aldrich St. Louis, MO, USA, code 434272; 40–48 µm; ultra-high molecular weight; density 0.94 g mL^{-1})were used as standardized material to ensure controlled and reproducible exposures (Figure 2A). Five individuals were placed in each beaker for 24 h (Figure 1). A total of 16 beakers (5 organisms each) were prepared for each experimental run: 8 beakers (n = 40 individuals) for the genotoxicity assessment and 8 beakers (n = 40 individuals) for energy biomarkers, obtained from two independent experimental runs. Replicates used for gut MP quantification followed the setup described in Section 2.5. Triton X-100, a non-ionic surfactant (0.00022%), was added at a concentration of 0.00022% to water to promote more even improve particle dispersion of microplastics in suspension. The applied compound is commonly used in ecotoxicological assays and is at levels far below those known to cause toxic or genotoxic effects [40–42]. However, since surfactants can potentially act as confounding factors, we performed preliminary controls

comparing treatments with and without this compound. Table 1 outlines the exposure types and the corresponding toxicological endpoints evaluated.

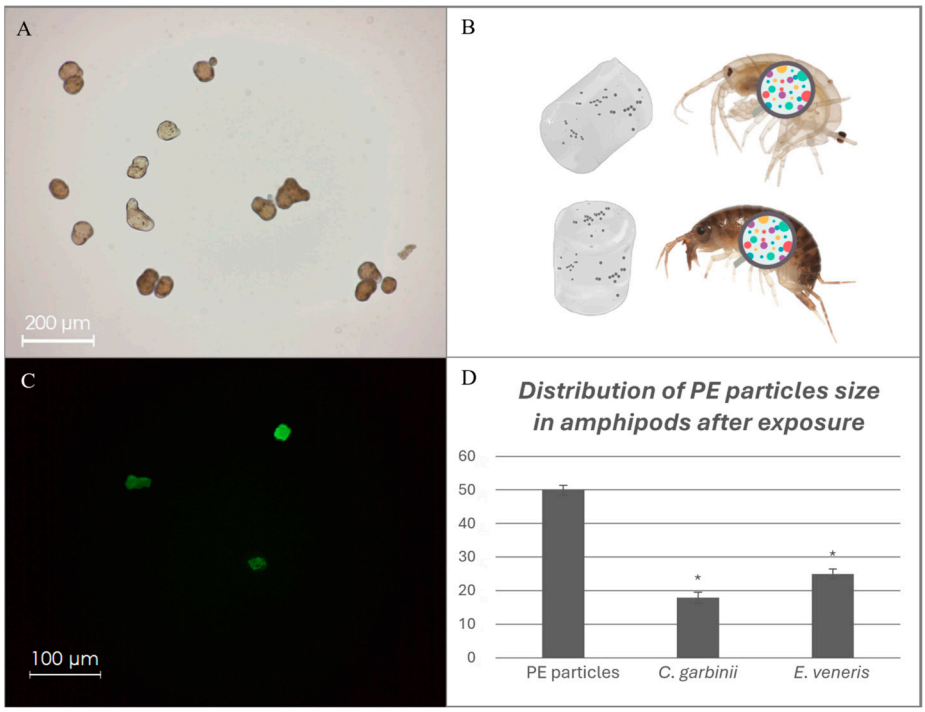

Figure 2. Polyethylene (PE) microplastic ingestion and fragmentation in amphipods. (**A**) Optical microscopy image of PE microparticles used in the exposure experiment (mean size 49.98 ± 1.43 µm); (**B**) Schematic representation of ingestion from DTs in *E. veneris* and *C. garbinii*. (**C**) Fluorescence microscopy image showing PE particles recovered from the digestive tract of exposed amphipods. (**D**) Mean particle size (± SE) of PE microplastics before exposure and after recovery from *C. garbinii* and *E. veneris*. Statistically significant differences are indicated with * ($p < 0.01$).

Prior to the main exposures conducted with PE microplastics dispersed in Triton X-100, preliminary tests were carried out to assess possible differences in the effects of more or less dispersed particles in the medium. Two negative controls (10 specimens each species) were processed: one with only water and another with water and Triton X-100, to evaluate any potential toxicity of the surfactant.

For microplastic quantification in the gut, additional individual exposures were performed using 15 exposed and 5 control beakers per run, each containing 1 organism per beaker. The setup was repeated in two independent runs, resulting in a total of 40 specimens analyzed.

Table 1 reports the exposure type and the toxicological endpoints evaluated.

2.4. Food Exposures

To assess the effects of microplastic ingestion, exposures with microplastics incorporated into special tabs, used to feed the animals, were prepared. These tabs were created following the procedure described by Kampfraath [34] for DTs (decomposition and consumption tablet, DT), with modifications as described in Ciotti et al., 2023 [32]. Briefly, the tablets containing microplastics were prepared, including the polymer (PE powder, 280 mg/L) into 4% agar with L-ascorbic acid (100 mg/L), melted in distilled water and poured into 96-well plates (200 µL/well) to solidify. The density of microplastics in each tab was estimated by weighing and microscopic counting.

For *C. garbinii*, individual exposures were performed using 1 organism + 1 DT per beaker containing 100 g of glass beads and 15 mL of dechlorinated tap water, reproducing

the semi-terrestrial substrate conditions typical of the species. For *E. veneris*, the same dietary exposure scheme was applied (1 organism + 1 DT per beaker) under aquatic conditions, with 40 g of glass beads and 150 mL of dechlorinated tap water, consistent with the setup used in the water exposure (Section 2.3). For both species, a total of 80 replicates (40 for genotoxicity and 40 for energy biomarkers) were prepared across two independent runs. For gut MP quantification, 15 exposed and 5 control beakers per run were prepared for each species (1 organism + 1 DT per beaker), and the experiment was repeated in two independent runs, yielding 40 analyzed specimens per species

The containers were maintained in a thermostatic cabinet at 20 °C with a 12 h light/12 h dark photoperiod throughout the experiment. After 24 h, the individuals were weighed and preserved in 80% ethanol. Simultaneously, a numerical estimate of the microplastic density in the tabs for each polymer was performed by counting microplastics under a microscope after weighing and pressing a section of a tab in distilled water on a microscopic slide. The number of microplastics per milligram of tab was calculated.

2.5. Extraction of Digestive Tracts for Ingested Microplastic Detection and Measurement

After each exposure period, the specimens were stored in 80% ethanol. The digestive tracts of each individual were carefully dissected under a stereomicroscope (Leica M80, Leica Microsystems, Wetzlar, Germany), then placed into a tube containing 1 mL of 30% hydrogen peroxide to break down organic material. These samples were kept at room temperature for 7 days before further processing and analysis.

The suspension obtained was filtered using black polycarbonate filters (Cyclopore Track Etched Membrane, 0.2 UM, 47 mm, Whatman^{TM} Cytiva, Marlborough, MA, USA) following the method described by Ciotti et al. [32]. The filters were then washed with 100 µL of n-hexane under a fume hood and subsequently stained with Nile red solution [5]. After staining, the filters were observed under a fluorescence microscope (Figure 2C) in the green (excitation wavelength 450–490 nm and emission wavelength 515–565 nm). The microplastic particles were photographed and measured at 10× and 20× in length (the longest dimension) and width (perpendicular to the length) using LAS software, Leica Application Suite, version 49.

Quality assurance and control (QA/QC) measures were applied during the analytical procedure to minimize contamination risk. All materials and glassware were rinsed with filtered deionized water and covered with aluminum foil. Sample preparation and filtration steps were conducted under a laminar flow hood to prevent airborne contamination. Operators wore cotton laboratory coats and nitrile gloves. All reagents and solutions were pre-filtered through glass fiber filters (0.2 µm), and procedural blanks were processed. Three negative procedural control samples were processed following the same protocol to detect potential contamination, and no microplastics were detected.

2.6. Glucose, Glycogen, and Lipid Concentration Measures

To assess the effects of microplastic (MP) exposure on metabolic processes, an analysis of energy reserves, such as glucose, glycogen, and lipids, was performed following the exposure period, both for dietary and matrix exposures.

At the end of the exposure period, each specimen was weighed, and energy reserves were evaluated according to the protocol by Foray et al. [43] and adapted by Ciotti et al. [32] for amphipod tissues. For glucose and glycogen quantification, we used the anthrone method and for quantification of lipids we used vanillin method [32]. Each specimen was individually homogenized with 200 µL of ultrapure water using a Potter–Elvehjem tissue homogenizer (Merck KGaA, Darmstadt, Germany). For glucose and glycogen analysis, absorbance was measured at 625 nm instead for lipid analysis the absorbance was measured

at 525 nm, using a photometer (Hach Lange DR1900, Hach Company, Loveland, CO, USA). All biochemical measurements were normalized to the individual dry weight of each specimen and expressed as milligrams of substance per milligram of animal dry weight (mg/mg d.w.), allowing comparison among treatments and species.

2.7. Genotoxicity Assay

The Comet assay was performed to determine the direct DNA damage caused by exposure to microplastics (MPs). It was used as a biomarker of exposure for both dietary and matrix exposures. It is a fast and sensitive tool that can be applied to various tissues and cell types and is increasingly utilized in genotoxicity testing. We performed the alkaline version of the Comet assay, which, through a gel electrophoresis-based method, quantifies DNA damage by detecting both single- and double-strand DNA breaks. *E. veneris* and *C. garbinii* specimens were exposed following the above-described setup. The Comet assay was performed after 24 h of exposure on circulant hemolymph cells (haemocytes) extracted from each specimen immediately after exposure, following the procedure described by Cosentino et al. [30]. The slides stained with ethidium bromide (20 µg/mL) were blindly scored, evaluating of 100 randomly selected nuclei each treatment, to determine the DNA damage, and photographed at 40× magnification by a Digital HD camera (Leica ICC50HD) and the software LAS V4.9. The images obtained were analyzed by the software © 2017 TriTekCorp™ CometScore (Sumerduck, VA, USA), version 2.0 measuring the Tail Moment (TM), defined as the product of the tail length and the fraction of total DNA in the tail. This widely used parameter reflects the size of migrating DNA and the number of broken DNA fragments [44]. In addition to Tail Moment, we also analyzed the percentage of DNA in the tail (%DNA in tail). It represents the proportion of total cellular DNA that has migrated from the nucleus into the comet tail during electrophoresis. It serves as a sensitive indicator of the extent of DNA strand breaks, with higher values corresponding to greater DNA damage.

2.8. Statistical Analysis

Analyses were performed to determine the significance of differences between the values of the energy reserves after the exposures and the untreated samples, and between the two exposure times; *p*-values below a confidence level of 0.05 were considered statistically significant. After checking for the data normal distribution, t-tests were carried out on pairs of groups of values resulting from the dosages of the energy reserves. When the data did not follow a normal distribution, the Mann–Whitney test was used. The data analysis was carried out using the software PAST (version 4.06).

The results of the comet assay and MPs fragmentation were presented as means ± SE and analyzed using the statistical analysis software PAST (version 4.06b). Since the data were not normally distributed (Shapiro–Wilk test), we used a nonparametric test (Kruskal–Wallis) to compare each treatment with the relative control group and considered it significant for $p \leq 0.01$.

3. Results and Discussion

3.1. Microplastics Ingestion and Fragmentation

In recent years, several studies have documented MP ingestion by amphipods in both natural environments and laboratory experiments, confirming their ability to interact with and uptake plastic particles. To further investigate this process, we conducted a short-term exposure using DTs enriched with MPs. After 24 h *C. garbinii* ingested 9.6 ± 1.23 MPs, consistent with previous findings reported for the same species (4.5 ± 0.7) exposed to larger PE particles (100 µm). The lower ingestion observed in the previous study was probably due

to the larger particle size (100 µm) used in that experiment, since bigger particles are less likely to be encountered and ingested by amphipods. This also aligns with the functional morphology of amphipod mouthparts, which are adapted to grasp and triturate fine detrital particles. (typically about 50 µm [45]), thus promoting the ingestion of smaller microplastics. The same *C. garbinii* was also used as a proxy to assess microplastic contamination in Lake Garda [25], observing a mean of 7.13 ± 2.11 microplastics per specimen analyzed, and previously, in volcanic lakes of central Italy, Lake Albano: 2.2–1.8 MP/ind, Lake Bracciano: 5–4.6 MP/ind [5]. These results are consistent across both field and laboratory studies, backing the use of this species as a bioindicator of MP contamination and as an entry point for MPs into the food chain. The numerical consistency between field and laboratory data indicates that the simulated exposure closely reflects real ecological conditions, as amphipods effectively ingest microplastics under both situations, showing comparable ingestion rates that support the realism and effectiveness of the protocol.

In the present study, *E. veneris* ingested 12.5 ± 2.83 MPs/ind when exposed to contaminated DTs. These results are comparable to those obtained in the same species exposed to fronds of the floating plant *Spirodela polyrhiza* contaminated with the same type of PE (7.6 ± 2.6) [27]. Possibly, the greater number of MPs ingested reflects the higher concentration in DTs compared to *Spirodela*. The species has been successfully used as a model organism for ecotoxicological assessments. It has been employed as a model to evaluate the toxicity of herbicides [46], plastic additives [30], microplastics ([27] and present work), metals [47] and particulate matter [29]. Being widely distributed across the circummediterranean area, it can be considered an alternative to other gammarid species not occurring in this region.

The last aspect considered was the size of particles found in the digestive tubes of both species, compared to the size of the particles provided as food. The mean particle size in DTs was 49.98 µm (±1.43). In digestive tubes, the mean size decreased to 17.9 µm (±1.69) in *C. garbinii* and 24.99 µm (±1.42) in *E. veneris* (respectively, by 64% and 50%) (Figure 2C). These results confirm what was obtained in *E. veneris* in our previous work, where using the same PE microparticles, we observed particles of a comparable size (26.2 ± 1.6 µm) in the digestive tube. These results strongly suggest that the size reduction is due to digestive fragmentation, similar to that observed in *G. duebeni* [48]. However, given the irregular shape of the MPs (Figure 2A) and a minor contribution from pre-existing small particles in the commercial PE stock cannot be excluded a contribution from selective ingestion cannot be entirely excluded. Nevertheless, in our earlier work, we observed the same phenomenon after ingestion of microplastics dispersed in water, a condition in which it is easier to select the size. While some degree of selective ingestion may occur when microplastics are freely suspended in water, this possibility is negligible for dietary exposure through DTs, where the particles are embedded within the food matrix (Figure 2B) and are not visible or separable during feeding. However, further tests will be necessary to confirm these findings using regular-shaped microplastics. The observations made for *C. garbinii* from Albano and Bracciano lakes [5] highlighted that the mean size of particles ingested in a natural context was 55 µm, and from Garda Lake [25] the size ranges between 9.09 and 706.16 µm. Therefore, this species is fully capable of ingesting larger particles and does not need to select smaller ones; we can reasonably assume that the observed reduction in size results from biofragmentation. The ability to fragment microplastics, as demonstrated in *G. duebeni* [48] has also been shown in *Hyalella azteca* [49]. In an earlier study, individuals of *H. azteca* were shown to ingest polystyrene (PS) spheres (24.5 µm in diameter) and reduce their size by approximately 25% after seven days of exposure to environmentally relevant concentrations. Additionally, alterations in the surface morphology of the PS particles were also observed [37]. Amphipods typically possess a mouthpart structure and diges-

tive system adapted to break down particulate organic matter, both through mechanical grinding and enzymatic digestion [45]. A reduction in the mean particle size of MPs was also observed in other crustacean species, such as the freshwater *Daphnia similis*, which can modify the properties of microplastics during their passage through the digestive tract [50], and in *Chironomus* sp., where a marked decrease in PS particle size was detected, accompanied by changes in oxidative stress biomarkers that indicate the potential for microplastic biofragmentation [51]. All these findings suggest that aquatic organisms play an active role in shaping the dynamics of this contaminant, and by increasing particle numbers through fragmentation, they also generate smaller, potentially more hazardous particles that contribute to microplastic pollution. From an ecological point of view, amphipods' capacity to break down microplastics into tinier particles holds significant implications. The creation of smaller debris increases the surface area for chemical attachment and microbial growth, which can raise pollutant transfer within the food web. Additionally, these smaller fragments are more likely to be ingested by lower trophic levels or pass through biological barriers, potentially amplifying the ecological and toxic impacts of microplastic pollution in freshwater environments.

3.2. Genotoxic Effects

Following the ingestion experiments, we investigated whether short-term exposure to PE microplastics could also induce genotoxic effects in the two amphipod species. Both *E. veneris* and *C. garbinii* exhibited a significant increase in DNA damage after exposure to PE MPs, whether suspended in water or provided as food (Figure 3). The increase in both Tail Moment and % tail DNA values observed through the comet assay indicates a clear genotoxic response.

Importantly, this study presents the first evidence of genotoxicity ever documented in *C. garbinii*. Although the sensitivity of *C. garbinii* to MPs has been assessed using other physiological endpoints, there was previously no data on DNA damage. The significant DNA damage observed in exposed individuals (Figure 3) emphasizes the high sensitivity of *C. garbinii* to plastic particles, confirming its potential as a model organism for future genotoxicity studies. This helps to address a significant gap in current research on the effects of MPs in semi-terrestrial amphipods, which inhabit transitional ecosystems between aquatic and terrestrial environments, and remain largely unexplored despite their ecological importance and vulnerability to plastic pollution.

Before attributing the observed effects solely to MPs, preliminary tests were conducted to assess the potential contribution of Triton X-100, used to enhance the dispersion of microplastics in water. This non-ionic surfactant was employed to ensure a homogeneous distribution of the particles within the exposure medium, preventing aggregation and sedimentation. No difference was found between treatments with and without this compound, confirming that the use of Triton X-100 does not affect genotoxicity at the concentrations applied.

Our findings align with Cosentino et al. [15], who documented genotoxic damage in *E. veneris* and *G. aequicauda*, after exposure to plastic additives (bisphenols BPA, BPF, BPS), both in somatic and germ cells, highlighting a dose–response trend in both cell types. Similarly, a remarkable genotoxic effect was reported in the hemocytes of *E. veneris* exposed to PE [27], and also documented that MPs can undergo trophic transfer from aquatic plants (*Spirodela polyrhiza*) to amphipods, highlighting how microplastic contamination can spread throughout the food web.

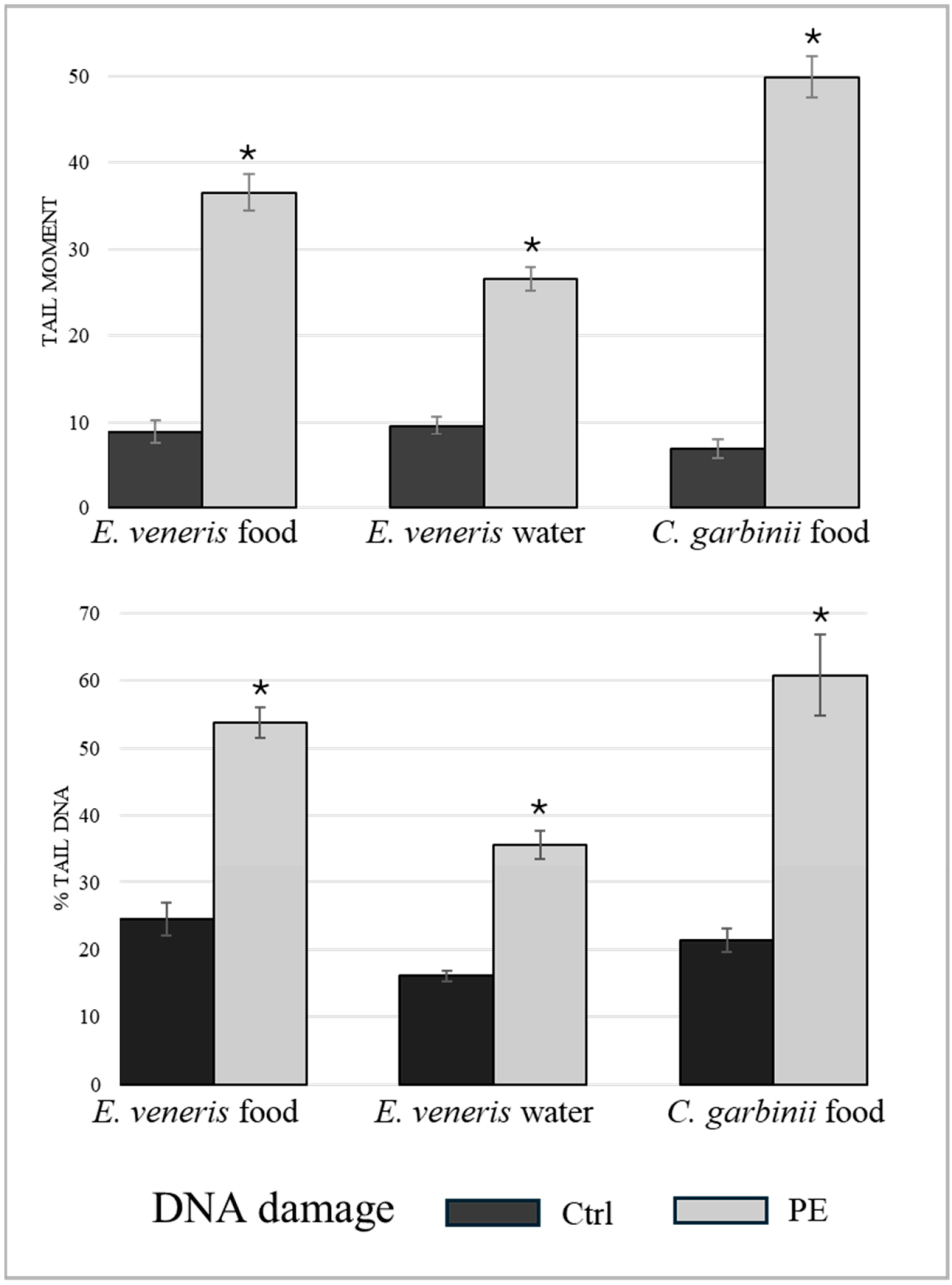

Figure 3. DNA damage expressed as Tail Moment (mean ± standard error) and % tail DNA measured by comet assay in *E. veneris* and *C. garbinii* after 24 h exposure to polyethylene (PE) microplastics via food or water, compared to unexposed controls (ctrl). Asterisks indicate statistically significant differences between PE and control ($p < 0.05$). A significant increase in DNA fragmentation was observed in all PE-treated groups, regardless of the exposure route or species, indicating the genotoxic potential of polyethylene microplastics (Kruskal–Wallis test, $p < 0.01$).

In general, the genotoxic potential of MPs on invertebrates remains poorly explored, and the findings from this study represent one of the few in amphipods. However, similar DNA damage patterns have been observed in *Neocaridina davidi* exposed to polystyrene (PS) particles [49], *Scrobicularia plana* and in *Mytilus galloprovincialis* hemocytes, exposed to polyethylene (PE) [52]. In vertebrates, PS, PET, and PE microparticles induced micronuclei and nuclear buds in *Salmo trutta* larvae, with PE causing the most pronounced damage. Additionally, environmental MPs triggered DNA fragmentation in *Oryzias latipes* [53]. Despite limited data, these data collectively suggest that MPs and their additives are bioavailable and capable of causing direct genotoxic effects. The leading hypothesis for

the observed genotoxicity involves the production of reactive oxygen species (ROS), as a result of the body's oxidative stress and inflammatory response to the plastic particles [54]. However, the exact cause of the DNA damage is still under investigation, and it remains unclear whether the damage is caused by the plastic polymer itself or the chemical additives and plasticisers used in its production.

3.3. Metabolic Biomarkers and Energy Reserves

Biomarkers related to energy allocation were used as effective endpoints assessing the impact of emerging contaminants. They reveal how these pollutants affect the energy balance of organisms, potentially damaging vital functions such as growth and reproduction [55]. In our study, a shift in energy allocation was already evident after 24 h of exposure (Figure 4). Glucose levels remained relatively stable in most treatments, except for a notable decrease in *E. veneris* exposed to MPs via DTs (Figure 4). In contrast, glycogen and lipid concentrations showed more pronounced variations across both species and conditions. Both glycogen and lipid reserves tended to decrease compared to the respective negative controls, suggesting a metabolic response likely associated with stress and the mobilization of stored energy to cope with the contamination.

These changes, while not statistically significant, indicate the mobilization of internal energy stores as a physiological reaction to the contaminant load. These results are consistent with an acute stress response, where energy reserves, particularly glycogen and later lipids, are mobilized and broken down into glucose to cope with the stress and maintain physiological functions at similar levels [56]. As a result, glucose levels remain high or increase, even as reserves begin to decline [57]. Similar trend were reported by Ciotti et al. [32]. After 24 h of exposure to microplastics via DTs, they observed a reduction in energy reserves, even if this effect tended to stabilize by 48 h, suggesting a possible metabolic compensation in response to short-term stress (Figure 4). It is important to note that the 24 h exposure period limits the assessment of metabolic endpoints, since the observed changes probably indicate initial, short-term physiological responses rather than stable long-lasting metabolic alterations. This assumption is supported by findings from long-term exposure studies, such as that of *Gammarus pulex*, where exposure over 48 days to PET MPs did not significantly affect feeding activity, energy reserves, molting, or mortality [58]. Although these results may depend on the polymer types (PET vs. PE) used for exposure [59]. According to Cong and Pham [60], MPs originating from PS, PP, and PVC pose a potential direct toxic risk to aquatic organisms due to their emission of toxic monomers. In another study, the chronic exposure (42 days) of *Hyalella azteca* to PE particles led to toxic effects on growth, reproduction, and mortality [19], while Wright et al. [61] highlighted reductions in energy reserves by as much as 50% in marine worms. This depletion appears to result from a combination of decreased feeding activity and prolonged retention of ingested material in the gut. Since effects were also observed in *E. veneris* exposed solely to the contaminated water matrix (and therefore not via food), we must consider both direct ingestion from water and the potential effects of this, similar to those observed with DTs, as well as a possible direct effect, probably mediated by inflammatory responses [54]. The results we obtained after dietary and water exposure are very similar. This supports the idea that MPs toxicity may involve multiple factors, including both direct ingestion and physiological responses, and that the type of polymer is crucial in determining its effect.

A comparison of the current findings with those reported in previous studies on amphipods and other aquatic invertebrates is summarized in Table 2, emphasizing consistent evidence of ingestion, genotoxicity, and metabolic responses to microplastic exposure.

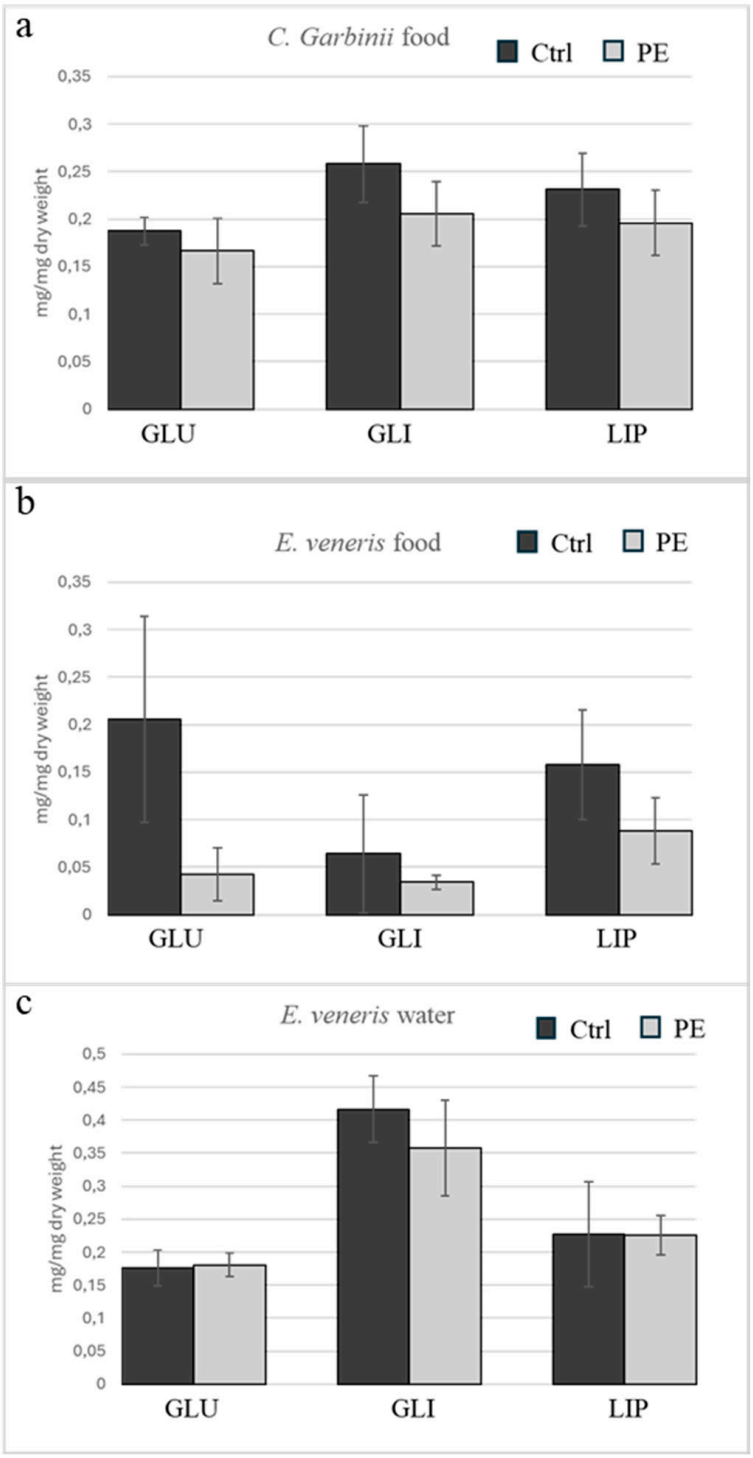

Figure 4. Mean concentrations (± standard error) of glucose (GLU), glycogen (GLI), and lipids (LIP) in *C. garbinii* and *E. veneris* after 24 h exposure to polyethylene (PE) microplastics via food (DTs) and in *E. veneris* after exposure to PE microplastics suspended in water, compared to the respective control groups (CTRL). Energy reserve concentrations (glucose, glycogen, and lipids) are expressed as mg per mg dry weight (mean ± SE). No statistically significant differences were observed between treatments (Kruskal–Wallis test, $p < 0.01$).

Table 2. Comparison of present findings with relevant studies on microplastic effects. Summary of findings from previous studies on ingestion, genotoxicity, and metabolic effects of microplastics in invertebrates and vertebrates, compared with results from the present study.

Endpoint	Study/Reference	Species	Polymer/Exposure	Duration	Main Findings in Literature	Comparison with Present Study
Ingestion & fragmentation	Mateos-Cárdenas et al. (2020) [48]	*Gammarus duebeni*	PE MPs	24–96 h	Amphipods fragmented MPs during digestion.	Confirms digestive fragmentation in *E. veneris* and *C. garbinii*.
	Iannilli et al. (2023) [27]	*E. veneris*	PE MPs via *Spirodela polyrhiza*	24 h	Ingestion and trophic transfer of MPs.	Comparable ingestion rate and fragmentation evidence.
	Ciotti et al. (2023) [32]	*C. garbinii*	PE MPs via DECOTABs	24–48 h	MP ingestion and reduction in energy reserves.	Similar ingestion levels and metabolic trends.
	Rani-Borges et al. (2023) [37]	*Hyalella azteca*	PS MPs	24 h–168 h	Fragmentation and oxidative stress increase.	Supports biofragmentation and metabolic stress.
	Rani-Borges et al. (2023) [50]	*Daphnia similis*	PS MPs	48 h–144 h	Reduction in MP size and altered surface morphology.	Similar digestive modification of MPs.
	Queiroz et al. (2024) [51]	*Chironomus sancticaroli*	PS MPs	144 h	MP fragmentation linked to oxidative stress and deformities	Suggests similar biofragmentation mechanisms.
Genotoxicity	Cosentino et al. (2022) [30]	*E. veneris*, *G. aequicauda*	Bisphenols (BPA, BPF, BPS)	24–48 h	DNA strand breaks in somatic and germ cells.	Confirms *E. veneris* sensitivity to plastic-related compounds.
	Iannilli et al. (2023) [27]	*E. veneris*	PE MPs via *Spirodela polyrhiza*	24 h	Genotoxic effects and trophic transfer.	Comparable DNA damage patterns.
	Berber (2019) [62]	*Neocaridina davidi*	PS MPs	24 h	Significant DNA strand breaks detected by Comet assay.	Consistent with PE-induced DNA damage.
	Ribeiro et al. (2017) [52]	*Scrobicularia plana*	PE MPs	14 d	DNA damage and ROS increase.	Supports genotoxic potential of PE MPs.
	Pannetier et al. (2020) [53]	*Oryzias latipes* larvae	PS, PET, PE environmental MPs	30 d	DNA damage.	Confirms strong genotoxic potential of MPs.
Metabolic/Energy biomarkers	Ciotti et al. (2023) [32]	*C. garbinii*	MPs via DECOTABs	24–48 h	Decrease in glycogen and lipid reserves.	Similar metabolic stress pattern.
	Weber et al. (2018) [58]	*Gammarus pulex*	PET MPs	48 d	No significant metabolic changes.	Suggests transient acute effects in present study.
	Au et al. (2015) [19]	*Hyalella azteca*	PE, PP MPs	42 d	Growth and reproduction impairment.	Chronic effects may extend from early responses.

Table 2. *Cont.*

Endpoint	Study/Reference	Species	Polymer/Exposure	Duration	Main Findings in Literature	Comparison with Present Study
	Wright et al. (2013) [61]	*Arenicola marina*	PVC MPs	28 d	Depletion of energy reserves (~50%) and reduced feeding.	Comparable trend of energy mobilization.
	Cong and Pham (2020) [60]	—	PS, PP, PVC MPs	—	Polymer-specific toxicity due to monomer release.	Supports polymer-dependent effects observed here.

Overall, these results show early signs of metabolic stress, indicating that energy mobilization occurs rapidly after microplastic exposure, even in the absence of statistically significant variations. This underlines the complexity of organismal responses to microplastics and the need to evaluate multiple biological levels. Although preliminary, these results highlight the need for integrated biomarker approaches to better comprehend the sublethal effects of microplastics on organisms. While the short exposure duration and the limited biological material available from individual amphipods restricted the inclusion of additional assays, the selected endpoints offered sensitive indicators of early genotoxic and metabolic responses.

4. Conclusions

This study demonstrated that *E. veneris* and *C. garbinii* are susceptible to microplastic exposure in terms of genotoxicity and metabolic responses, exhibiting genotoxic and metabolic effects within just 24 h, regardless of the exposure route. Although changes in energy biomarkers do not achieve statistical significance, the observed variations suggest a mobilization of reserves (glycogen and lipids) consistent with an acute stress response. Notably, this study provides the first evidence of DNA damage in *C. garbinii*, tested for the first time using the comet assay, which proves to be a highly sensitive tool for detecting genotoxicity. Due to its detritivorous ecology and occurrence in transitional environments, *C. garbinii* is proposed as a promising model for environmental biomonitoring.

The ingestion rate observed and the reduction in particle size observed in both species indicate digestive fragmentation of polyethylene particles, supporting the role of amphipods in the biofragmentation and trophic transfer of microplastics. Overall, the results of this study contribute valuable information to the emerging field of microplastic ecotoxicology by identifying sensitive early biological responses in freshwater amphipods.

These findings emphasize the importance of integrating multiple species and sublethal biomarkers when evaluating the biological effects of microplastics, particularly in vulnerable freshwater and transitional ecosystems. Extending exposure durations, adding complementary biochemical markers and including environmentally relevant concentrations will be essential for future ecotoxicological and biomonitoring studies.

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