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Increased assimilation efficiency and mortality rate in *Gammarus fossarum* exposed to PVC microplastics☆

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ABSTRACT

Shredder organisms play a key role in rivers by feeding and fragmenting coarse organic matter that will then be exploited by other consumers. The effects of microplastics (MPs) on *Gammarus* sp., an ubiquitous genus of freshwater amphipods, and its shredding activity have been broadly investigated. However, the potential behavioral and physiological effects of different sizes of MPs on *Gammarus* sp. remain overlooked despite the recognized influence of MP size on MP toxicity. This study investigated the effects of a 28-day exposure to four different concentrations of two size fractions of PVC-microplastics (PVC-MPs), on *Gammarus fossarum* mortality rate, feeding rate, assimilation efficiency, and expression of proteins involved in key processes. Increased mortality was observed for all treatments exposed to PVC-MPs, with higher mortality in the presence of smaller PVC-MPs at the highest concentration. No protein biomarker modulation was observed in presence of PVC-MPs, suggesting that no metabolic stress but direct physical damages of PVC-MPs might have led to the observed mortalities. No difference was observed for feeding rates, but a higher assimilation efficiency was measured for individuals exposed to PVC-MPs, regardless of the concentration. This could be due to energy reallocation towards defense mechanisms or indicate a potential shift in digestive microbiota. This study highlighted the toxicity of PVC-MPs, particularly of smaller sizes and even at relatively low concentration, for *Gammarus fossarum*. PVC-MP pollution may therefore alter the functional integrity of river ecosystems by reducing the abundance of shredder organisms and, subsequently, the process of leaf litter decomposition.

1. Introduction

Microplastics (MPs) are raising global concerns due to their widespread environmental dispersal, ranging from the deep ocean to high altitude area, the multiple and complex pathways leading to their release into the environment as well as the threat they represent for ecosystem functioning and human health (Díaz-Galiano et al., 2023; Free et al., 2014; Krause et al., 2021; Nyberg et al., 2023; Weston et al., 2020). Due to their ubiquity and small sizes (<5 mm), MPs are readily ingested by a wide range of organisms (Frias and Nash, 2019; Hurley et al., 2017; Zantis et al., 2021). Upon ingestion, MPs can cause a wide range of detrimental effects, including increased mortality, impaired growth, reproductive disturbance, metabolic dysfunction and microbiome alterations (Fackelmann et al., 2023; Funke et al., 2024; Green et al., 2019; Kratina et al., 2019; Park et al., 2023). Consequently, these adverse effects can have repercussions on the roles played by organisms in ecosystems, subsequently affecting ecosystem health and functioning (López-Rojo et al., 2020; Silva et al., 2022; Wazne et al., 2023).

The ecological functioning of most rivers is dependent on allochthonous inputs of organic matter (Baxter et al., 2005; Vannote et al., 1980). In temperate regions these inputs are mainly composed of organic matter of vegetal origins that will be processed by a set of organisms and which serves as the foundation of river food webs (Cummins, 1974; Vannote et al., 1980). Among these organisms,

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members of the shredder feeding guild play a crucial role by feeding on and fragmenting coarse vegetal organic matter, thus, making it available for other consumers (Wallace and Webster, 1996). One of the most common shredder taxa in European temperate rivers is the benthic amphipod genus *Gammarus*, which regroups over a hundred of freshwater species with wide geographical distributions that contribute significantly to leaf litter breakdown processes and constitute a key food source for various invertebrates, fish and birds (Dangles and Guerold, 2000; Gerhardt et al., 2011; Macneil et al., 1999, 1997). Due to their contributions to ecosystem functioning, their small size, their widespread distribution and their ease of maintenance under laboratory conditions, several *Gammarus* species are frequently used as model and sentinel organisms in ecotoxicological studies (Kunz et al., 2010). Consequently, the effects of MP exposure on these organisms have been extensively investigated, with multiple studies reporting the ingestion and egestion of MPs of different sizes, polymer types and shapes by *Gammarus* species (Redondo-Hasselerharm et al., 2018; Straub et al., 2017; Weber et al., 2018).

The ecotoxicological impacts of MPs on gammarids have previously been analyzed with quantification of mortality rates but also of early non-lethal responses like metabolic regulation, predatory behavior, growth, feeding and assimilation efficiency rates (Griffith et al., 2023; Kratina et al., 2019; Weber et al., 2018). These studies showed contrasting impacts of MPs on *Gammarus* species, but, overall, gammarids appeared to be less affected by MP exposure than other freshwater invertebrate model organisms like the planktonic Daphniidae (Funke et al., 2024; Jemec et al., 2016). Indeed, most studies assessing the impacts of MP exposure on gammarid survival reported no, or moderate, effects of plastic particles (Blarer and Burkhardt-Holm, 2016; Redondo-Hasselerharm et al., 2018; Straub et al., 2017). Similar results were observed on metabolic responses and feeding activities of gammarids (Erni-Cassola et al., 2024; Griffith et al., 2024; Redondo-Hasselerharm et al., 2018; Weber et al., 2018). Nevertheless, most studies using *Gammarus* species as model organisms focus on relatively commonly used polymers and on a specific MP size, whereas these characteristics are main determinants of MP toxicity. For example, particles of polyvinyl chloride (PVC), a highly produced polymer, remain understudied in ecotoxicology despite their apparent toxicity to aquatic organisms (Pelegrini et al., 2023; PlasticEurope, 2022; Zhang et al., 2023). Indeed, PVC microplastics have been, to our knowledge, taken into consideration in only one study on the *Gammarus* genus (Gerhardt et al., 2019). Moreover, this work was performed with PVC particles of a size range of 2–3 mm which was too large to be ingested and recovered in the gut of *G. fossarum* (see Table 1 in Gerhardt et al., 2019). Thus, experimental data are lacking to test the impacts of ingestible particles of an understudied and potentially more toxic polymer like PVC on survival, metabolism, and feeding activities of *Gammarus* species. In view of the wide diversity of MPs present in the environment (Gao et al., 2024; Krause et al., 2021), it is crucial to investigate the effects of different MP characteristics on organisms and their ability to maintain their ecological functions in order to better assess the environmental impacts of MP pollution.

The present study aimed to evaluate the effects of a 28-day exposure to four concentrations (0 particles/L, 500 particles/L, 5000 particles/L, and 50 000 particles/L) of microplastics PVC particles (PVC-MPs) of two different size fractions (20–63 and 125–160 μm) on *Gammarus fossarum*. First, we measured mortality rates to evaluate the toxic effects of PVC-MP exposure on gammarids. As the literature reported low mortality of gammarids exposed to MPs (Griffith et al., 2024; Straub et al., 2017), we hypothesized that the presence of PVC-MPs will not lead to increased mortality (H1). We secondly assessed the ecophysiological effects of PVC-MP exposure by measuring the metabolic response of surviving organisms after the 28-day exposure period, to detect whether the exposition disrupted key ecophysiological functions. Leveraging a recently established quantitative targeted mass spectrometry assay in *G. fossarum* (Leprêtre et al., 2022a), we used proteomic analysis to obtain information about the impacts of MP exposure on organism health status, monitored through the expression of proteins involved in osmoregulation, stress response, digestion and molting. The effects of MP exposure on gammarids proteome remain underexplored and we reported only one study (Erni-Cassola et al., 2024) showing no major proteome modulation in *G. fossarum* as assessed by comparative shotgun proteomics. Based on this previous study, we hypothesize that the presence of PVC-MPs would not stress the organisms and not result in any proteomic responses (H2). Finally, we measured the feeding rate and assimilation efficiency of gammarids exposed to PVC-MPs to investigate the potential effects of PVC-MP exposure on *Gammarus fossarum* shredding functions, associated with leaf litter processing in rivers and streams. As reported in the literature (Redondo-Hasselerharm et al., 2018; Weber et al., 2018), we hypothesized that presence of PVC-MPs will not alter the leaf decomposition process performed by the gammarids (H3). However, the three previous hypotheses were based on experimental studies performed with other plastic polymers than PVC. Thus, alternative hypotheses could be emitted with negative impacts of PVC-MP exposure on gammarid survival, metabolism and feeding activities. In this case, we expected the highest impacts of PVC-MPs for the highest concentrations and for the smallest particle size fractions, as classically reported in the literature (Berlino et al., 2021; Kukkola et al., 2021).

2. Materials and methods

2.1. Model organisms

The benthic amphipod *Gammarus fossarum* was selected due to the its common use in ecotoxicological studies as well as for the important number of ecotoxicoproteomics markers available for this species (Charnot et al., 2017; Faugere et al., 2020; Gouveia et al., 2017). *G. fossarum* individuals were collected in May 2023 from the reference population used by the INRAE lab, located in a watercress farm near Saint-Maurice-de-Rémens in Eastern France (45°57′26.2″N 5°15′43.6″E). Gammarids were sorted in the laboratory. To minimize the potential influence of organism size and suppress the potential influence of sex on results, only male adults of similar size were selected for the experiment (Alric et al., 2019; Leprêtre et al., 2022b). Once selected, the gammarids were then placed in aerated tanks containing a mix of groundwater and

Table 1
Summary of the PVC-MP exposure treatments used in the exposure experiment, the number of replicates per treatment is expressed in-between parenthesis.

Organism	PVC-MP Concentrations	Size fractions		Control (CG)
		20–63 μm (S)	125–160 μm (L)	
With gammarids (G)	**Low (L)** (500 particles/L)	**GLS** (×20)	**GLL** (×20)	**G-CG** (×20)
	High (H) (5000 particles/L)	**GHS** (×20)	**GHL** (×20)	
	Extreme (E) (50 000 particles/L)	**GEL** (×20)	**GEL** (×20)	
Without gammarids (M)	**Low (L)** (500 particles/L)	**MLS** (×5)	**MLL** (×5)	**M-CG** (×5)
	High (H) (5000 particles/L)	**MHS** (×5)	**MHL** (×5)	
	Extreme (E) (50 000 particles/L)	**MES** (×5)	**MEL** (×5)	

osmosis-filtered water (GW-OW) maintained at 14–16 °C with a 12:12 hours light-dark cycle for acclimation to laboratory conditions. Similar conditions were used for the following exposure experiment. Decaying alder leaves were harvested in the Isère department on the bank of the Bourbre stream (45°33′33.9″N 5°32′31.2″E). In order to allow microbial colonization of the leaves to enhance their palatability to the gammarids, the leaves were incubated for two weeks in ponds located in the Lyon 1 University campus experimental platform "Les étangs" (45°46′47.5″N 4°52′05.6″E). The leaves were then retrieved and rinsed with tap water before being provided *ad libitum* to gammarids for the entirety of the acclimation period. Following acclimation, gammarids were individually transferred into 400 mL glass beakers filled with 200 mL of filtered GW-OW (filtered using GF/F filter of 0.7 µm porosity) for 48 h. Pebbles previously burned at 500 °C were provided as shelters in beakers. This step allowed gut clearance and led to similar satiety levels among individuals.

2.2. Microplastic preparation

In addition to the aforementioned lack of consideration of PVC-MPs in ecotoxicological studies, this polymer type was also chosen due to its density which is higher than water (1.3–1.45 g/cm^3) allowing MPs to sink (Horton and Dixon, 2018). This property facilitates the contact of PVC-MPs with benthic organisms, whereas lighter polymers like polyethylene or polystyrene may not provide opportunity for direct physical interactions with these organisms. PVC-MPs were obtained by grinding a PVC pipe using a bench electric grinder resulting in the formation of fine particle fragments of a light grey color. The resulting PVC particles were collected and placed in a separation funnel filled with a solution of filtered $ZnCl_2$ (1.5 g/cm^3, GF/F filters) for 24 h in order to remove any bench grinder residual. The supernatant was then passed through a sieve tower consisting of 200 µm, 160 µm 125 µm, 100 µm, 80 µm, 63 µm and 20 µm sieves, with two 160 µm sieves stacked together to minimize the potential passage of larger particles (Fig. S1). The supernatant within the sieve column was abundantly rinsed with several liters of vacuum filtered ultrapure water (VF-UF) then with several liters of 96% ethanol to clean MPs from residuals of $ZnCl_2$ solution. The sieve column was then covered in aluminum foil and left to dry at room temperature, and a final dry sieving was performed. A total of 12 sub-samples were taken, from the 20–63 µm (n = 6) and 125–160 µm (n = 6) size ranges, weighing in average 0.011 and 0.012 (±0.001) mg, respectively. These size ranges were selected as they can be differentially ingested by gammarids. MPs from the 20–63 µm size range can be easily ingested due to their small size, whereas MPs from the larger 125–160 µm size range are less prone to be ingested by gammarids (Mateos-Cárdenas et al., 2020; Straub et al., 2017). Each sub-sample was sealed between a couple of glass microscope slides and MPs counting was performed with a Nikon SMZ1270 fluorescent stereo microscope (magnification ×15, exposure time 100ms). Images of all PVC-MPs present in between the microscope slides were captured and the abundances of PVC-MPs present in each sub sample were determined using the ImageJ software (See supplementary information, Figs. S2–S4). The average PVC-MP abundances of the weighed subsamples were 412 (20–63 µm) and 64 (125–160 µm, Fig. S4). This work allowed us to establish the relationships between the mass, abundance and size of the PVC-MPs that have been subsequently used for the preparation of the different PVC-MP concentrations used in the experiment. Scanning electron microscope (SEM) images on the produced PVC-MPs showed that the bench grinding process led to the formation of roughly shaped PVC-MP fragments (Fig. 1).

2.3. Exposure experiment

A 28-day long exposure experiment was conducted to assess the effects of continuous exposure to different concentrations of two size classes (20–63 µm and 125–160 µm) of PVC-MPs on *G. fossarum* by monitoring their mortality rate, their proteomic responses as well as shredding activities through feeding-rate and assimilation efficiency assessment. For facilitating comparisons with the literature, the duration of the exposure experiment was chosen to fit with several studies which assessed the impacts of MP exposure on *Gammarus* species (Blarer and Burkhardt-Holm, 2016; Redondo-Hasselerharm et al., 2018; Straub et al., 2017). A total of six stock solutions with concentrations of 500, 5000, 50 000 MP/L of the two size classes were made by mixing a previously determined mass of PVC-MPs to filtered GW-OW. A total of 150 400 mL glass beakers were filled with 200 mL of each stock solution (n = 25 beakers per treatment). An additional 25 beakers filled only with filtered GW-OW were used as control. Finally, seven different PVC-MP exposure conditions were tested: Low (500 MP/L), High (5000 MP/L) and Extreme (50 000 MP/L) concentrations of small (20–63 µm) and large (125–160 µm) PVC-MPs, plus a negative control treatment without added particles (Table 1). This concentration gradient was used to determine whether the presence of PVC-MPs impacts *G. fossarum* individuals, both in the presence of a non-environmental relevant extreme

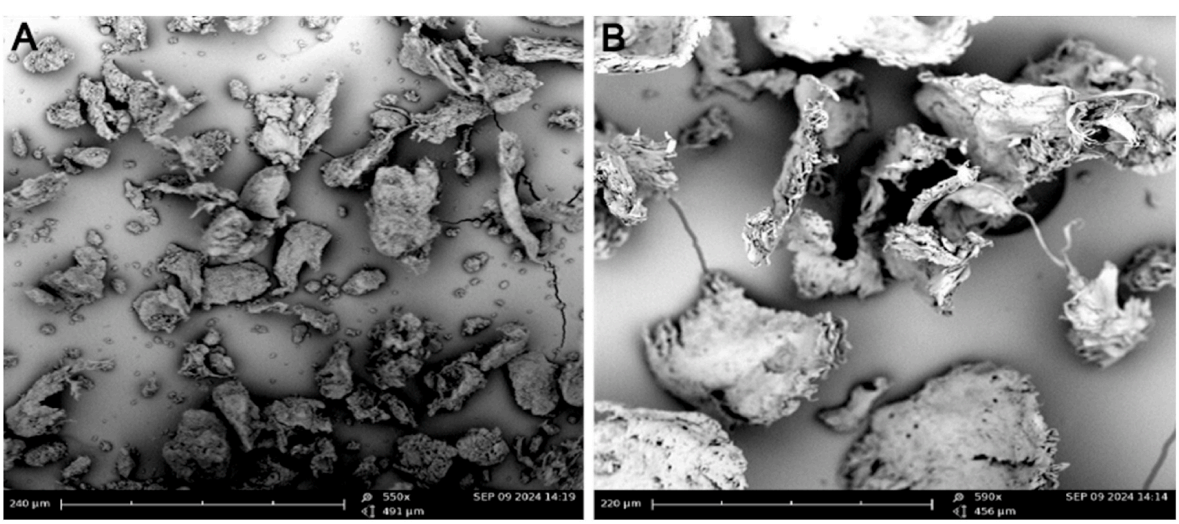

Fig. 1. SEM images showing the rough surface of PVC-MP particles of the two selected size fractions: 20–63 µm (A) and 120–165 µm (B). Images were taken using a Phenom Pure G2 scanning electron microscope. Note that the magnification was different in image (A) and image (B) in order to illustrate in details the asperities present on the edge of particles having contrasting sizes.

concentration (50 000 MP/L) and in the presence of an environmental relevant concentration, albeit elevated (500 MP/L, Devereux et al., 2022; Leslie et al., 2017). The “High” and “Extreme” concentration used in the present experiment were within the same order of magnitude as concentrations used in previous ecotoxicological studies focusing on freshwater model organisms (e.g. Griffith et al., 2024; Guimarães et al., 2021; López-Rojo et al., 2020).

Incubated *A. glutinosa* leaves disks were cut avoiding the stalk and main veins, oven dried at 50 °C for 24 h and then individually weighed in order to determine their initial dry mass. In each beaker, pebbles were added in addition to two leave disks. After the 48 h of gut clearance, 140 gammarids were individually transferred into the prepared beakers (n = 20 replicates with one individual per MP exposure treatment). Then, all beakers were lightly sealed with aluminum foil to avoid airborne MP contamination. The 5 beakers without gammarids of each MP exposure treatment were used as microbial control treatments. These microbial control treatments (i.e. MLS, MHS, MES, MLL, MHL, MEL and M-CG, Table 1) were used to determine the leaf change correction factor (CF; the effect of microbial activity and handling on the leaves disk mass loss). CF was calculated based on the formula provided by Blarer and Burkhardt-Holm (2016), where $\mathbf{C_f}$ and $\mathbf{C_i}$ represent the final and initial leaf disc dry mass of the microbial control treatments, respectively and **n** represents the number of replicates (Eq (1)).

$$CF = \frac{\sum\left(\frac{C_f}{C_i}\right)}{n} \qquad \text{Eq 1}$$

Gammarids were transferred to new beakers with similar exposure conditions on a weekly basis, to ensure a constant supply of water and food. During the entire duration of the experiment, daily observations were performed to monitor gammarid mortality and molting.

On day-28, five gammarids per treatment (n = 35) were wet weighed and then immediately shock-frozen in liquid nitrogen and stored at −80 °C for proteomic analysis. Gammarid individuals that had molted during the last 7 days were not considered for the proteomic analysis to avoid any confounding effects caused by the molting process. Following the Multiple-Reaction Monitoring assay developed by Leprêtre et al. (2022a), we measured whole-body concentrations of 39 peptide biomarkers selected to monitor the expression of 27 proteins notably involved in osmoregulation, molting, digestion and stress response (Table S1). The sample preparation for proteomic analysis was conducted following the protocol described by Leprêtre et al. (2022b), without any modification. Briefly, protein extracts obtained from individual organisms were digested using TPCK trypsin (Fisher Scientist, France). Then a mixing solution containing internal standards, consisting of 39 peptides isotopically labeled, was spiked in each sample. A peptide purification step was performed by solid phase extraction (SPE) using 60 mg hydrophilic-lipophilic balance (HLB) cartridge Oasis from Waters (Millford, MA). The protein digestion and SPE steps were performed using an automated liquid handler (Biomek NXP, Beckman Coulter). Finally, evaporation under nitrogen flow was carried out to concentrate the peptide content prior to high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) analysis. Peptide solutions were analyzed using an Agilent 1290 Infinity II HPLC system with an Agilent 6495B triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) in dynamic multiple reaction monitoring mode (dMRM) with the same parameters as described by Leprêtre et al. (2022b). The data acquisition and quantification were conducted using MassHunter software (version B.09.00; Agilent Technologies). Quantified peptides with concentration below the quantification threshold (i.e. signal to noise ratio >5, Leprêtre et al., 2022b) were removed from the analysis (Table S1). Only the peptides for which the quantification rate was greater than 50% of replicates were conserved for further analyses (i.e. peptides for which the values obtained on more than 50% of all sampled organisms were above the quantification threshold).

On day-21, prior to their transfer to new beakers, and on day-28 at the end of the experiment, wet weight measurements of the gammarids were taken. This allowed the monitoring of the individual wet mass during the last week of exposure. On day-28 the leaf disks were also recovered, oven dried at 50 °C for 24 h and weighed to determine the leaf mass loss over the last week of the exposure experiment. The pebbles were removed, rinsed and the water filtered on previously weighed GF/F filters. Filters were oven dried for 48 h and then weighed in order to obtain the dry feces mass. These measurements enabled the calculation of weekly feeding rate (FR; i.e. the daily amount of ingested leaf mass, corrected for the average autogenous leaf weight change, in relation to the wet weight of the animal, (Blarer and Burkhardt-Holm, 2016), Eq (2)) calculated as follow:

$$FR = \frac{L_i^*(CF) - L_f}{w^*t} \qquad \text{Eq 2}$$

$\mathbf{L_f}$ and $\mathbf{L_i}$ represents the final and initial dry masses of the leaf discs, whereas **w** is the average wet weight of the gammarids between the measures taken at day-21 and day-28 and **t** represents the feeding time in days (i.e. 7 days). The assimilation efficiency was also calculated (AE, Eq (3)), it corresponds to the ratio between assimilated (A, Eq (4)) and ingested (IL, Eq (5)) leaf mass (Blarer and Burkhardt-Holm, 2016). $\mathbf{F_f}$ and $\mathbf{F_i}$ are the final and initial dry masses of the filters used to measure the feces mass and $\mathbf{MP_w}$ represents the average mass of the PVC-MPs on the filter.

$$AE = \frac{A}{IL} \qquad \text{Eq 3}$$

$$A = IL - \left(F_f - F_i - MP_W\right) \qquad \text{Eq 4}$$

$$IL = L_i^*(CF) - L_f \qquad \text{Eq 5}$$

2.4. Statistical analysis

The influences of PVC-MP exposure treatments on gammarid mortality (H1) were statistically compared using survival curve comparison based on the log-rank Mantel-Haenszel test (“survdiff” function in the R studio survival package version 3.5–7; (Therneau et al., 2024)). To evaluate the potential influences of PVC-MP exposure treatments on gammarids ecotoxicological response (H2), Permutational Multivariate Analysis of Variance using distance matrices (PERMANOVA) with the Bray-Curtis dissimilarity index was used with the VEGAN package to compare total peptide contents among the different exposure treatments (Oksanen et al., 2024). PERMANOVA analyses were performed instead of MANOVA analyses because multivariate normality was not verified (Shapiro-Wilk multivariate normality tests, $p > 0.05$). Non-Metric Multidimensional Scaling (NMDS) ordination plot was used as graphical representation. PERMANOVA analyses were only conducted on peptides with a 100% quantification rate (i.e. peptides for which the obtained values were above the quantification threshold for all sampled organisms). Then for the same set of peptides, the potential differences in each peptide concentration among the different exposure treatments were assessed using one-way ANOVA followed by Tukey-HSD tests when the assumptions of normality and variance homogeneity were verified using the Shapiro-Wilk’s test and the Bartlett’s test, respectively. Non-parametric Kruskall-Wallis tests were performed, using the agricolae package, when these assumptions were not met (de Mendiburu, 2023). Similar analyses were performed for all peptides with a quantification rate between 50 and 99%, but only the potential differences in peptides concentration depending on PVC-MP size or concentration were explored, due to the exclusion of individuals from the analysis for which the detected peptides values were below the quantification threshold. Finally, the effects of PVC-MP concentration and size fractions as well as their interaction on assimilation efficiency (AE, H3) and feeding rate (FR, H3) were analyzed using Two-Way ANOVA followed by multiple comparison using Tukey's HSD tests with the agricolae package. Prior to these analyses, normality of the residuals and variance homogeneity were verified for both tested variables using the Shapiro-Wilk's test and the Levene's test, respectively. As homogeneity of variance was not verified for the FR values (Levene's test, $p = 0.041$), data were log-transformed (Ln) before statistical analyses to fit with this assumption (Levene's test on transformed data, $p > 0.05$). Replicates with aberrant AE value (i.e. $AE \geq 1$ or $AE \leq 0$) were removed from the analysis as well as replicates where the individual died or molted during the last week of experiment ($n = 10$ and $n = 23$, respectively, Table S2). To obtain a balanced design for two-way ANOVA analysis, control replicates (to which no PVC particles were added) were randomly split into two groups of 10 individuals: one group assigned to small MP treatments (CG-S) and one group to large MP treatments (CG-L). Finally, AE and FR analyses were performed on a total of 84 gammarids. One gammarid from the GLS treatment was damaged during handling and was removed from the analysis. All statistical analyses were performed using R Studio [R version 4.4.0 (2024-04-24)] (R Core Team, 2024).

3. Results

3.1. Effects of MP exposure on Gammarus fossarum mortality (H1)

An increased mortality was observed in all treatments containing MPs, the highest mortality rates were observed in the GLS and GES treatment (42% and 55% at D28, respectively), whereas zero mortality was observed in the control treatment (Fig. 2). All treatments containing MPs had mortality curves that were statistically different from the control (Mantel-Haenszel tests, $p < 0.05$ for all), except for a non-significant inferior survival in the GEL treatment ($p = 0.08$). The concentration of large MPs (i.e. GLL, GHL, GEL) had no significant effect on the survival curves of gammarids (all $p > 0.05$). In contrast, for organisms exposed to small PVC-MPs, the survival curve obtained with the Extreme concentration was statistically different from those obtained with the High concentration ($p < 0.05$), but it was comparable to the curve obtained with the Low concentration ($p > 0.05$). When considering all treatments, gammarid mortality was higher in the presence of small MPs than in the presence of larger ones ($p = 0.03$). However, this effect was largely due to a higher mortality rate of gammarids exposed to the Extreme concentration of small PVC-MPs ($p < 0.05$) because MP size had no significant effect on mortality at low and high MP concentrations (GLS vs. GLL and GHS vs. GHL, both $p > 0.05$).

3.2. Effects of MP exposure on the ecophysiology of Gammarus fossarum (H2)

Out of the 39 peptides selected for this analysis, 16 were detected and only eight had values that were above the quantification threshold for all sampled organisms (i.e. 100% quantification rate; Fig. 3, Table S1). Among the remaining eight peptides, five had a quantification rate that was greater than 50% (ELFDFADAHR, EVNGDASEAALLK, GTLAVIPVQNR, LADNIAGHVINTQEFIR and LGSNFLQIPVNCPYR; Table S1) while the last three detected peptides had quantification rates that were below 50% and were removed from the analysis (APILEGYFSK, FSESSAILR and LSAWLAACK; Table S1). The analysis was thus performed on 13 peptides, among which six were related to proteins playing a role in osmoregulatory functions (FVGLISLIDPPR, VIMVTGDHPITAK, SGQDGVPILK, LIFDNLK, LIFDNLK and EVNGDASEAALLK), two were linked to proteins associated with molt-related functions (EFWIATDHNEVR and LVLGTATYGR) and five were related to general biomarkers such as the catalase enzyme (ADPALGQAIQER, LADNIAGHVINTQEFIR and LGSNFLQIPVNCPYR), the transglutaminase enzyme (GTLAVIPVQNR) and the cellulase enzyme (ELFDFADAHR). There was no significant effect of the different exposure treatments on the overall expression rate of fully quantified peptide (100% quantification rate) in the gammarids after the 28-day exposure period (PERMANOVA, all $p > 0.05$, Fig. S5). No significant difference in peptide concentration was observed between the exposure conditions for each quantified peptide regardless of their quantification rate (One-Way ANOVA/Kruskal-Wallis tests, Fig. 3, Fig. S6).

3.3. Effects of MP exposure on Gammarus fossarum feeding rate and assimilation efficiency (H3)

MP concentration affected both FR and AE of gammarids (two-way ANOVA, $p < 0.05$ for FR and AE), whereas MP size did not have a significant effect on these two variables ($p > 0.05$ for FR and AE). When only considering PVC-MP concentration, FR was lower at extreme MP concentration than at low concentration ($p < 0.05$; Fig. 4A). However, the low, high and extreme MP concentrations did not statistically differ from the control treatment (all $p > 0.05$). The highest average FR in gammarids was measured in the GLS treatment which was statistically different from its control (CG-S) treatment ($p < 0.05$; Fig. 4A). AE of gammarids was higher in presence of MPs than without MPs, regardless of the MP concentration (all $p < 0.05$; Fig. 4B). The highest average AE was measured in the GEL treatment which was statistically different from the control (CG-L, $p < 0.05$; Fig. 4B), but all treatments containing

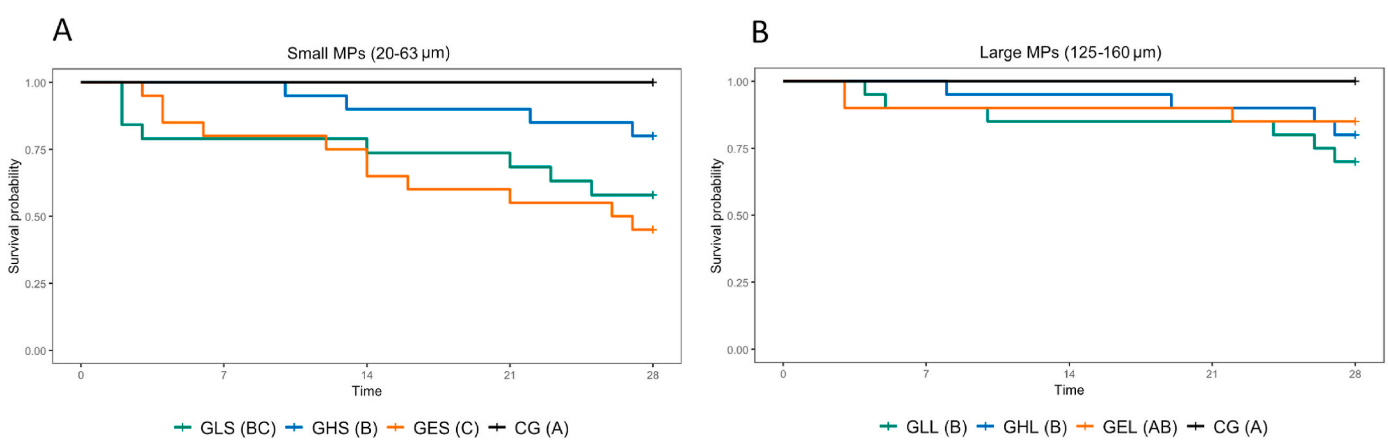

Fig. 2. Probability of gammarid survival across time depending on exposure to small (A) and large (B) PVC-MPs. GLS, GHS and GES refer to treatments where gammarids were exposed to Low (500 MP/L), High (5000 MP/L) and Extreme (50 000 MP/L) concentrations of small PVC-MPs (20–63 µm), respectively. GLL, GHL and GEL refer to treatments where gammarids were exposed to Low, High and Extreme concentrations of large PVC-MPs (125–160 µm), respectively. CG indicates control treatment (no exposure to added PVC particles). Different letters under parenthesis in the legend indicate significant differences between treatments ($p < 0.05$).

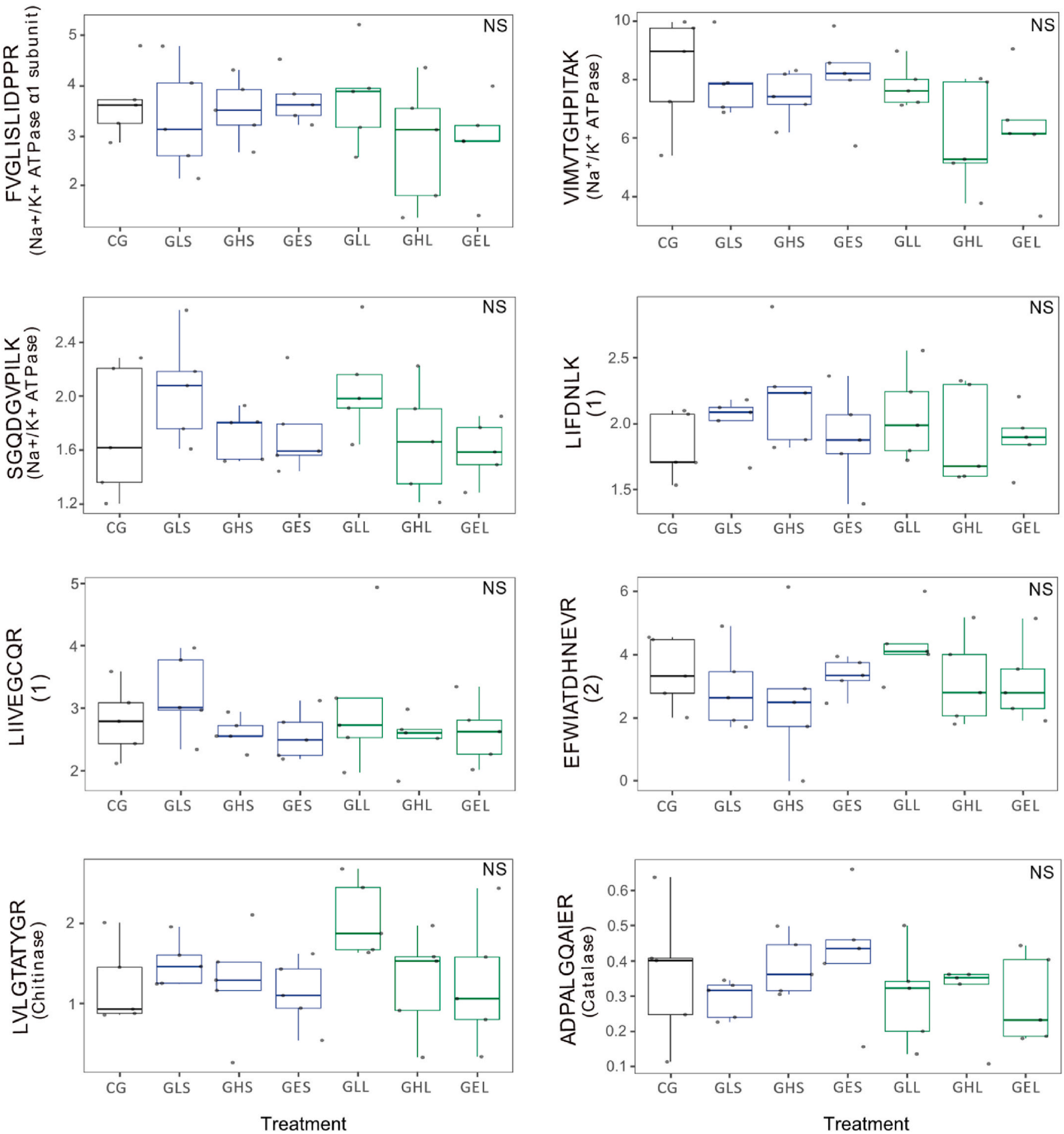

Fig. 3. Average concentration of the fully quantified peptides (100% quantification rate) and their respective function in gammarids exposed to different PVC-MPs exposure treatments. The concentrations are expressed in pmol/mg of wet individual. GLS, GHS and GES refer to treatments with gammarid exposures to Low (500 MP/L), High (5000 MP/L) and Extreme (50 000 MP/L) concentrations of small PVC-MPs (20–63 μm), respectively. GLL, GHL and GEL refer to treatments gammarid exposures to Low, High and Extreme concentrations of large PVC-MPs (125–160 μm), respectively. CG indicates control treatment (gammarids non-exposed to added particles). (1): sodium-potassium-activated adenosine triphosphatase alpha subunit D isoform; (2): Farnesoic acid methyltransferase.

MPs resulted in comparable average AE in gammarids (all $p > 0.05$). Interestingly, we detected a marginally significant result for the interaction between MP sizes and MP concentrations, indicating that the effects of MP concentration on AE tended to increase with MP size (two-way ANOVA statistical interaction between MP size and MP concentration, $p = 0.051$).

4. Discussion

4.1. PVC-MP effects on *Gammarus fossarum*

The first hypothesis stating that gammarids were tolerant to PVC-MPs (H1) was not validated. PVC-MP exposure led to increased

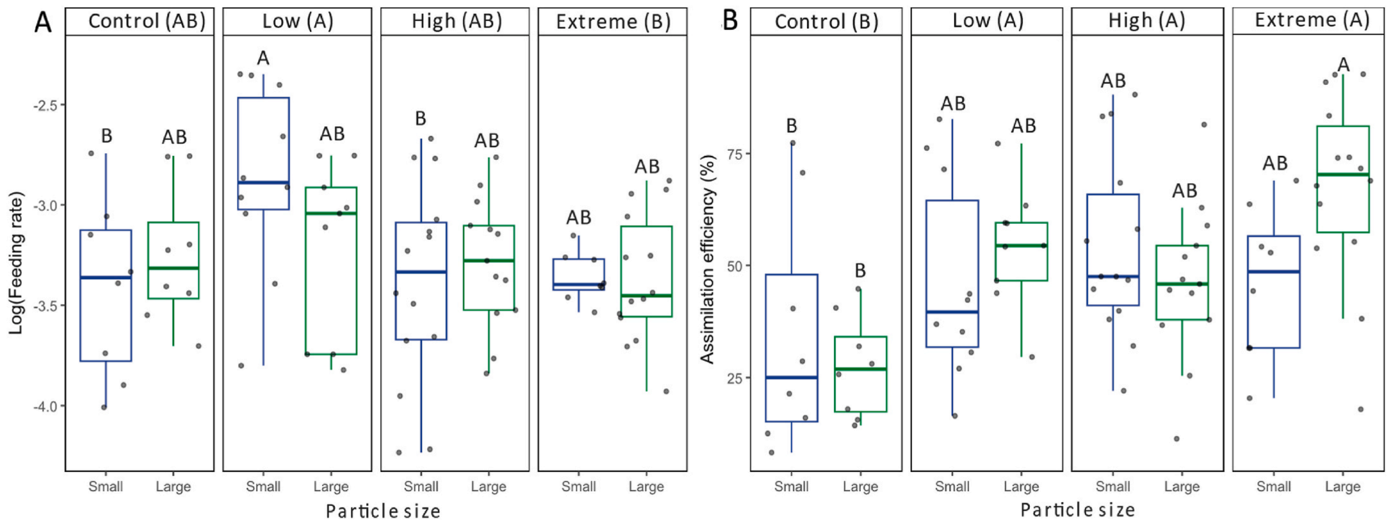

Fig. 4. Average Feeding-Rate (FR; A) and Assimilation-Efficiency (AE; B) of the gammarids measured for the last week of exposure. GLS, GHS and GES refer to treatments where gammarids were exposed to Low (500 MP/L), High (5000 MP/L) and Extreme (50 000 MP/L) concentrations of small PVC-MPs (20–63 μm), respectively. GLL, GHL and GEL refer to treatments where gammarids were exposed to Low, High and Extreme concentrations of large PVC-MPs (125–160 μm), respectively. CG indicates control treatment (non-exposed to added particles), CG-S and CG-L refer to replicates from the CG treatment that were randomly assigned to small and large MP treatment, respectively. Different letters displayed on the graphs indicate significant differences between the treatments ($p < 0.05$).

mortality in gammarids, especially in the presence of the small size fraction of MP particles. In comparison, other studies using freshwater *Gammarus* sp. as model organisms did not find an increased mortality in presence of MPs, even at similar or higher concentrations and of comparable or lower particle size ranges than MPs used in the present experiment (Redondo-Hasselerharm et al., 2018; Straub et al., 2017; Weber et al., 2018). Such dissimilarities can be explained by the use of PVC, a polymer that has never been used in the form of plastic particles <1 mm in previous studies focusing on *Gammarus* sp., as MP toxicity can depend on polymer types (Pelegrini et al., 2023; Vo and Pham, 2021). The fact that MP toxicity increases with decreasing particle size was more coherent with literature (e.g., An et al., 2021; Bobori et al., 2022; Pelegrini et al., 2023). More precisely, the small PVC-MPs used in the experiment were within a size range (20–63 μm) that was easily ingestible by the gammarids, in contrast with the larger ones (125–160 μm; Mateos-Cárdenas et al., 2020; Straub et al., 2017), suggesting that particles ingestion was a key factor in PVC-MP toxicity. It is also worth noting that comparable mortality rates were observed in gammarids exposed to relatively low (500 MP/L) and very high (50 000 MP/L) concentrations of PVC-MPs for both MP sizes. Therefore, even at low and realistic concentration, PVC-MPs were toxic for the gammarids. Although freshwater gammarids seemed to be relatively tolerant to MP exposure in comparison with other freshwater organisms, this tolerance depends strongly on MP characteristics, and particularly polymer type. This suggests that a given MP concentration is likely to have different ecotoxicological effects on gammarids depending on the type of polymer and the additives associated with these MPs, highlighting the importance of considering these characteristics in future studies assessing the toxicity of these pollutants and their subsequent effects on ecosystem functioning.

We did not find any modulation of protein involved in osmoregulation, oxidative stress responses, digestive and molting process in presence of PVC-MPs, partially validating our second hypothesis (H2). However, only a small number of proteins have been quantified and perhaps, the proteins not detected in our study could have been impacted by the presence of MPs, calling for more research. Furthermore, despite the mortality of organisms, none of the analyzed proteins were related to any physiological processes linked to mortality (e.g. apoptosis, Gismondi et al., 2017), which could explain the lack of proteomic response. Additionally, the proteomic analyses were performed on whole organisms, an approach that may lack sensitivity in comparison to targeted organ specific proteomic analysis (Degli Esposti et al., 2019; Koenig et al., 2024). Nevertheless, the lack of proteomic response was consistent with the work of Erni-Cassola et al. (2024) which showed that a 111 days exposure period to three different concentrations of irregular PET fragments (0, 0.04 and 40 mg PET g^{-1} sediment) led to minimal proteome modulations in *G. fossarum*. In this study, only 8 out of the 2519 proteins detected in male gammarids were differently expressed in individuals exposed to MPs, among which two proteins were related to immune response function. However, Erni-Cassola et al. (2024) did not report an increase in mortality in gammarids exposed to MPs, contrary to our study. MP toxicity can be related to physical force, when particles cause direct physical damage or lesions, or of chemical origin due to the additives associated with MP particles (i.e. chemical compounds added during the fabrication process of the plastic item to enhance its properties, Chouchene et al., 2023; Wiesinger et al., 2021; Wright et al., 2013). PVC is known for being commonly associated with a wide range of additives such as phthalates (phthalic acid esters) which are notorious for being highly toxic and easily leached in the environment (Henkel et al., 2022). Phthalate exposure has been linked to endocrine disrupting, reproductive and oxidative stress effects among a wide range of aquatic and terrestrial organisms (Kashara et al., 2002; Oehlmann et al., 2009; Schrank et al., 2019). Therefore, one would expect that chemical effects associated with the leaching of additives from PVC particles would increase the expression of proteins involved in oxidative stress response like the antioxidant enzyme catalase (e.g., Trestrail et al., 2020). Nevertheless, the lack of proteomic response, like catalase expression, in gammarids exposed to PVC-MPs suggests that the chemical effects associated with the leaching of additives were not responsible of the adverse effects of PVC-MPs on gammarid survival. It is thus most probable that gammarid mortality in the present experiment was the result of direct physical damages caused by the PVC particles. In this direction, PVC-MPs used in the present study were irregularly shaped and rough particles (Fig. 1, Fig. S7) which were prone to cause physical damages to the organisms (Casagrande et al., 2024). Physical damages by MPs can be external, leading to exoskeleton surface fractures in Crustaceans (Tong et al., 2024). Here, potential effects of exoskeleton surface fractures might be of relevance, particularly if they impacted gammarid pleopods (i.e. swimming limbs attached to the abdomen). By beating, pleopods increase the current of water towards gammarid gills to sustain oxygen demand (Sutcliffe, 1984). Thus, injured pleopods could largely impact the fitness of gammarids by limiting their dissolved oxygen supply. However, internal physical damages caused by MP particles ingestion that can lead to intestinal occlusion or injuries are more commonly reported in the literature (Alexiadou et al., 2019; Murray and Cowie, 2011). In the present experiment, mortality of gammarids exposed to PVC-MPs was observed for both size fractions, but the observed mortalities tended to be higher in gammarids exposed to the smallest fraction of particles. This could reflect differences in ingestion rates of MPs between the two size fractions of MPs. The gammarids exposed to smaller PVC-MPs could have easily ingested the particles, resulting in high mortality, while gammarids exposed to larger particles were only able to ingest a limited number of particles, reducing the risk of intestinal physical damage in comparison with the smallest fraction of MP particles. In conclusion, our study highlights that the impact of PVC-MPs on *Gammarus fossarum* can be highly significant and that the effects were likely mainly associated with physical impacts rather than chemical impacts on organisms. Indeed, although these results suggest that the physical impacts of PVC-MPs were the main drivers of gammarid mortality, it is not possible to totally exclude potential chemical effects due to residual PVC-MP additives. Such chemical effects would be associated with contaminants which did not have any influence on the metabolic markers tested in the study. Although it is unlikely, future experimentations based on chemically inert particles using either additives free PVC-MPs or natural inorganic particles such as clay or silica would be highly pertinent to clearly discriminate the chemical impacts from the physical impacts of MPs on gammarids. Furthermore, the investigation of the effects of MP exposure on protein modulation of the intestinal tract of *Gammarus* sp. is also a relevant perspective to explore in order to improve the understanding of the mechanisms behind the toxicity induced by the ingestion of MPs.

4.2. PVC-MP effects on *Gammarus fossarum* shredding activities

Despite increased mortality, the feeding rate (FR) of living gammarids was not affected by PVC-MPs as hypothesized in the third hypothesis (H3). Nevertheless, the assimilation efficiency (AE) of gammarids increased in presence of PVC-MPs, regardless of the concentration, invalidating (H3). The lack of PVC-MP exposure effects on the FR of *Gammarus fossarum* was not surprising as FR was the least affected variable across studies focusing on the effects of MPs on gammarids feeding activities (Blarer and Burkhardt-Holm, 2016; Redondo-Hasselerharm et al., 2018; Straub et al., 2017; Weber et al., 2018). Although significant reductions of the FR were measured in *G. duebeni celticus* when exposed to high concentration of polyethylene MPs (2 000 and 200 000 MP/L), similar findings were not observed for other species of the *Gammarus* genus like *G. pulex* even at comparable MP concentrations (Griffith et al., 2024). In comparison, AE appeared to be more sensible than FR to MP exposure especially for *G. fossarum.* For example, a significant decrease in AE was reported for this species in presence of polyamide fibers (Blarer and Burkhardt-Holm, 2016) or polymethylmethacrylate fragments (Straub et al., 2017). FR and AE are intrinsically linked variables, in part due to their quantification method that used similar metrics (Eqs (2)–(5)) and mostly due to the fact that these variables are tightly linked to the feeding activity of the gammarids. Nevertheless, if the quantity of ingested leaves (FR) is comparable in presence or absence of PVC-MPs but the assimilation of this resource (AE) is higher in gammarids exposed to PVC-MPs than in non-exposed gammarids, more energy resources and energetic reserves would be available in gammarids exposed to PVC-MPs (Sokolova et al., 2012). To test this hypothesis, a complementary experiment of 28-days duration has been performed on *Gammarus fossarum* individuals exposed to extreme concentration (50 000 MP/L, Table 1) of PVC-MPs of both size fractions and to individuals not exposed to added PVC-MPs to assess the effects of this exposure on gammarids energy storage (experimental design detailed in supplementary material). However, triglyceride reserves, the main form of energy storage in Crustaceans (Becker et al., 2013; Hervant et al., 1999), were comparable in gammarids exposed and not exposed to PVC-MPs (Fig. S8). This suggests that the higher observed AE in gammarids exposed to PVC-MPs compared with gammarids not exposed to PVC-MPs induced an energy surplus which was not allocated towards the energy reserves. This energy surplus was potentially used to maintain physiological functions, as observed in several organisms exposed to pollutants. Indeed, increased level of AE in presence of fungicide (tebuconazole), polycyclic aromatic hydrocarbons (PAH) and engineered inorganic nanoparticles have been previously reported for different terrestrial and aquatic organisms including *Gammarus fossarum* (Lüderwald et al., 2019; Van Straalen and Verweij, 1991; Zubrod et al., 2010). Increased AE were likely due to the implementation of energy costly response mechanisms in reaction to the presence of pollutants that led to potential shift in energetic allocation within the organisms (Zubrod et al., 2011, 2010). As such, the increase in AE observed in the present experiment likely results from similar processes that were induced by PVC-MPs exposure. As aforementioned, the proteomic analyses did not detect a response of gammarids to chemical exposure associated with PVC. Consequently, the reallocated energy might have potentially been used towards wound healings and pathogen control mechanisms related to the potential physical damages caused by the particles. Thus, in the present study, the reallocated energy might rather have been used towards immune response or repair mechanisms due to physical damages caused by the particles rather than towards an antioxidant response process. However, measurement of phenoloxidase activity or other immunobiological relevant biomarkers are necessary to investigate such claims. Furthermore, it cannot be ruled out that the potential presence of PVC-MPs within the digestive tract of the gammarids might have led to an increased AE, either due to physical effects or gut microbiome modifications. Particularly as MP ingestion has been linked with gut microbiome modification in several organisms (Fackelmann et al., 2023; Park et al., 2023; Varg et al., 2021). In any case, the present study highlights the toxicity of PVC-MPs towards *Gammarus fossarum* even at relatively low concentration, but mechanisms behind said toxicity remain to be further explored in order to fully encapsulate how MPs can affect these organisms which play a crucial role in the functioning of European rivers.

5. Conclusion

Drawing conclusions about the impacts of pollutants in natural environments based solely on experiments conducted under laboratory-controlled conditions is challenging. Studies focusing on MP pollution are no exceptions as MPs are generally found in the environment at low concentrations and with heterogeneous characteristics such as size, forms, weathering and polymer type, creating a "cocktail effect" that is challenging to study (Krause et al., 2021; Tibbetts et al., 2018). Although PVC is one of the world's most produced polymers, it is usually not the most represented within MPs sampled in rivers (Kukkola et al., 2021; Kumar et al., 2021; PlasticEurope, 2022). However, PVC-MPs can locally be the predominant type of polymer recovered in rivers. For example, Margenat et al. (2021) found that 53% of the recovered MPs downstream of a wastewater treatment plant were PVC, which likely originated from PVC pipes used in the operating system. This suggests that local pollution sources of MPs can lead to the overrepresentation of a specific polymer type in restricted areas, which might result in contrasting impacts on local invertebrate communities depending on the polymers contaminating the area. This underlines the importance of studying the potential effects on organisms of polymers that are not the most commonly found in rivers like PVC, polyethylene terephthalate (PET) and polyamide (PA).

Concerning *Gammarus fossarum* and other freshwater species from the genus *Gammarus*, laboratory studies showed that they are tolerant, to a certain degree, to MP exposure at environmental and non-environmental relevant concentration for several polymer types (Erni-Cassola et al., 2024; Griffith et al., 2024; Weber et al., 2018). However, the present study challenges this premise. Although the lowest concentration used in this experiment still represents a high environmental concentration and that laboratory exposure clearly differs from natural exposure, the observed gammarid mortality was significant. Especially due to the key role that *Gammarus fossarum* plays in rivers as a leaf litter shredder, increased mortality due to the presence of PVC-MPs can lead to a reduction in population sizes resulting in negative consequences on the process of leaf litter breakdown and, then, on the whole river ecosystem functioning. This study focused solely on adult male gammarids, thus omitting the potential negative effects that PVC-MPs could have on juveniles and their growth rate, as well as on females and reproduction. Indeed, the possibility to observe contrasting effects of PVC-MP exposure, or more broadly of MP exposure, on gammarids depending on the sex or the growth stages of individuals is not negligible. As a result, the presence of MPs in the environment could have a different impact on individuals depending on their own characteristics, which could emphasize the impact of MP pollution on *Gammarus fossarum* population. It is also important to consider that gammarids cannot only be restricted to their role as shredder organisms because they also predate on other invertebrates and are preyed on by multiple organisms (Kelly et al., 2002). As such, a diminution in gammarids population size could affect the whole aquatic food-web, especially considering the important biomass that gammarids can represent in certain areas (e.g. more than 25% of the total macroinvertebrate biomass, Dangles et al., 2004). The results of this study advocate for further investigations towards the impacts of relatively understudied MP polymer types and shapes on freshwater shredder organisms as their impacts may strongly differ from the ones reported with MPs which are more conventionally used in preliminary laboratory experiments such as polystyrene and polyethylene.

CRediT authorship contribution statement

Nans Barthelemy: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Florian Mermillod-Blondin:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Anabelle Espeyte:** Writing – review & editing, Methodology, Data curation. **Mohammad Wazne:** Writing – review & editing, Methodology, Formal analysis. **Frédéric Hervant:** Writing – review & editing, Methodology, Formal analysis. **Ghislaine Broillet:** Writing – review & editing, Methodology, Conceptualization. **Davide Degli-Esposti:** Writing – review & editing, Validation. **Arnaud Chaumot:** Writing – review & editing, Validation, Conceptualization. **Stefan Krause:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Laurent Simon:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Thibault Datry:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2025.126029.

Data availability

Data are available through the following link: https://github.com/NansBarthelemy/PVC_MPs_G.fossarum.

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