



Physiological and behavioural responses of aquatic organisms to microplastics and experimental warming[☆]

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ABSTRACT

Microplastics are an emerging contaminant of concern because of their potential to cause harm to aquatic biota, such as reproduction, growth, and survival, and there is a lack of knowledge about how microplastics can affect other sub-lethal responses, such as movement behaviour and respiration rates, which may have consequences for species interactions. Additionally, there is little evidence for the effects of microplastics under different climate warming scenarios. To address this knowledge gap, the effects of high-density polyethylene (HDPE) microplastics, in combination with different constant temperature regimes (10 °C, 15 °C, and 20 °C) and a fluctuating regime (10–20 °C over a 24h diel cycle) on the respiration rates, feeding rates, and movement speeds of *Gammarus pulex* and *Asellus aquaticus* were assessed. Respiration rates of *G. pulex* increased with temperature according to metabolic theory, but there was no evidence for increased respiration rates of *A. aquaticus* at higher temperatures. Overall, the respiration rates and movement speeds of *G. pulex* were higher than *A. aquaticus* but there was no evidence that microplastics independently, or in combination with experimental warming, influenced any of the responses tested. There is increasing evidence that some microplastic particles may not be harmful to aquatic biota, and the findings presented in this study indicated that further evidence about the effects of different microplastic types, in combination with other human-induced pressures, is required to better understand the hazards and risks associated with microplastic particles in the environment.

1. Introduction

A growing body of evidence suggests that the ingestion of microplastics can lead to adverse health effects on freshwater organisms. Microplastics (particle size ranging between 1 µm–5 mm) can negatively impact several vital rates of freshwater organisms, including feeding and metabolic rates (Kratina et al., 2019; Rist et al., 2017; Watts et al., 2016), growth and reproduction (Redondo-Hasselerharm et al., 2018), or survival (Boyer et al., 2020; Ziajahromi et al., 2018). Microplastic pollution can also induce other sub-lethal responses of aquatic organisms, including changes in movement behaviour. For instance, microplastics have been recently shown to negatively affect the locomotory activity of copepods (Suwaki et al., 2020), cladocerans (Pan et al., 2022) and hermit crabs (Cunningham et al., 2021), and social behaviour of fish (Mattsson et al., 2015), likely through mechanisms such as reduced feeding (Ogonowski et al., 2016), nutritional deficiencies (Blarer and Burkhardt-Holm, 2016; Wright et al., 2013), or blockages in the digestive tract causing immobilization (Au et al., 2015). Such changes in movement behaviour could lead to changes in species interactions, because movement behaviour determines the ability of animals to feed

and reproduce, modifying processes such as predator-prey interactions (Crowl and Covich, 1990; Peckarsky et al., 1993). However, the effects of microplastics on sub-lethal responses that may have implications for key ecological interactions, such as those between predators and their prey, are still poorly understood.

Microplastic pollution will affect ecological communities in the context of ongoing and future climate change, which includes rising mean annual surface temperatures (IPCC et al., 2023; Webb et al., 2008), increases in temperature fluctuations (Schär et al., 2004) and extreme temperature events such as heatwaves (Jentsch et al., 2007). Biological responses to climate warming are complex due to the multifaceted nature of changing temperature regimes, yet the majority of previous studies have focused solely on increases in mean constant temperatures (Cabrerizo and Maranon, 2021). These studies have provided valuable insights into how warming can increase individual metabolism up to a thermal optimum, and in turn influence key biological processes such as feeding rates, developmental rate, and behaviour (Brown et al., 2004; Rall et al., 2012). However, many organisms currently face increases in thermal fluctuations in their natural environment, ranging from diel fluctuations over a 24-h period to

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

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seasonal fluctuations across a year (Dell et al., 2011; Steinberg et al., 2021). More variable thermal regimes can be more stressful for ectotherms than raises in constant mean temperature, depending on the magnitude of temperature fluctuation (Colinet et al., 2015). Therefore, experimental comparisons between mean constant temperature increases and fluctuating thermal regimes are important for providing ecologically realistic assessments of the broader climate change impacts on ecological communities.

Microplastic body burden could be higher as environmental temperatures increase because warming enhances the metabolic demands and feeding rates of ectotherms, and subsequently the uptake rates of microplastic particles. However, only few studies have addressed how microplastics and climate warming interactively influence sub-lethal responses of freshwater organisms. So far, there is experimental evidence showing that rising temperatures can enhance the negative effects of microplastics on metabolic rates of freshwater detritivores (Kratina et al., 2019), and the survival rates of different cladoceran species (Jaikumar et al., 2018). In contrast to this, warmer temperatures alleviated the negative effects of microplastics on larval growth, development, and adult weight of chironomids (Silva et al., 2022). Other studies have shown additive effects, whereby freshwater organisms respond more negatively to warming, whilst microplastics only have minor effects (Sadler et al., 2019; Scherer et al., 2017). These discrepancies could be due to different species sensitivities to multiple pressures, or the different responses tested. For example, reduced metabolic rates caused by increasing microplastic pollution in a warmer environment (Kratina et al., 2019) could result in reduced physical activity or movement because the two traits are tightly linked (Beamish, 1978; Brett and Groves, 1979). Whether and how the interactive effects of microplastic pollution and warming on vital rates of animals can cascade to changes in species interactions is still poorly understood.

To address this gap in microplastic research, this study experimentally tested the effects of constant temperature (10 °C, 15 °C, 20 °C), a fluctuating thermal regime, and the absence/presence of microplastics (low and high concentrations) on the respiration rates, feeding rates and movement speed of two widely distributed freshwater detritivores, *Gammarus pulex* and *Asellus aquaticus*. A fluctuating thermal regime (ranging from 10 to 20 °C over a 24h period) was included as an additional temperature treatment to determine the effects of microplastics on sub-lethal responses of *G. pulex* and *A. aquaticus* under more thermally stressful climatic scenarios, in comparison with the constant ambient treatment (15 °C). This study tested the following hypotheses: (1) that the respiration and feeding rates of *G. pulex* and *A. aquaticus* would be higher at warmer temperatures; (2), that organisms exposed to the fluctuating temperature regime would have higher respiration and feeding rates than those with the same mean temperature (15 °C); (3) exposure to high microplastic concentrations would suppress the respiration and feeding rates of *G. pulex* and *A. aquaticus*; and (4) that warming would thus enhance the negative effects of high microplastic concentrations on freshwater organisms.

2. Materials and methods

2.1. Model organisms and their maintenance

Gammarus pulex and *Asellus aquaticus* are ubiquitous benthic shredders found throughout freshwater habitats in Europe (Hynes, 1955; Hynes et al., 1960; Lafuente et al., 2022). They play important ecological roles in breaking down coarse particulate matter and aiding the transfer of nutrients to higher trophic levels (Wallace et al., 1997). *Gammarus pulex* generally move short distances by swimming, whereby *A. aquaticus* move through the benthic habitat by crawling. Both species are commonly used for testing the effects of different stressors under laboratory conditions (Kratina et al., 2019; Redondo-Hasselerharm et al., 2018; Yardy and Callaghan, 2020).

Several hundred *G. pulex* and *A. aquaticus* were collected from the

source of the Hogsmill river in South-West London in November 2022 by kick sampling. The organisms were transported to the laboratory and visually sorted, removing individuals smaller than 1 cm or egg-bearing females (Kratina et al., 2019). Gammarids infected with Acanthocephalan parasites were also removed, since these parasites have been shown to alter amphipod behaviour (Labaude et al., 2015; Tain et al., 2006). The remaining organisms were housed in a temperature controlled incubator (15 °C, 12h:12h light:dark photoperiod) in two aerated glass beakers (2 L), each containing 1.5 L filtered river water (25 µm mesh size). They were fed *ad libitum* with oak leaves (*Quercus robur*) and left to acclimatise for 7 days prior to any experimentation.

2.2. Microplastic and temperature exposure

For the microplastics exposure, commercially available high-density polyethylene (HDPE) pellets (3–4 mm) were purchased from an online retailer (Poli Plastic Pellets). HDPE is widely used in the packaging industry and is amongst one of the most commonly produced plastic polymers in Europe (PlasticsEurope, 2022). The HDPE pellets were ground into smaller microplastic particles using a spice grinder to obtain a final size range of 12.5–500 µm (median 87.5 µm, IQR: 62.5–150 µm). It has previously been reported that freshwater macroinvertebrates are able to ingest microplastic particles within this size range (Mateos-Cardenas et al., 2020; Yardy and Callaghan, 2020; Yildiz et al., 2022). For full details on the production and characterisation of the microplastic particles, and determination of microplastic concentrations see (Marchant et al., 2023).

Experimental microcosms containing 50 ml filtered river water were each inoculated with microplastic particles and three pre-weighed oak leaf discs (see below for more details) prior to the start of the experiment. Two different microplastic concentrations were used: 1 particle ml⁻¹ and 23 particles ml⁻¹ representing 'low' and 'high' concentrations respectively, following Marchant et al. (2023). No microplastic 'controls' were also included in the experimental design. Low and high microplastic treatments were chosen based on concentrations of microplastics that are readily found in the environment, and those that have been shown to have negative effects on ecotoxicological endpoints of several aquatic taxa (Jones et al., 2019). Microplastic stock solutions were prepared by calculating the required particle weight to achieve concentrations of 1 and 23 particles ml⁻¹. The concentrations of both the low and high stock concentrations were verified by counting the microplastic particles under a microscope using a haemocytometer. Once set up, the microcosms were transferred to temperature-controlled incubators (Lovibond TS255, Germany) set to the target constant experimental temperatures of 10, 15 and 20 °C and fluctuating cabinets (Mettmert IPP^{plus}, Germany) were used for the fluctuating temperature treatment ranging between 10 and 20 °C over a 24h diel cycle (Fig. S1), totalling 24 experimental treatment combinations. The microcosms were left for a conditioning period of 3 days to allow for better distribution of the microplastic particles (Green et al., 2017) and for microbial colonisation of the leaf discs, which increases palatability for benthic shredders (Bärlocher and Kendrick, 1975). Following the 3-day conditioning period, *G. pulex* and *A. aquaticus* individuals (one per microcosm) were transferred to the microcosms and left for a 2-week exposure period to the experimental treatments. Animal-free microcosms (n = 5) containing 50 ml filtered stream water and three pre-weighed leaf discs were also set-up at each temperature treatment to correct for microbial decomposition. Each treatment combination was replicated 5 times, where organisms were exposed to the experimental treatments in individual microcosms. This resulted in a total of 120 experimental microcosms and associated organisms.

2.3. Respiration rates

Respiration rates (µmol O₂ h⁻¹) of *G. pulex* and *A. aquaticus* individuals exposed to each treatment combination were measured after

the 2-week exposure period using constant volume respirometry. Organisms from each treatment combination were placed individually in 2-ml vials (Presens, SV-PSt5) pre-fitted with an optode oxygen sensor and half filled with filtered stream water. The vials were left to acclimatise at the respective measurement temperature for 5 min before being topped up with filtered stream water and sealed with screw top caps. Care was taken to ensure that there were no air bubbles within the vials, as introduction of air bubbles could add variability to oxygen measurements. The vials were placed on 24-well SensorDish readers (PreSens SDR Oxodish, AS1 Ltd, Regensburg, Germany), where dissolved oxygen was non-invasively measured in 15-s intervals until there was a measurable decrease in oxygen concentration (between 3 and 4 h depending on the experimental temperature regime). Up to 960 measurements of oxygen consumption were made for each individual organism, depending on the measurement duration. Animal free controls ($n = 5$) were also set up in the same way for each treatment combination to account for background decreases in oxygen concentration through microbial respiration. The animals were able to move freely in the vials, therefore making it unlikely that an oxygen gradient would form. For the fluctuating temperature treatment, respiration rates were measured at the mean temperature of the diurnal fluctuation (15 °C).

2.4. Movement rates

The movement activity of *G. pulex* and *A. aquaticus* was recorded following the 2-week exposure period. Individuals were placed in glass Petri dishes (diameter 8 cm, height, 1 cm), each containing 50 ml filtered stream water. The organisms were left to acclimatise for 5 min within a temperature-controlled incubator which was set to the target measurement temperature. Video footage was recorded using high-definition cameras (Raspberry Pi 3 with Camera Module v2) positioned 10 cm above the bottom of the Petri dishes. The movement activity of animals was recorded for 30 min, and the animals were left undisturbed throughout the duration of the recordings. Although *G. pulex* and *A. aquaticus* are both commonly found beneath stones or hidden in the detritus, shelters were not provided for the animals as this would have made it challenging to measure movement rates if the animals remained hidden during the recordings. Movement activity was measured as the mean swimming speeds of individual animals. These responses were calculated by first extracting the trajectories from the videos using a modified software written in Python based on the “opencv” library (Bradski, 2000), which provides information based on “tracked particles”. Trajectories for individual animals were grouped based on particles within a defined region of interest ($n = 4$ per video), where each region of interest represents an individual Petri dish housing one organism. Where two sets of trajectories were recorded in a single frame, the trajectories furthest away from the centre of the Petri dishes were removed because these were likely to be animal reflections. Multiple speed measurements were made during the 30-min recordings, where speed was measured based on how far the centre of each individual organism moved every 1/10 of a second, corresponding to three frames captured in that time. This resulted in multiple instantaneous speed measurements per individual organism. Mean swimming speeds (mm s^{-1}) were then calculated from these instantaneous speeds along the organism's entire trajectory. To minimize the potential impact of stress-related movement on the analysis, only the 75th percentile of the movement data was analysed. By using this approach, the analysis captured more typical movement patterns and reduced the influence of extreme or potentially erratic movements that could have been a result of stress.

2.5. Feeding rates

Feeding rates of *G. pulex* and *A. aquaticus* individuals were quantified during the 2-week experimental exposure period to microplastic and warming treatment combinations. Each animal was provided with three

leaf discs (15 mm each), cut from whole oak leaves (*Quercus robur*) using a metal cork borer (Kratina et al., 2019). Leaf discs were dried and weighed to the nearest 0.01 mg using an ultra-micro-balance and conditioned in filtered stream water 3 days to prevent them floating on the surface during the experiment. After the exposure period, the leaf discs were removed from the microcosms and rinsed with de-ionised water before being individually wrapped in foil and left to oven-dry at 60 °C for 48 h. The leaf discs were then re-weighed to the nearest 0.01 mg using an ultra-micro-balance.

2.6. Statistical analysis

Linear models (LM) were used to assess the effects of microplastic concentrations and constant temperature regimes (10, 15, and 20 °C) on the respiration rates, feeding rates and movement activity of *G. pulex* and *A. aquaticus*. Separate LM models were also constructed to determine whether there was a difference in sub-lethal responses between the constant ambient (15 °C) and fluctuating temperature regimes (with a mean of 15 °C). Organism (two levels: *G. pulex* and *A. aquaticus*), microplastic concentration (three levels: none, low and high) and constant temperature regime (three levels: 10 °C, 15 °C, and 20 °C) as well as their interactions were included as fixed effects. In the constant vs fluctuating model, temperature regime had two levels: constant (15 °C) and fluctuating (around a mean of 15 °C). Significance of the fixed effects were estimated by extracting p-values. All statistical analyses were carried out in R Statistical Software v4.0.2 (R Development Core Team, 2021).

3. Results

3.1. Respiration rates

Constant increase in the mean water temperature had a strong influence on the respiration rates of *G. pulex* and *A. aquaticus* (LM; $F_{(2, 72)} = 999.96$; $p < 0.001$; Fig. 1a and c) and this effect differed between the two species (LM; $F_{(2, 72)} = 6.018$; $p < 0.001$). Generally, the respiration rates of *G. pulex* increased with temperature, with the highest rates at the warmest temperature of 20 °C (Fig. 1a). However, the respiration rates of *A. aquaticus* were relatively high at 10 °C and 20 °C, with the lowest respiration rates at 15 °C (Fig. 1c). Overall, there was little evidence for the independent effect of microplastics (LM; $F_{(2, 72)} = 2.047$, $p = 0.137$), the two-way interactions between microplastics and constant temperature increase (LM; $F_{(2, 72)} = 2.384$, $p = 0.059$), organism and microplastics (LM; $F_{(2, 72)} = 1.844$; $p = 0.166$), or the three-way interaction between organism, constant temperature increase, and microplastics (LM; $F_{(4, 72)} = 2.400$; $p = 0.057$; Table S1) on the respiration rates.

There was no evidence for a difference in the respiration rates of either *G. pulex* or *A. aquaticus* between the constant ambient (15 °C) and fluctuating temperature treatment (LM; $F_{(1, 47)} = 0.019$, $p = 0.892$; Fig. 1b and d). Similarly, there was little evidence of an effect of microplastic concentration (LM; $F_{(2, 47)} = 0.910$, $p = 0.409$), the two-way interactions between microplastics and constant temperature increase (LM; $F_{(2, 47)} = 0.353$, $p = 0.704$), organism and microplastics (LM; $F_{(2, 47)} = 0.616$; $p = 0.544$), or the three-way interaction between organism, constant temperature increase, and microplastics (LM; $F_{(2, 47)} = 1.437$; $p = 0.247$; Table S2) on the respiration rates.

3.2. Movement activity

Mean movement speeds of *G. pulex* were higher than mean movement speeds of *A. aquaticus* (LM; $F_{(1, 66)} = 85.236$, $p < 0.001$). However, there was no evidence for a difference in the mean movement speeds of either *G. pulex* or *A. aquaticus* across constant temperature regimes (LM; $F_{(2, 66)} = 0.504$, $p = 0.606$; Fig. 2a and c). Additionally there was little evidence for the effect of microplastic concentration (LM; $F_{(2, 66)} = 1.223$, $p = 0.301$), or the two-way interactions between constant water

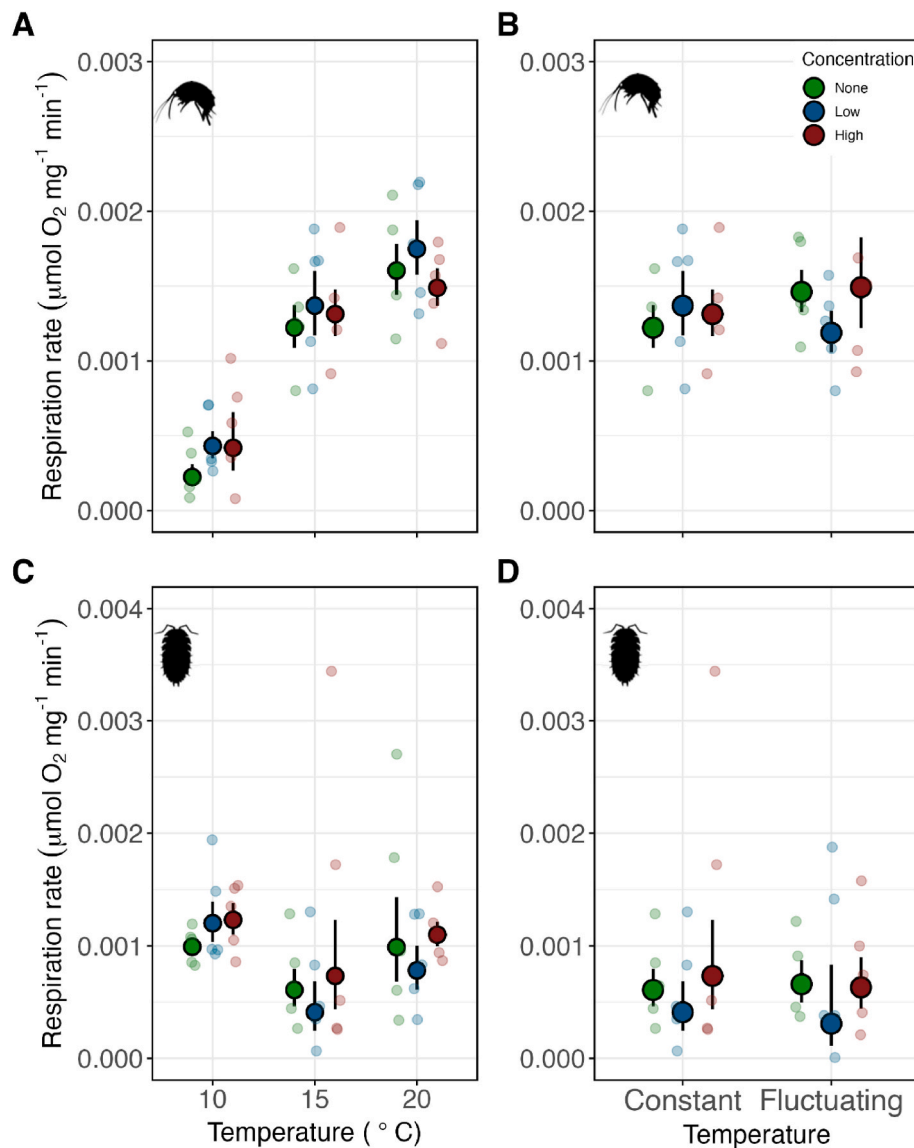


Fig. 1. Mass-corrected respiration rates of *G. pulex* and *A. aquaticus* following pre-exposure to microplastics and experimental warming. A) A) and C) Comparison between mass specific respiration rates at constant 10, 15 and 20 °C and B) and D) comparison between the pre-exposure to microplastics at the constant ambient (15 °C) and fluctuating temperature regime that ranged between 10 and 20 °C over a 24h diel cycle, with measurements made at the mean daily temperature of 15 °C. Bold data points are mean respiration rates of five replicated animals \pm 1 SE and shaded data points are respiration rates of individual animals (n = 5 per treatment combination) following backwards ln-transformation. Error bars are asymmetrical due to the non-linear transformation from the log-scale used in the analysis. Point and line colours represent microplastic concentrations (none, low, and high). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

temperature and microplastic concentration (LM; $F_{(4, 66)} = 1.256$, $p = 0.296$), organism and temperature (LM; $F_{(2, 66)} = 0.044$, $p = 0.957$), organism and microplastic concentration (LM; $F_{(2, 66)} = 0.186$, $p = 0.830$), or the three-way interaction between organism, temperature, and microplastics (LM; $F_{(4, 66)} = 1.498$, $p = 0.213$; Table S3), on the movement speeds of the model animals.

Mean movement speeds of *G. pulex* were higher than mean movement speeds of *A. aquaticus* LME under the constant ambient (15 °C) and fluctuating temperature treatments (LM; $F_{(1, 42)} = 99.786$, $p < 0.001$). There was no evidence for a difference in the movement speeds of *G. pulex* and *A. aquaticus* between constant ambient and fluctuating temperature regime (LM; $F_{(1, 42)} = 0.918$, $p = 0.344$; Fig. 1b and d). There was weak evidence for the effect of microplastic concentration on the swimming speeds of either *G. pulex* or *A. aquaticus* (LM; $F_{(2, 42)} = 3.413$, $p = 0.041$), but no evidence for a two-way interaction between microplastic concentration and temperature (LM; $F_{(2, 42)} = 0.142$, $p =$

0.868), organism and temperature (LM; $F_{(1, 42)} = 1.019$, $p = 0.319$), or organism and microplastic concentration (LM; $F_{(2, 42)} = 3.024$, $p = 0.059$), or a three-way interaction between organism, temperature, and microplastics (LME; $F_{(2, 42)} = 5.539$, $p = 0.072$; Table S4).

3.3. Feeding rates

The effects of constant experimental warming on feeding rates differed between *G. pulex* and *A. aquaticus* (LME; $F_{(2, 66)} = 85.236$, $p < 0.001$; Fig. 3a and c). There was a trend towards increased feeding rates of *A. aquaticus* with increasing temperature, but the feeding rates of *G. pulex* were similar across all experimental temperatures (Fig. 3a and c). There was no effect of microplastic concentration (LM; $F_{(2, 66)} = 1.223$, $p = 0.301$) or the two-way interactions between constant water temperature and microplastic concentration (LM; $F_{(4, 66)} = 1.256$, $p = 0.296$) or the three-way interaction between organism, constant

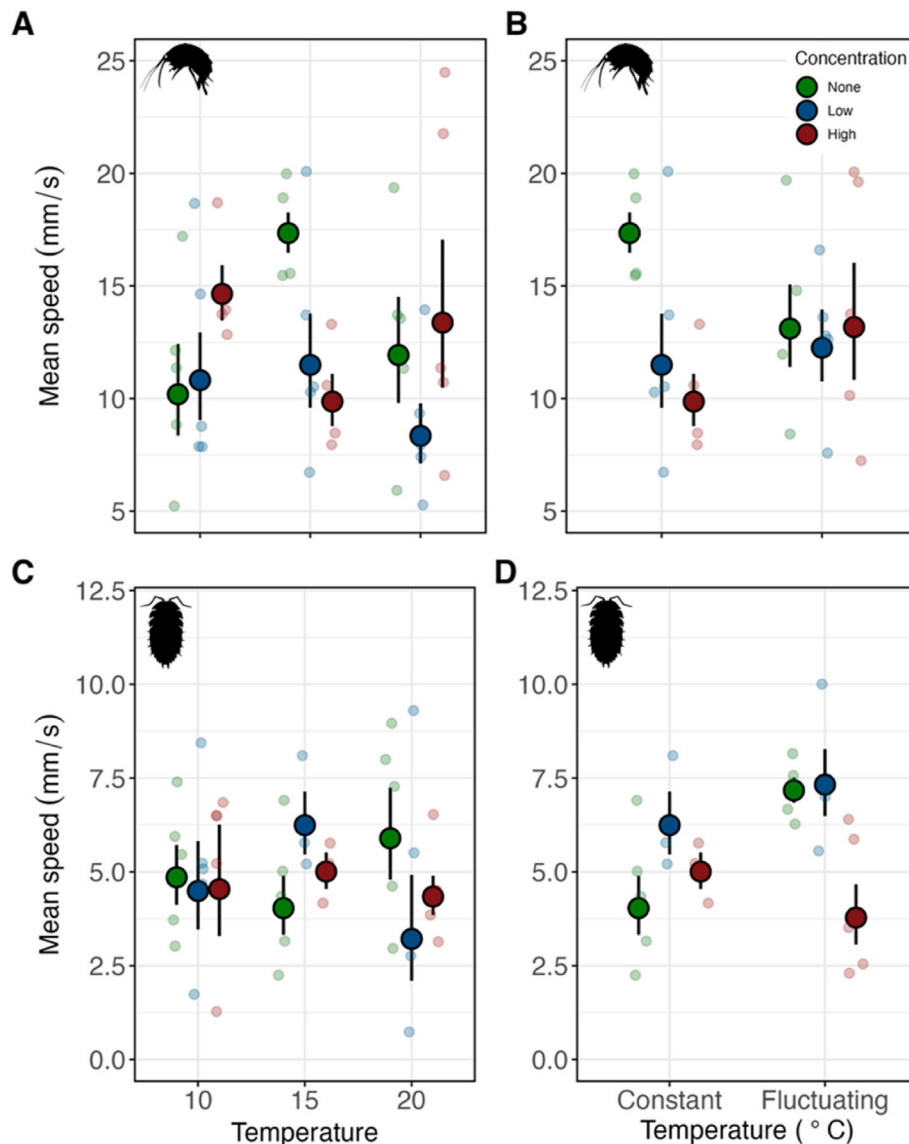


Fig. 2. Mean swimming speeds of *G. pulex* and *A. aquaticus* individuals measured after a pre-exposure to microplastics and experimental warming. A) and C) Comparison between mean swimming speeds at constant 10, 15, and 20 °C and B) and D) comparison between the pre-exposure to microplastics at the constant ambient (15 °C) and fluctuating temperature regime that ranged between 10 and 20 °C over a 24h diel cycle, with measurements made at the mean daily temperature of 15 °C. Bold data points are mean swimming speeds of five organisms \pm 1SE and shaded data points show swimming speeds of individual organisms ($n = 5$ per treatment combination) following backwards ln-transformation. Error bars are asymmetrical due to the non-linear transformation from the log-scale used in the analysis. Point and line colours represent microplastic concentrations (none, low, and high). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

temperature increase, and microplastics (LM; $F_{(4, 66)} = 1.498$; $p = 0.213$) on the feeding rates of the model organisms.

Feeding rates of *G. pulex* and *A. aquaticus* differed between the constant ambient (15 °C) and fluctuating temperature exposures (LM; $F_{(1, 43)} = 17.092$, $p < 0.001$; Fig. 3b and d). The feeding rates of *G. pulex* were lower during the fluctuating temperature regime (Fig. 3b), whereas the feeding rates of *A. aquaticus* were similar between the constant ambient and fluctuating regime (Fig. 3d). There was no effect of microplastic concentration (LM; $F_{(2, 43)} = 0.398$, $p = 0.674$) or the two-way interactions between temperature (constant and fluctuating regime) and microplastics (LM; $F_{(2, 43)} = 0.435$, $p = 0.650$), organism and microplastics (LM; $F_{(2, 43)} = 1.754$, $p = 0.185$), or the three-way interaction between organism, temperature, and microplastics (LM; $F_{(2, 43)} = 1.111$, $p = 0.338$; Table S6) on the feeding rates of the model organisms.

4. Discussion

Currently, little is known about the effects of microplastics on physiological (e.g., respiration rates) and behavioural responses of freshwater biota, which may have implications for species interactions and community structure. Therefore, this study experimentally tested the effects of HDPE microplastics and different warming scenarios on the respiration rates, feeding rates and swimming behaviour of two important freshwater macroinvertebrates, *G. pulex* and *A. aquaticus*. For all of the sub-lethal responses tested in this study, exposure to microplastics had negligible ecological effects at either of the concentrations tested, or in combination with experimental warming. This is an intriguing result, considering that the microplastic concentrations used in this study are known to cause effects on ecotoxicological endpoints of freshwater organisms under laboratory conditions (Jones et al., 2019), and were within the ingestible size range for both *G. pulex* and *A. aquaticus*.

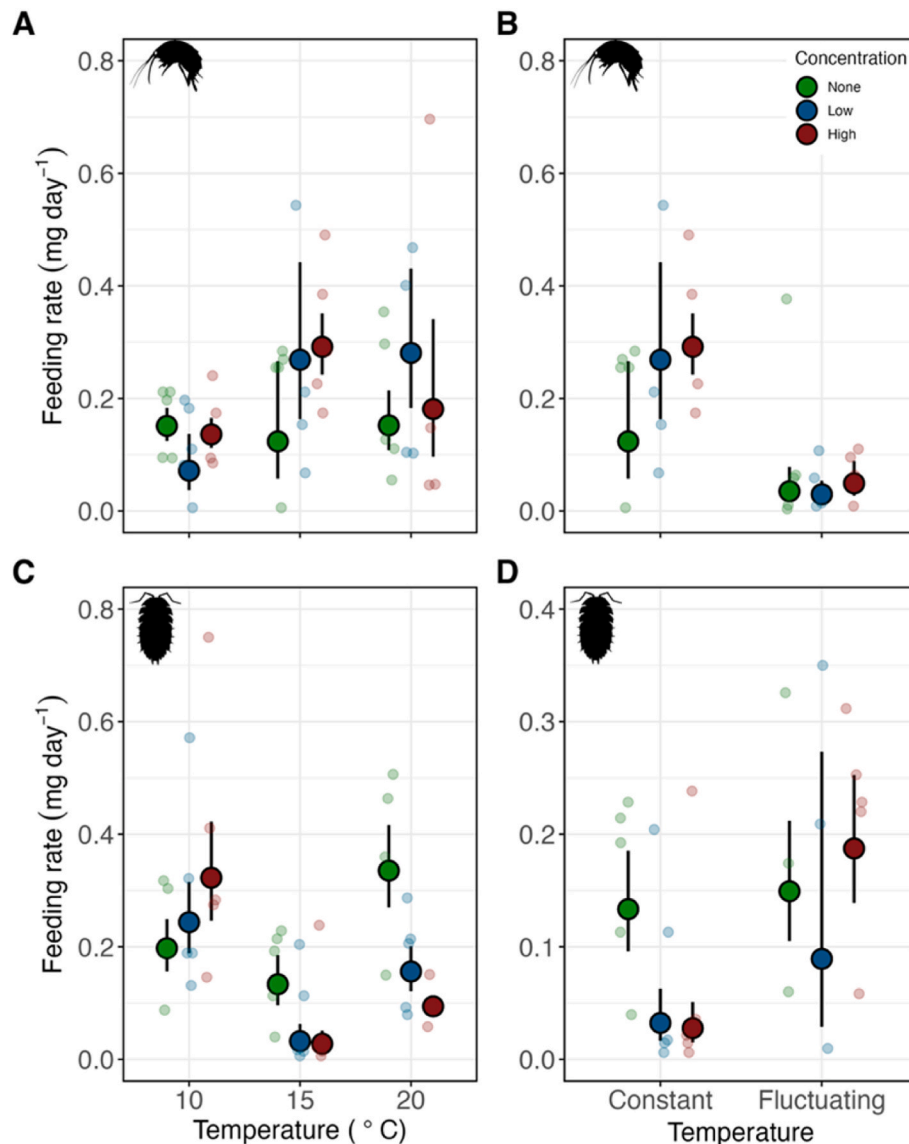


Fig. 3. Feeding rates of *G. pulex* and *A. aquaticus* individuals measured after a pre-exposure to microplastics and experimental warming. A) and C) Comparison between mean swimming speeds at constant 10, 15, and 20 °C and B) and D) comparison between the pre-exposure to microplastics at the constant ambient (15 °C) and fluctuating temperature regime that ranged between 10 and 20 °C over a 24h diel cycle, with measurements made at the mean daily temperature of 15 °C. Bold data points are mean feeding rates of five organisms \pm 1SE and shaded data points swimming speeds of individual organisms ($n = 5$ per treatment combination) following backwards ln-transformation. Error bars are asymmetrical due to the non-linear transformation from the log-scale used in the analysis. Point and line colours represent microplastic concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Redondo-Hasselerharm et al., 2018; Scherer et al., 2017).

Metabolic demands of ectotherms increase with temperature according to well-established metabolic principles (Brown et al., 2004), leading to higher energy requirements and increased consumption rates (Rall et al., 2012). In agreement with this theory, the respiration rates of *G. pulex* increased with temperature in the current study. However, in contrast to our first hypothesis, increased respiration rates of *G. pulex* did not translate into higher feeding rates or mean swimming speeds of *G. pulex* at higher temperatures, and *A. aquaticus* showed little response to increased temperature. Feeding rates of ectotherms are influenced by a range of other factors, including digestion rates (Wallace et al., 1973), which could have affected the feeding rates of *G. pulex* and *A. aquaticus* in this study. Movement activity is another important response that is tightly linked to metabolism in ectotherms (Issartel et al., 2005; Maa-zouzi et al., 2011), but there were no changes in the mean swimming speeds of either *G. pulex* or *A. aquaticus* individuals at higher

temperatures. However, there was a trend towards increasing swimming speeds of *G. pulex* between 10 and 15 °C before decreasing again at 20 °C. A reduction in performance parameters at warmer temperatures is indicative of organisms approaching their thermal limits (Bruno et al., 2013; Huey, 1979), therefore this could indicate that gammarids used in this study were approaching their thermal limit for movement at 20 °C. *A. aquaticus* are considered as tolerant species to different environmental pressures, including organic pollution (Rotvit and Jacobsen, 2013), high temperatures (Verberk et al., 2018), and low oxygen levels (Maltby, 1995). This could have resulted in better regulation of respiration rates at higher temperatures, in comparison with *G. pulex* which have a lower thermal tolerance limit (Verberk et al., 2018), and are less tolerant of lower oxygen levels (Rotvit and Jacobsen, 2013). Moreover, oxygen solubility in water increases at lower temperatures, which could have made it difficult to detect any differences in oxygen concentrations at 10 °C, particularly for *A. aquaticus* as visual observations revealed that

they mostly remained on the bottom of the vials. By contrast, *G. pulex* swam freely, likely agitating the media and maintaining uniform oxygen concentrations within the vials.

Many studies have been criticised for exposing organisms to simple constant temperature increases, rather than incorporating diurnal fluctuating regimes which are more representative of thermal regimes in the natural environment (Paaijmans et al., 2013). Therefore, in this study, the respiration rates, feeding rates, and swimming behaviour of *G. pulex* and *A. aquaticus* under constant temperatures (10, 15, and 20 °C) were directly compared to these rates measured in a fluctuating thermal regime (10–20 °C over a 24h cycle). The magnitude of temperature fluctuation in the current study was quite large (10 °C each day) and could be considered stressful for ectothermic organisms. However, there were no clear differences in any of the sub-lethal responses of *G. pulex* and *A. aquaticus* between the constant ambient and fluctuating thermal regimes in the current study, rejecting the second hypothesis. Moreover, there were no effects of microplastics on any of the sub-lethal responses measured following pre-exposure to constant 15 °C and fluctuating thermal regimes. These are interesting findings, considering that previous studies have shown that microplastics can affect life-history traits of freshwater organisms under more challenging thermal conditions, such as large daily temperature variations (Chang et al., 2022). Testing the effects of microplastics in combination with other human-induced pressures, such as climate warming, can be challenging due to the complexity of microplastics as a contaminant and replication level can sometimes be limited when there are many experimental treatments. Nevertheless, other studies with similar level of replication were able to detect effects of microplastics and warming on sub-lethal responses of aquatic biota (Kratina et al., 2019; Sadler et al., 2019).

There was no evidence supporting individual-level effects of microplastics at any of the concentrations tested, or in combination with experimental warming scenarios, rejecting the third and fourth hypotheses. This is in partial agreement with previous studies showing that exposure to microplastics does not always result in negative effects on ecotoxicological responses of freshwater biota (Aljaibachi and Callaghan, 2018; Marchant et al., 2023; Weber et al., 2018). No effects of microplastic exposure on feeding rates has commonly been reported for many freshwater macroinvertebrates (Kratina et al., 2019; Redondo-Hasselerharm et al., 2018; Weber et al., 2018). Yet, the negative effects of microplastics on respiration rates and swimming behaviour are commonly attributed to blockages in the digestive tract that cause organisms to become stressed and lead to more rapid breathing and movement rates (Hebel et al., 1997). Still, microplastic particles can be readily egested and have short retention times in the digestive system of freshwater biota (Frydkjaer et al., 2017; Straub et al., 2017; Weber et al., 2018). Freshwater detritivores are also morphologically adapted to feed on non-digestible materials (Agrawal, 1965; Shmitz, 1967). Although not directly assessed here, rapid egestion of non-digestible materials could explain the lack of effects of HDPE microplastics on the sub-lethal responses of *G. pulex* and *A. aquaticus* individuals in the current study.

Lastly and in contrast to our expectations, there was no evidence for an interaction between microplastics and temperature regime on the sub-lethal responses tested here. This is intriguing, considering that previous works have shown that the negative effects of microplastics on sub-lethal responses of freshwater organisms only manifest at higher temperatures (Kratina et al., 2019; Sadler et al., 2019). For instance, the respiration rates of *G. pulex* were suppressed following exposure to microplastics but only at the highest experimental temperature (Kratina et al., 2019), and daphnids were more sensitive to microplastics as temperatures increased (Jaikumar et al., 2018). These negative effects are commonly attributed to increased feeding rates at higher temperatures, and therefore increased uptake and exposure to microplastic particles (Rist et al., 2017). However, feeding rates of *G. pulex* and *A. aquaticus* on leaf litter did not increase with temperature in this study, and it has been shown that microplastic uptake does not always increase with temperature, due to rapid clearance rates of microplastic particles

(Sadler et al., 2019). These previous studies were conducted over relatively short durations (up to 72h exposure), in comparison with 14 days in the current study, whereas the effects of microplastics on ecological communities and ecosystems may only be apparent over the long term and across multiple generations (Schur et al., 2020; Silva et al., 2022). However, the short-term effects of microplastics may be more apparent than the long-term responses for respiration rates and movement behaviour if organisms are able to rapidly egest microplastic particles.

This study provides important insights into the effects of microplastics on the physiological and behavioural responses of important macroinvertebrate taxa under different experimental warming scenarios. Published data on the effects of microplastics on such sub-lethal responses of freshwater biota is lacking (Ockenden et al., 2021), despite their key role in determining species interactions (Huey and Kingsolver, 1989). Such behavioural alterations have previously been shown to alter the strength of trophic cascades in freshwater communities through changes in the swimming behaviour of aquatic grazers (Pan et al., 2022), revealing the importance of investigating sub-lethal responses of organisms to microplastic pollution in the context of other environmental stressors which is lacking from current research (Castro-Castellon et al., 2021). Even though there were no effects of microplastics, experimental warming or their combination in this study, microplastics are found in various types, shapes, and sizes in the environment. Therefore, further evidence about the properties of microplastics that cause toxicity to freshwater biota, and in combination with other environmental pressures, are urgently needed to fully determine the consequences for trophic interactions and ecosystem functioning. A better understanding of microplastic pollution in combination with other environmental pressures is vital for determining a proper risk assessment for microplastics in all ecosystem types.

CRedit authorship contribution statement

Danielle J. Marchant: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daniel M. Perkins:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **J. Iwan Jones:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Pavel Kratina:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

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

Acknowledgements

This research was supported by the EU INTERREG France (Channel) England project “Preventing Plastic Pollution” co-financed by the European Regional Development Fund. We thank Lindsey Cheeseman for help with the experimental setup and general technical guidance. We also thank Mark Trimmer, Kate Spencer, and Alice Horton for comments on an earlier draft.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2025.126182>.

Data availability

Data will be made available on request.

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