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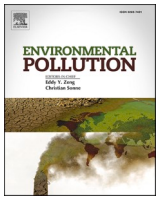

Interactive effects between water temperature, microparticle compositions, and fiber types on the marine keystone species *Americamysis bahia*☆

F. Biefel [a,b,d,*], J. Geist [a], R.E. Connon [b], B. Harper [c], S.M. Brander [d]

[a] *Aquatic Systems Biology Unit, TUM School of Life Sciences, Technical University of Munich, 85354, Germany*
[b] *School of Veterinary Medicine, Department of Anatomy, Physiology and Cell Biology, University of California Davis, 95616, CA, USA*
[c] *Environmental and Molecular Toxicology, College of Agricultural and Life Sciences, Oregon State University, 97331, OR, USA*
[d] *Fisheries, Wildlife, and Conservation Sciences, Coastal Oregon Marine Experiment Station, College of Agricultural and Life Sciences, Oregon State University, 97365, OR, USA*

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ABSTRACT

Recently, there has been an increasing emphasis on examining the ecotoxicological effects of anthropogenic microparticles (MPs), especially microplastic particles, and related issues. Nevertheless, a notable deficiency exists in our understanding of the consequences on marine organisms, specifically in relation to microfibers and the combined influence of MPs and temperature.

In this investigation, mysid shrimp (*Americamysis bahia*), an important species and prey item in estuarine and marine food webs, were subjected to four separate experimental trials involving fibers (cotton, nylon, polyester, hemp; 3 particles/ml; approximately 200 μm in length) or fragments (low-density Polyethylene: LDPE, polylactic acid: PLA, and their leachates; 5, 50, 200, 500 particles/ml; 1–20 μm). To consider the effects in the context of climate change, three different temperatures (22, 25, and 28 °C) were examined. Organismal growth and swimming behavior were measured following exposure to fragments and microfibers, and reactive oxygen species and particle uptake were investigated after microfiber exposure. To simulate the physical characteristics of MP exposure, such as microfibers obstructing the gills, we also assessed the post-fiber-exposure swimming behavior in an oxygen-depleted environment.

Data revealed negligible fragment, but fiber exposure effects on growth. PLA leachate triggered higher activity at 25 °C and 28 °C; LDPE exposures led to decreased activity at 28 °C. Cotton exposures led to fewer behavioral differences compared to controls than other fiber types. The exposure to hemp fibers resulted in significant ROS increases at 28 °C. Microfibers were predominantly located within the gastric and upper gastrointestinal tract, suggesting extended periods of residence and the potential for obstructive phenomena over the longer term. The combination of increasing water temperatures, microplastic influx, and oxidative stress has the potential to pose risks to all components of marine and aquatic food webs.

1. Introduction

In the media and the scientific community, the subject of plastic pollution has garnered increasing attention over the past decade. However, it is essential to distinguish between plastic types and the context of exposure, as consequences for the environment can be different (Triebskorn et al., 2019). The variety of plastic types and limited comparability of laboratory experiments to environmental conditions make research challenging. We adopt the term "microparticles" (MPs) to refer to microplastics, acknowledging that not all MPs exhibit a synthetic composition, in accordance with the suggested terminology (Miller et al., 2021; Lasdin et al., 2023; Torres et al., 2023). MPs, commonly defined as particles under 5 mm in size, are introduced into the environment through multiple pathways. Primary MPs are small particles intentionally manufactured for commercial purposes, such as those found in cosmetics. Once in the environment, larger plastic components can undergo additional fragmentation, leading to the formation of secondary MPs. The main sources of secondary MPs are textiles

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* Corresponding author. Aquatic Systems Biology Unit, Department of Ecology and Ecosystem Management, Technische Universität München, Mühlenweg 22, D-85354, Freising, Germany.
E-mail address: felix.biefel@tum.de (F. Biefel).

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(35%), tire wear (28%), and city dust (24%) (Boucher and Friot, 2017). Stormwater runoff transports MPs to rivers and oceans, resulting in common concentrations of 1.1–24.6 particles/l, with fibers being the most common shape detected (Werbowski et al., 2021). MPs are ubiquitously present across diverse ecosystems, and it is challenging to evaluate their impact at representative environmental conditions to assess their risk to organismal health. The predominant portion of above 80% of evidenced impacts resulted from plastic in comparison to alternative materials (e.g., metals), with a significant proportion occurring primarily at suborganismal levels; in contrast, marine debris composed of plastic particles larger than 1 mm, including items like ropes, straws, and fragments, exerts an impact on higher organismal levels (Rochman et al., 2016). As a result of mainly sublethal effects of MPs and the requirement of sensitive endpoints to determine their effect, researchers often evaluate mechanistic responses by intentionally inducing effects at high concentrations. However, there is a common limitation in these studies, as they frequently fall short of examining conditions that mirror the concentrations and sizes prevalent in the natural environment (Bucci et al., 2020).

MPs exposure can have age- and size-specific effects on feeding rates, oxidative stress, and offspring production of mysids (*Neomysis awatschensis*, Lee et al., 2021). Studies show that MPs can have various effects that are further influenced by particle size and shape, as well as specific abiotic parameters. Natural and synthetic microfibers between 3 and 30 particles/ml were shown to impact growth and swimming behavior on *Menidia beryllina* and *Americamysis bahia* (Siddiqui et al., 2023), and salinity further impacted tire particle internalization of fish and mysids (*M. beryllina, A. bahia,* Siddiqui et al., 2022). Concentration-dependent effects of polystyrene on feeding and swimming behavior were also reported for *Neomysis japonica*, a filter-feeding mysid crustacean (Wang et al., 2017, 2020). Ten-days acute toxicity studies on *H. azteca* exposed to fluorescent polyethylene MPs (4.64×10^4 particles/ml) and polypropylene fibers (71.43 particles/ml) showed that fibers were significantly the most toxic of the three types tested (Au et al., 2015). In *Daphnia magna*, the number of particles (polyethylene microbeads, 63–75 μm) taken up was shown to be concentration-dependent; however, it was observed that even if guts were full of these MPs, there was no effect on survival (Canniff and Hoang, 2018).

As described by Athey & Erdle (2022), anthropogenically altered natural and semi-synthetic fibers need greater consideration due to their prevalent distribution, persistent presence in the environment, and potential repercussions on biota. The consequences of anthropogenically altered natural fibers, such as cotton and hemp, have not been thoroughly investigated, leading to a general lack of understanding regarding potential distinctions in the toxicological effects between non-synthetic microfibers and their synthetic counterparts (Athey & Erdle, 2022). Nevertheless, Kim et al. (2021) and Mateos-Cárdenas et al. (2021) report that both non-synthetic and synthetic microfibers induce comparable impacts on freshwater and marine invertebrates.

Increasing temperatures due to global warming have profound implications for aquatic organisms and ecosystems. Temperature acts as a possible stressor that can interact with other factors, such as pollutants, to modulate their toxicity through various mechanisms. Dissolved oxygen is a main indicator of the health of an aquatic ecosystem, and its direct link to temperature makes it a parameter of high interest for exposure studies. Temperature can influence the lipid, protein, and overall energy status of, for example, the mysid *Neomysis integer* (Reyes et al., 2008; Verslycke and Janssen, 2002). It has been shown that exposure to polystyrene at different temperatures can have combined adverse effects on brine shrimp *Artemia franciscana* and that exposure at higher temperatures leads to reduced growth and increased mortalities (Han et al., 2021).

Small crustaceans serve as crucial intermediaries in the food web, functioning both as primary and secondary consumers, rendering them suitable candidates for ecotoxicological evaluations; their role in transferring energy from primary producers, notably algae to higher-level consumers like fish, situates them prominently within the food web structure (Luigi et al., 2012). Mysid crustaceans live across diverse aquatic environments. They are well-suited for ecotoxicological research due to their broad distribution, short life cycle, ecological significance as a food source for fish, and ease of culture in aquarium settings (Lussier et al., 1985, 1999; Wortham-Neal and Price, 2002; Sardo et al., 2005). Mysids play an important role as plankton predators and are an important keystone species in estuarine and marine food webs (de Almeida Prado, 1973). Specifically, *Americamysis bahia* (formerly *Mysidopsis bahia*), first described in 1969, is commonly used in toxicology studies because of early sexual maturity and high sensitivity (Molenock, 1969; Nimmo et al., 1977; Stephan et al., 1985; Lussier et al., 1999; Hirano et al., 2004).

Behavioral testing of invertebrate organisms holds the promise of becoming a powerful approach within the realm of aquatic toxicology and monitoring of water quality (Hasenbein et al., 2015; Kristofco et al., 2016). Besides more classical endpoints such as mortality and growth, photomotor/locomotion assays, measuring, for example, total distance moved, are widely used in ecotoxicological studies (Grillitsch et al., 1999; Little and Finger, 1990; Mundy et al., 2020, 2021; Segarra et al., 2021). Behavior results from the cumulative interplay of various biotic and abiotic elements and serves as the primary mechanism through which organisms adapt to alterations in their surroundings, including exposure to contaminants (Evans, 1994). Behavior offers a distinct viewpoint that connects an organism's physiology and ecology with its surroundings (Little and Brewer, 2010). As such, behavioral endpoints bridge the gap across different levels of biological organization, connecting subcellular, usually neurological processes, with ecological consequences from exposure to contaminants. Altered behavior can lead to modified responses such as deficiencies in feeding, predator avoidance, social interactions, and reproductive success, ultimately influencing individual survival and population dynamics (Bridges, 1997; Dell'Omo, 2002).

In biological systems, oxygen-derived radicals are collectively known as reactive oxygen species (ROS; Freeman and Crapo, 1982). MPs have the potential to induce oxidative stress and in response, stimulate the upregulation of antioxidant defenses in invertebrates (Jeong et al., 2017; Trestrail et al., 2020). ROS assays and behavior in an oxygen-decreasing environment can be valuable tools for investigating oxidative stress. In the context of global warming and the field of aquatic ecotoxicology, there is an increasing prevalence of research focusing on the impact of oxidative stress, often in conjunction with additional variables such as temperature. Organismal responses to oxidative stress can be influenced by temperature, salinity, and oxygen content in the water, which ultimately has the potential to influence fish responses to environmental changes (Birnie-Gauvin et al., 2017). Furthermore, oxidative metabolism is highly dependent on the differentiation and development status of an individual, while the identification of particular genes and pathways influenced by oxidants has given rise to the hypothesis that ROS function as subcellular messengers in gene regulatory and signal transduction pathways (Allen and Tresini, 2000).

This study endeavors to assess the interactive effects of MPs and temperature, aiming to provide insights that contribute to determining the risks associated with environmentally relevant particle concentrations and their impact on a keystone species, especially in the context of increasing water temperatures. Polylactic acid (PLA) and low-density polyethylene (LDPE) represent commonly utilized industrial polymer types; however, environmental samples frequently exhibit fibers, eliciting distinct responses compared to fragments. To address this shape effect, representative fiber polymer types were selected, encompassing both anthropogenic fibers derived from natural materials (cotton and hemp) and synthetic counterparts (nylon and polyester, PES). This approach ensures a comprehensive understanding of the diverse effects that different polymer types and shapes may exert, shedding light on the intricate interplay between MPs and temperature in the ecological context.

The objective of this study was to investigate the potential effects of MPs fragments, leachates thereof, and microfiber exposure on the growth and behavior of *A. bahia*. Leachates were used to distinguish whether observed effects stem from the particles per se or from leachate molecules effectively surpassing cellular barriers, resulting in distinct modes of action. Tire leachate was shown to affect swimming behavior of *A. bahia* (Siddiqui et al., 2022). As shown by Au et al., (2015), Ziajahromi et al., (2017), Stienbarger et al., (2021), Granek et al., (2022), Brander et al. (2024) among others, fibers are expected to have more significant ecotoxicological effects than fragments and beads. Therefore, we hypothesize that cotton, nylon, PES and hemp fiber exposures have more pronounced effects on *A. bahia* and investigated endpoints compared to fragment exposures. Additionally, we anticipate that higher temperature treatments will amplify the stress-related effects of the exposure treatments. Our research aims to investigate whether exposure to particle concentrations of sublethal doses leads to impaired swimming activities and reduced growth. Furthermore, we seek to determine if fiber exposure contributes to oxidative metabolism related stress. Finally, we aim to explore potential interactions between the measured parameters of particle type and concentration with temperature.

2. Methods

2.1. Microplastic solutions

The LDPE and PLA fragment solutions utilized in the study were obtained from the Harper lab at OSU, USA, where they had been cryomilled from larger plastic items per methods described in McColley et al. (2023). LDPE was purchased from Alfa Aesar (Ward Hill, MA, USA, Mfr. #42607) and was further reduced in size using liquid nitrogen cryomilling (Retsch CryoMill, Haan, Germany). PLA particles were made from 2 to 4 mm pieces of drinking straws (Open Nature, Pleasanton, CA, USA) which were also subsequently cryomilled. After cryomilling, materials were suspended in water and the suspension was passed through a 20 μm filter (Merck Millipore, Tullagreen, Cork, IRL, Mfr. # NY2004700), followed by a 1 μm filter (Advantec mixed cellulose ester filter, Mfr. #A100A047A). The 1 μm filter was then backflushed with fresh particle free water to get a solution of plastic microparticles between 1 and 20 μm. Particle counts were taken for the 1–20 μm fraction using a flow cytometer (Accuri C6 Flow Cytometer, BD Biosciences, San Jose, CA) calibrated with size standards. The via basic dilution equation C1V1=C2V2 ($V1 = \frac{Stock\ concentration\ x\ 1l}{Goal\ concentration}$) calculated volumes, representing the nominal concentrations, were added to Erlenmeyer flasks (1l) after manual shaking. Water changes (50%) were carried out every other day with spiked water from the Erlenmeyer flasks resulting in goal concentrations of 5–500 p/ml for fragment treatments.

Commercially available fiber rolls (for cotton and PES see Siddiqui et al., 2022; hemp and nylon: JO-ANN STORES, LLC.) were carefully cut using dissection knives, subsequently cleaned with ethanol, and subjected to evaporation. The fibers were then stored in RO water, and their lengths were measured using a dissecting scope equipped with Moticam visual software. Water changes replacing 50% of the water volume, were performed every other day, after which fibers of calculated volume ($V1 = \frac{Stock\ concentration\ x\ 200\ ml}{3\ p/ml}$) were added from stock solutions via pipetting to reach a 3 p/ml concentration. Due to fragmentation, the concentration of fibers in post-exposure measurements was higher (Table S1).

2.2. Source of organisms

Females of *A. bahia* were selected from the initial lab culture maintained in four separate 20-gallon aquaria at a temperature of 25 °C. These females were then sorted by size and transferred to three experimental aquaria set at 22 °C, 25 °C, and 28 °C. In order to minimize cannibalism and facilitate acclimation to the experimental temperature, approximately 30 females were kept within each of these systems. When a sufficient number of offspring was produced by the females at each temperature, offspring of similar size were distributed into treatment-specific beakers. If the number of offspring generated by the experimental females was inadequate, offspring from the stock culture with similar size and age were randomly introduced across the different treatment groups.

2.3. Exposure setup

The main protocols and practices used in this study were adapted from the U.S. Environmental Protection Agency (US EPA, 2009) and "Techniques for the laboratory culture of *Mysidopsis* species (crustacea: mysidacea)" (Lussier et al., 1988). Multiple runs of 7-day exposures (Initial Trial: Temperature comparison; Trial 1: PLA; Trial 2: PLA leachate; Trial 3: LDPE; Trial 4: Fibers: Cotton, nylon, PES, hemp) were carried out with fragment particles of 1–20 μm and fibers of in average 200 μm in length. Four plastic fragment concentrations were tested: 5, 50, 200, and 500 particles/ml (p/ml). Stock solutions of the target concentrations were set up with reverse osmosis (RO) water, which was adjusted to 15 ppt via filtered ocean water. Then, 50% of the water of the exposure beakers was replaced with this stock solution, which was prepared before each water change every other day. Organismal waste was also removed during water changes. Beakers were positioned in water baths to provide a temperature gradient of 22, 25, and 28 °C at a light-dark cycle of 16:8 h. For the fragments, three replicate beakers (200 ml, n = 3) per group with 5 individuals per beaker (N = 15) were exposed and continuously aerated. For the microfibers tested at one concentration of 3 p/ml, three replicate beakers (400 ml, n = 3) per group with 10 Mysids per beaker (N = 30) were exposed and continuously aerated in 15 ppt water. In the fiber trial, we opted for an increased water volume and a higher number of individuals to attain an adequate population size for the analysis of ROS and oxygen challenge. Alive brine shrimp were fed once daily (ca. 150 per mysid shrimp per day). Temperature, dissolved oxygen, salinity, and pH were monitored and recorded from a pool of each treatment. Fiber (size range, concentrations before and after exposure) and water parameters are shown in Tables S1 and S2.

2.4. Contamination controls

To mitigate the potential presence of MP contamination, precautionary measures were implemented prior to conducting and during the exposures, including the application of covers to beakers, the use of laboratory cotton coats dyed in orange, and the filtration of water (5 μm polycarbonate filters) prior to its use. Air samples collected during the exposures showed minimal contamination; the number of items found were PLA trial: 15; PLA leachate trial:17; LDPE trial: 24; Fiber trial: 12 (Fig. S1). The main type found was blue fibers. In the samples from controls, only one fiber was found during the exposure. All protocols for working with mysids and QA/QC procedures were adapted from Brander et al. (2020) and Siddiqui et al. (2022, 2023).

2.5. Specific endpoint measurement approaches

2.5.1. Survival and growth

Mortality assessments were conducted during each water change, which occurred every other day. The cumulative survival across all control treatments was >80%, meeting the expectations of a sublethal exposure. The total lengths and widths of mysids were measured using a dissecting scope equipped with Moticam visual software and ImageJ version 1.53k (Schneider, Rasband, and Eliceiri, 2012). The width (W) was measured in the stomach region, and the length (L) was measured from the tip of the thorax to the end of the last segment of the abdomen per methods described before (Wilhelm and Lasenby, 1998). A

segmented line with multiple points was measured if the shrimp was not photographed in a straight position. The growth index was calculated according to Siddiqui et al., (2022) as $\frac{W}{L}$ *x d,* where d is the number of days the organism was exposed for. Each pool of 3 individuals from length measurements was also used for dry weight measurements to determine the dry weight per individual. For preparation, 1.5 ml Falcon tubes were dried at 60 °C, cooled down for at least 30 min in a desiccator at room temperature, and measured without samples. After adding samples, tubes were baked for 24 h at 60 °C. After cooling down in the desiccator, the total weight was measured via Sartorius Quintix Analytical Balance (0.01 mg readability), and the weight difference was divided by the number of individuals. The biological replication was n = 3 (N = 9).

2.5.2. Locomotor behavior assay

For each trial, behavioral tests were performed at the time point of 7 days. As described before (Mundy et al., 2020, 2021; Siddiqui et al., 2022, 2023), a 35 min Light:Dark (LD) cycle test was performed in a DanioVision Observation Chamber (Wageningen, the Netherlands). The light-dark cycle protocol and arena settings (outer and inner arena) were adapted from a recent study (Siddiqui et al., 2022). As an acclimation time, 5 min were used following three alternating LD cycles of 5-min durations. Costume 12-well plates made from glass and filled with 2 ml un-spiked, filtered, and aerated 15 ppt RO-water were used. Behavioral assays were performed at treatment temperature. From each treatment, nine individuals were tested coming from 3 different beakers (n = 3, N = 9). For video tracking, EthoVision XT 15 software (version 15, Noldus, Wageningen, the Netherlands) was used. The resolution was set at 1280 × 960, light cycles were programmed at 10,000 lux, and the frame rate was set at 25/s.

In this study, we focused on two commonly used endpoints: Total distance moved TDM and Thigmotaxis ("wall hugging," which describes the ratio of staying in the outer ("hiding") to inner ("boldness") arena). Additionally, seven further locomotion endpoints (maximal acceleration, velocity, meander, movement, crossing frequency, turn angle, and mobility) were recorded. Therefore, a virtual center zone (1.6 cm diameter) was established in the glass well (2.2 cm diameter). Behavioral tests were conducted over one day between 9 a.m. and 6 p.m.

2.5.3. Respirometry challenge: swimming behavior during dissolved oxygen reduction

To observe stress-dependent swimming behavior after simulating an oxygen-deficient environment, mysids (n = 3, N > 2 individuals per run) were challenged on day 8 of the fiber exposure. Two to four mysids of each beaker were acclimated in a 1-L Zebrafish-culture tank at an oxygen concentration of 65–70%. Water chemistry was measured via YSI Professional Plus Quatro water quality meter (YSI Incorporated, Yellow Springs, OH, USA) in order to keep the temperature in range and to observe the oxygen drop. After 5 min of acclimation, nitrogen was introduced via an airstone plate. The rate of nitrogen inflow was observed via a bubble indicator at the cylinder. Videos were recorded between 11 a.m. and 11 p.m. The trials took about 3–10 min depending on the water temperature. Because of many treatments and replications, longer trials on one day were not possible. A similar procedure for lowering oxygen levels in water has been used in other studies of shrimp species and has no effect on the pH over 8 h (Dean and Richardson, 1999; Eriksson and Baden, 1997; Renaud, 1986; Roast et al., 2002). The experimental zebrafish tank did not have a lid to prevent nitrogen gas buildup. This procedure is similar to previous studies of hypoxia on crustaceans (Dean and Richardson, 1999; Landman, Van Den Heuvel, and Ling, 2005; Larkin, Closs, and Peake, 2007).

The oxygen content was measured at two endpoints: 1. Behavior: Stressed behavior indicated by bursting. 2. Mortality: Swimming disabled; Individual stops swimming for at least 5 s. This is described as ecological "mortality" as shrimp cannot perform escape responses (Hamilton, Russo, and Thurston, 1977; Roast et al., 2002).

2.5.4. Reactive oxygen species

To determine reactive oxygen species, the "Total Reactive Oxygen Species (ROS) Assay Kit 520 nm (Thermofisher, cat. 88–5930)" was used. The protocol used here varies slightly to the provided procedure and is based on pooled samples (n = 3) of 3 mysid shrimp each (8–10 days old, N = 9). Briefly, directly after the exposure, specimens were transferred into screw cap tubes, water was removed, and tubes were transferred into liquid nitrogen. Samples were then stored at −80 °C and processed within a month. After adding 500 μL PBS buffer, the samples were ground in the tubes. After spinning samples down for 10 min on high tempo, 10 μL of 1XROS were added into each well of a clean 96-well plate. Ninety microliters from the top of each sample (4 technical replicates) were added into one well with 10 μL 1XROS. PBS blanks were used as negative controls. The open well plates were incubated at 37 °C. To a water bath with 300 ml tap water, one Alka Seltzer tablet was added to reach an air CO_2 content of up to 5% in a 3l volume (Nyasulu, Paris, and Barlag, 2009). After 60 min, open plates were red on a fluorescent microplate reader.

2.5.5. Internalization

As previously described (Siddiqui et al., 2022), a CUBIC protocol was used to achieve tissue transparency. Subsequently, high-resolution images were obtained using microscopy to evaluate the presence of ingested particles. Animals were mounted in capillaries using a 2% w/v agarose solution dissolved in CUBIC R+ (for Animals, T3741, Tokyo Chemical Industry Co., Ltd). For this purpose, standard laboratory agarose was used with a modified protocol compared to the manufacturer's instructions. A mixture of 1 ml CUBIC R+ and 2% agarose was incubated on a heating block at medium temperature for 2 h, with thorough vortexing at least three times during the incubation period. High-resolution images were obtained using lightsheet-based microscopy (Zeiss Lightsheet 7) to examine the presence of ingested particles.

2.6. Statistics

To compare multiple treatments (temperature; concentration/polymer), two-way ANOVA analyses followed by Tukey multiple comparison tests with a single pooled variance were used if data allowed parametric tests. The assumptions normal distribution was verified via Shapiro-Wilk tests and visually via GG plots, and the homogeneity of variances was verified via Levene's tests (jamovi version 2.3.16.0 (Jamovi, 2022)). If assumptions were not met, non-parametric Kruskal-Wallis tests with multiple comparisons (Dunn's test) were used. ROS data did meet visual Q-Q plot satisfaction; consequently, ANOVA was applied. Spearman's correlations for light and dark were performed between treatment (concentration, polymer type) or temperature (22 °C, 25 °C, 28 °C), and total distance moved TDM or thigmotaxis, two main endpoints often described in literature. The locomotion endpoints TDM, maximal acceleration, thigmotaxis, velocity, meander, movement, crossing frequency, turn angle, and mobility were compared between treated and control animals. Data are presented as mean ± standard error (SEM); differences were called significant at $p < 0.05$; Analyses and graphs were run with GraphPad Prism (Version 10.1.2, © 1992–2021 Graphpad Software, LLC).

3. Results

3.1. Survival and growth

For environmentally relevant and low concentrations, exposure scenarios had limited effects on survival and growth. Overall, the survival rate at 22 °C was 92 ± 1.6%, at 25 °C 95 ± 1.8%, and at 28 °C survival was at 88 ± 3.2%. The highest survival rate was reached at 25 °C, which is the same temperature at which the laboratory culture

was set up. No significant differences between the temperatures nor concentrations for each trial were detected: PLA ($p = 0.56$), PLA leachate ($p = 0.13$), LDPE ($p = 0.15$), and fibers ($p = 0.21$) (Fig. S2). Growth-related endpoints (Figs. S3-S5) were impacted in the PLA leachate and fiber trial. In the PLA leachate trial, temperature and leachate led to interactive effects on length ($p = 0.013$) and width ($p = 0.023$), while the growth index in the fiber trial was influenced by fiber type ($p = 0.001$) (Fig. 1A).

Trends in the LDPE trial suggest that growth increased with temperature. However, these trends did not follow a linear pattern. In the LDPE trials, temperature significantly influenced the growth endpoints, accounting for 34.2% of the observed variation in dry weight ($P < 0.001$), 18.21% in width ($P < 0.001$), and 24.54% in total length ($P < 0.001$). This suggests that the differences observed in the LDPE trials were specific to those trials and did not occur in the other trials. Dry weight and width did not reveal differences in corresponding treatments (Figs. S3 and S4).

3.2. Locomotor behavior assay

A temperature test trial was used to determine the natural behavior of mysid shrimp at three different temperatures (Fig. S6A-D). The TDM in 22 °C treatments was significantly lower compared to 28 °C treatments in both dark ($p = 0.014$) and light ($p = <0.0001$) cycles. Thigmotaxis in 22 °C treatments was significantly higher compared to 28 °C treatments in both dark ($p = 0.0005$) and light ($p = <0.0001$) cycles. These strongly temperature-dependent differences led to the following analysis comparing treatments with corresponding control temperatures and not treatments across the three tested temperatures.

Responses to MP treatments led to hyper-as well as hypoactivity (Table S3). Exposure to PLA induced a greater number of locomotion endpoints that exhibited variations at 22 °C in comparison to 25 °C and 28 °C. PLA leachate exposure mainly led to an upregulation of activity at 25 °C and 28 °C. In the LDPE trial, PLA at 200 p/ml exposure led to more hyperactivity-related endpoints than LDPE at 200 p/ml, indicating that the polymer type triggered different behavioral responses. At 25 °C and 28 °C, especially LDPE of 5 and 50 p/ml led to differences in controls in a non-linear and non-concentration-dependent character. At 22 °C and 28 °C, only the cotton exposure did not significantly differ from controls in any behavioral endpoint, while other fiber types differed from controls at corresponding temperatures. At a temperature of 25 °C, fewer differences to the controls were noted.

Spearman correlation analysis revealed links in light cycles between temperature and TDM at all concentrations of PLA (Table S4). This was not observed in the LDPE trial. A similar correlation of temperature and TDM was observed in fiber treatments during the light cycle – PES excluded. Fiber type did not correlate with thigmotaxis. This lack of association implies that the specific characteristics or composition of the fibers, whether natural or synthetic, did not have a notable influence on the observed thigmotactic behavior.

3.3. Respirometry challenge: swimming behavior during dissolved oxygen reduction

The endpoint chosen to compare animals exposed to fibers with the control group was the moment when animals started bursting (Fig. 1B) and stopped swimming (Fig. 1C). The oxygen content of the event when individuals stopped swimming showed significant differences between 25 °C and 28 °C in control ($p = 0.024$) and cotton ($p = 0.039$) treatments. Temperature accounts for 50.42% of total variation ($p < 0$ 0.001), fiber type for 7.90% ($p = 0.42$), and their interaction for 3.07 ($p = 0.81$).

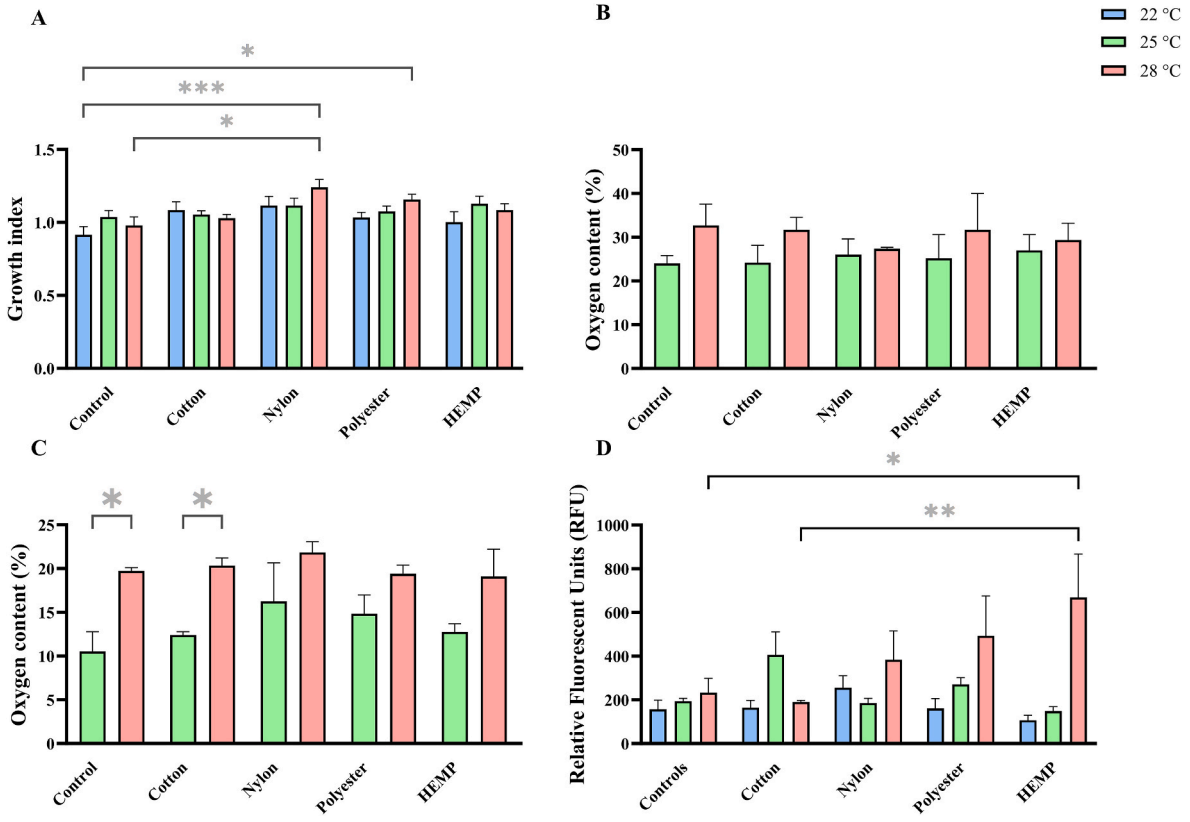

Fig. 1. Growth index (A), swimming behavior during dissolved oxygen reduction at the onset of bursting behavior (B) and termination of swimming (C), and ROS (D) of *Americamysis bahia* following a 7-day exposure to fibers (cotton, nylon, polyester, hemp) at three temperatures (22 °C in blue, 25 °C in green, and 28 °C in red); n = 3; *p < 0.05, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Reactive oxygen species

The average control signals were 22 °C: 157.2 ± 41.2, 25 °C: 193.8 ± 12.8, and 28 °C: 232.7 ± 65.8, indicating a wide range but a trend of ROS increase with temperature, albeit with no significant temperature-dependent difference. ROS differed significantly ($p = 0.014$, KW 27.98) and were influenced by temperature (23.09%, $p = 0.001$) and the interaction (31.50%, $p = 0.016$), but not by fiber type (4.67%, $p = 0.50$). At 28 °C, hemp exposure led to significantly higher ROS concentrations compared to controls ($p = 0.012$) and cotton ($p = 0.005$) of the same temperature (Fig. 1D).

3.5. Internalization

Fibers of all types were taken up and were visually detected in the stomach and upper gut regions (Fig. 2, Fig. S7). Lower gut areas were barely filled with fibers. Even controls meaning to account for background contamination showed fiber uptake, indicating that even lowest concentrations can cause fibers to remain in the stomach area, which also explains a lower clearance rate in comparison to fragments. While specimens from controls showed fewer fibers in their stomach, we also found controls with no obvious fiber uptake, indicating different levels of contamination or uptake variability caused by different behaviors.

4. Discussion

This study presents new insights into the impacts of MPs in combination with temperature on the growth, swimming behavior, and oxidative stress-related responses of *A. bahia*, contributing to a better understanding of the potential ecotoxicological consequences of fragment and fiber pollution. As expected, survival was not affected by fragments of up to 500 p/ml nor fibers of 3 p/ml, as this was intended to be a sublethal exposure. To our knowledge, there is a lack of reported data regarding the concentrations of MPs, especially PLA and LDPE, within the 1–20 μm size range, primarily due to technical constraints associated with isolating and characterizing MPs in environmental samples. The particle concentrations used in our study, however, should lay within the range of environmentally realistic concentrations and were expected to mainly cause sublethal effects. It is important to emphasize that environmentally relevant concentrations (comparable to tire wear concentrations of 60–60,000 p/ml used in Siddiqui et al., 2022) of MPs typically do not elicit severe effects, as observed under controlled laboratory conditions. This observation holds true even when PLA and LDPE fragment concentrations are increased by factors of up to 100 times. However, several factors such as environmental variables can contribute to the divergence between laboratory results and actual environmental impacts due to buffering or mitigating the effects of MPs.

Growth was also not impacted in this set of fragment exposures, although effects on growth have been observed previously (Siddiqui et al., 2022, 2023). The growth index, which combines length and width data, however, was influenced by the fiber type, suggesting pronounced growth effects in contrast to fragment exposures. In nylon treatments at 28 °C, these index values were higher than corresponding controls, indicating that this fiber type led to an increase in width or decrease in length. At the highest of the tested temperatures (22 °C, 25 °C, 28 °C), survival and growth-related effects were not altered at tested fragment concentrations of PLA and LDPE (5, 50, 200, 500 particles/ml; 1–20 μm).

Total distance moved, and thigmotaxis differed due to the LD cycle and temperature but not in response to fiber type or fragment concentration. Further differences in swimming behavior to controls do not seem to follow a concentration-dependent pattern. The particle concentrations used in our study are within the range of environmentally realistic concentrations and were expected to mainly cause sublethal

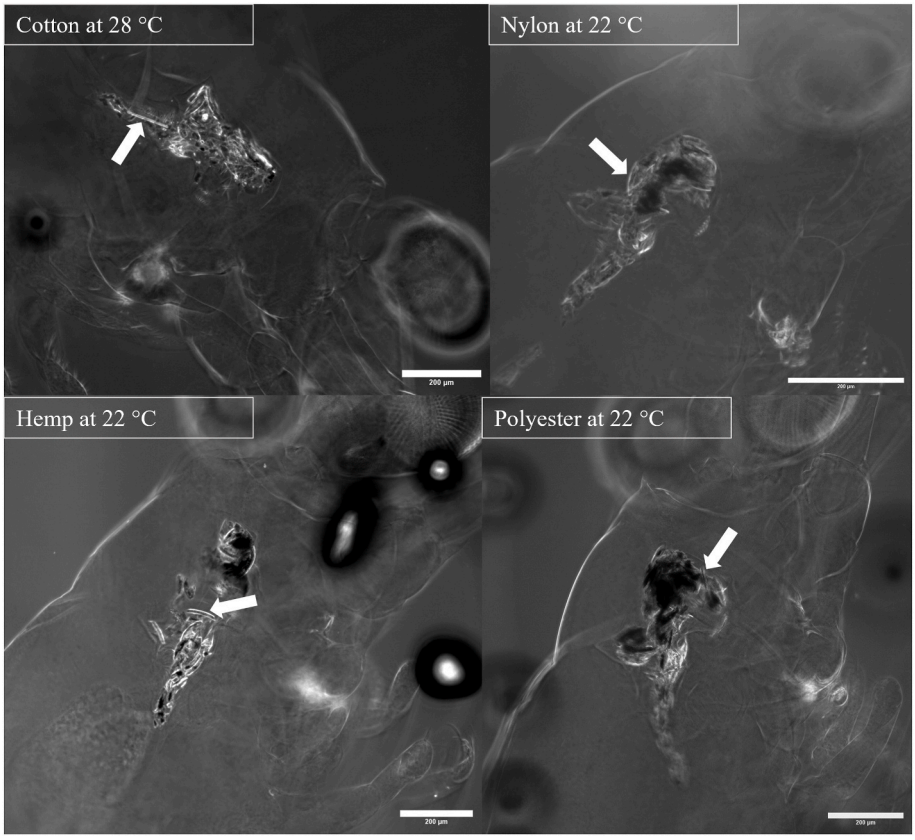

Fig. 2. Examples of Lightsheet microscopy imaging of the stomach region of *Americamysis bahia* following a 7-day exposure to fibers (cotton, nylon, polyester, hemp) at different water temperatures. Fibers ingested into the stomach are indicated with an arrow.

effects. Tire wear particles demonstrated an LC50 of 3426 ± 172 particles/ml in *Hyalella azteca*, an epibenthic filter-feeding isopod, while notably, the tire wear leachate failed to generate a sigmoidal dose-response curve, underscoring different toxicity mechanisms between particles and leachates (Khan et al., 2019). Although the mechanisms of action may vary between particles and leachates, our study focused on a singular concentration of leachate, preventing a comprehensive comparison of dose responses. In the LDPE trial (5, 50, and 200 p/ml) conducted at a temperature of 28 °C, a notable decrease in activity levels (e.g., TDM), characterized as hypoactivity, was observed in contrast to the conditions observed at lower temperatures. However, it is important to note that, aside from this hypoactive response at 28 °C, elevating the temperature did not induce a significantly greater array of distinct behavioral effects when compared to the responses observed at lower temperatures. Furthermore, fiber (cotton, nylon, PES, hemp) exposure at 3 p/ml and approximately 200 μm in length contributed to oxidative metabolism-related stress.

Animals exposed to nylon, PES, and hemp did not manifest statistically significant differences in oxygen levels between 25 °C and 28 °C when ceasing swimming activity (referred to as mortality in this context). The environment with decreasing oxygen content requires increasing gill activity in order to provide oxygen. The difference of this loss of equilibrium was significantly different between the two temperatures in cotton and control treatments, indicating that other fibers reduce gill functioning. As observed in our trials, tolerance to different stages of hypoxia in *T. novae-zealandiae* appears to involve a reduction in activity up to erratic behavior and escape responses just before losing equilibrium (Larkin et al., 2007). Metabolic rates may also be negatively impacted by MPs due to their capacity to impede oxygen uptake, potentially leading to alterations in swimming behavior (Rist et al., 2016; Siddiqui et al., 2023). Additionally, rising temperatures lead to a reduction in the oxygen capacity of water, which has significant implications for organisms with a high oxygen demand, such as cold-water salmonids (Chapra et al., 2021).

While the loss of equilibrium might have been caused by mechanical influences of fibers, increased ROS in hemp treatments at 28 °C also indicate higher oxidative stress at the cellular level. ROS play a role as subcellular messengers in gene regulatory and signal transduction pathways, while antioxidants have the ability to activate multiple genes and pathways (Allen and Tresini, 2000). MPs as well as temperature treatments might have contributed to combined ROS effects. MPs were shown to inhibit brine shrimp's cholinergic system and induce cell and oxidative stress (Eom et al., 2020). Furthermore, temperature was shown to be a main water parameter causing adverse effects on cellular energy allocation, for example, in the estuarine mysid shrimp *Neomysis integer* (Verslycke and Janssen, 2002), which can modify reactions to further stressors such as MPs. Further effects of MPs and temperature on oxidative metabolism and ROS can be modified by growth differences. The tendency of increased growth with rising temperatures in the LDPE trials can be explained by the Q10 effect (factor by which reaction rates are influenced by a 10 °C change) modifying biological reactions and the energy status of organisms (Reyes et al., 2008; Verslycke and Janssen, 2002). This could have had implications for *A. bahia* and their oxygen demand, leading to tentatively higher ROS concentrations at higher temperatures.

Microscopic examination revealed that fibers tended to become lodged in the foregut region, while their presence in the lower gut regions was relatively scarce. However, this clogging did not lead to reduced growth in response to cotton and PES as it has in mysids exposed to higher fiber concentrations previously (Siddiqui et al., 2023). Possibly, these fibers did not hinder the passage of nutrients and ingested brine shrimp, the environmentally relevant concentration of 3 p/ml was too low to trigger effects, or the exposure length was too short to observe impacts. As outlined in the findings reported by Siddiqui et al. (2023), which used mysid shrimp over the same exposure length in the same lab, none of the investigated fiber types (cotton, polyester, polypropylene; 80–150 μm length, 8–20 μm width, 3–30 particles/ml) were detected within mysid shrimps. This cannot be confirmed by the results of this study, as stomachs of investigated mysids showed fiber content and also variability in filling levels across individuals. This is surprising as 3 p/ml is on the lower spectrum of concentrations used in Siddiqui et al. (2023). Siddiqui et al. (2023) also reported a reduction in growth in mysids exposed to cotton microfibers, which could not be confirmed by the results of our study with cotton; however, we observed this with nylon. The lack of growth effects could be associated with high variability in uptake, potentially causing clogging but not necessarily diminished nutrient supply. In the present study, nylon showed significantly higher growth indices compared to controls at 22 °C and 28 °C, indicating differences to more natural fibers, cotton and hemp, which did not differ from controls. These nuanced responses underscore the species-specific variations in the behavioral and growth outcomes associated with microfiber exposure, highlighting the importance of considering the type and size of microfiber, abiotic factors, and the specific organism in ecological assessments. It is noteworthy to elaborate on the observation of minimal contamination of fibers in control samples, which might have led to fiber uptake. This finding was expected as fiber contamination via air (examples are shown in Fig. S6), clothes, and e.g., lab equipment is challenging to avoid. The low level of contamination in the control group and air samples, however, suggests that the experimental conditions were well-controlled, minimizing external factors that could have influenced the study results.

For *H. azteca*, higher concentrations of 71 polypropylene fibers/ml caused slower egestion of food, less growth, and longer residence time in the gut, which might cause lower abilities to process food (Au et al., 2015). Previous studies have also demonstrated that the crustacean Norway lobster (*Nephrops norvegicus)* struggles to fully eliminate polypropylene fibers, resulting in their retention within the chitinous foregut and subsequently leading to reduced growth of the organism (Murray and Cowie, 2011). The gastric filters of the Atlantic ditch shrimp (*Palaemon varians*), for example, play a crucial role in preventing larger MPs from reaching the midgut gland. This species' filters possess the capability to effectively filter and subsequently eliminate these particles through the process of egestion (Saborowski et al., 2022). In the case of brine shrimp larvae, ingestion of 10 μm polystyrene spheres resulted in the egestion of approximately 97% of the particles within 3 h, accompanied by deformities in the intestinal epithelia (Wang et al., 2019). In accordance to US EPA guidelines that advocate for sensitive experimental durations focusing on survival and growth endpoints, we conducted exposures lasting 7 days, aiming to avoid the development of secondary sexual characteristics, which typically commence at 12 days of age, and could potentially influence swimming behavior (US EPA, 2009). Depending on the length of the exposures and adaptation/avoidance mechanisms, effects during our 7-day exposure might have differed over time. Even short-term exposures of 3 h on grass shrimp (*Palaemonetes pugio*) showed that MP spheres and fragments below 50 μm are not acutely toxic, while fibers of 93 μm length caused higher mortalities than smaller sizes, largely being explained by the residence time of particles in the gut of 43.0 ± 13.8 h, and in the gills with 36.9 ± 5.4 h (Gray and Weinstein, 2017). PLA and LDPE particles were challenging to find in the organisms, possibly due to behavioral trials finding place in clear water leading to clearance. Not only can the shape of particles influence their effects, but smaller particles can cause higher bioconcentration and longer retention times (Liu et al., 2021). However, translocation of fibers, PLA, and LDPE particles from the gut was not observed, indicating sizes unable to penetrate gut surrounding tissue.

At 28 °C, only hypoactivity was observed in the case of LDPE, whereas PLA exposure, with a concentration of 200 particles/ml, resulted in hyperactivity at 25 °C. This difference might be due to the properties of the polymer, especially the density, leading to different positions in the water column. However, there is limited knowledge on the toxicity of LDPE and PLA. *Daphnia magna,* for example, did not exhibit any adverse effects in response to LDPE (1–100 mg/l) (Jemec

Kokalj et al., 2021). Di Giannantonio et al. (2022) found no discernible effects on the immobility of cnidarian Aurelia species (common jellyfish) following a 24-h PLA exposure but modulated swimming behavior (pulsation) across all exposure concentrations (1, 10, and 100 mg/l). This and our results show that perhaps hyperactive behavior represents a prevalent response to PLA.

5. Conclusion

In conclusion, our results reveal possible interactive effects between water temperature and fiber types. It is important to acknowledge that populations in natural environments and controlled laboratory settings may differ in their susceptibility to specific stressors (Clark et al., 2015). Laboratory populations, which are frequently exposed to plastics in laboratory and aquaculture settings, might exhibit lower sensitivity to these contaminants compared to their wild counterparts. Although the concentrations of PLA and LDPE utilized in our study exceeded environmental levels by a factor of 100, no significant adverse effects were observed. However, the extrapolation of these findings to real-world situations requires careful consideration of environmental context and conditions. With consideration of the ongoing global warming phenomenon, this research aimed to provide an evaluation of MPs and their risk to the welfare of *A. bahia*, a keystone species in marine food webs. It is crucial for future investigations to prioritize filling the existing knowledge gaps concerning the effects of fibers and their interactions with abiotic factors.

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Institutional review board statement

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CRediT authorship contribution statement

F. Biefel: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **J. Geist:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **R.E. Connon:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **B. Harper:** Writing – review & editing, Resources. **S.M. Brander:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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