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# Effects of natural and synthetic particles, and temperature on aquatic species

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***"Every aspect of nature reveals a deep mystery***

***and touches our sense of wonder and awe..."***

*Carl Sagan*

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# Preface

This thesis aims to enhance our comprehension of particle and temperature effects on aquatic organisms, as exemplified by the threatened fish Longfin Smelt (*Spirinchus thaleichthys*) and model crustacean organisms *Hyalella azteca* and *Americamysis bahia* (Figure 1). Chapter 1, the introduction, provides essential background information encompassing general information about natural and synthetic particles, turbidity and its effects, the global meaning of plastic pollution, particles and their biological effects, as well as multiple stressor scenarios, study organisms, and the study's goals and objectives.

Following the overview of the main applied methods in Chapter 2, Chapter 3 sums up key aspects vital to understanding the results of the three studies. Study one examined turbidity up to 11 NTU in combination with two temperatures on growth and gene expression of Longfin Smelt to understand physiological responses and determine rearing conditions. Study two investigated the impacts of polystyrene uptake on swimming behavior in *H. azteca*, specifically examining the interactions across three different temperature conditions. Study three investigated fragment and fiber effects of different polymer types on mysid shrimp *A. bahia*. Besides growth and swimming behavior, oxidative stress following fiber exposure was investigated. All three studies have undergone peer review and have been published.

Following the results of the three studies, chapter 4 provides a comprehensive discussion addressing the relevance and significance of confounding factors and scenarios involving temperature and particles for aquatic organisms. The study results were synthesized and compared with existing literature, culminating in an outlook for future research.

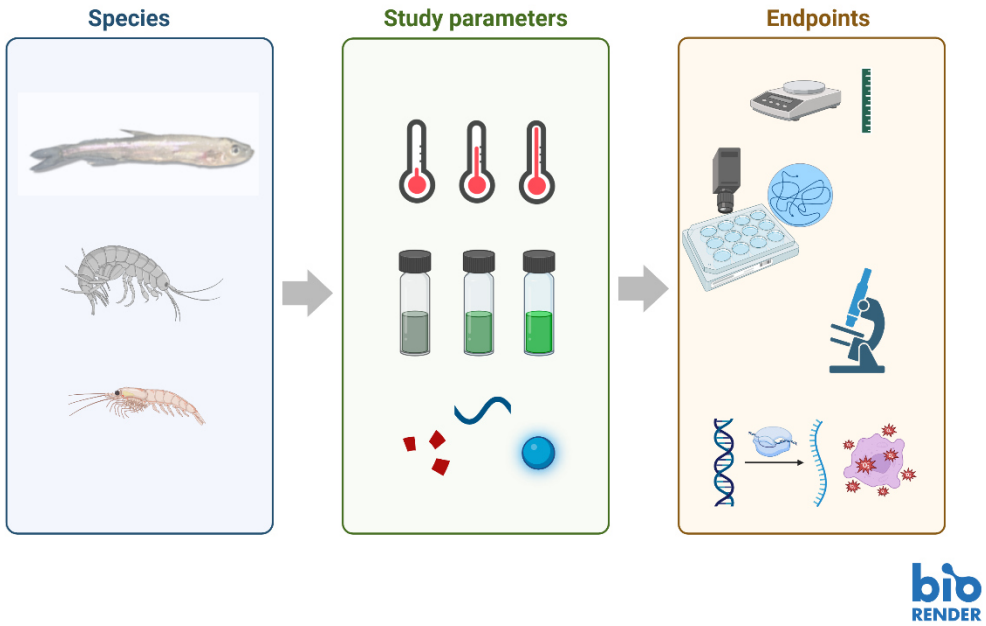


*Figure 1 Study overview with investigated species, parameters, and endpoints. Created with BioRender.com.*

# Summary

Particles can affect aquatic organisms through, e.g., uptake or turbidity. Variations in land use and water flow influence turbidity levels in rivers and estuaries, alongside the presence of plastic pollution. Both natural and synthetic particles impact organisms differently, with their effects modulated by abiotic factors such as temperature. These environmental changes compress habitats, necessitating that organisms adapt to avoid surpassing their niche limits.

The Longfin Smelt (*Spirinchus thaleichthys*) in the San Francisco Estuary is experiencing rapid population declines, highlighting the urgency to comprehend their sensitivity and stress responses. Gene expression analysis in juvenile Longfin Smelt exposed to two temperatures and three turbidity levels for four weeks indicated that while temperature significantly affected growth, turbidity levels up to 11 NTU did not directly impact gene expression or growth. The results suggest that the tested turbidity levels did not surpass the threshold needed to induce significant physiological changes. Based on these results, the parameters used are considered suitable for aquaculture.

Next to a lack of research on the ecotoxicological effects of natural particles in the form of turbidity, significant knowledge gaps remain regarding the effects of particulate matter, especially synthetic plastics in the shape of fibers, on aquatic organisms. Research on *Hyalella azteca* has shown increased polystyrene bead uptake and higher mortality rates at elevated temperatures. The study on mysid shrimp (*Americamysis bahia*) exposed to microfibers at different temperatures revealed minimal differences in mortality and growth, though there were variations in the production of reactive oxygen species, indicating differing stress responses. These results imply that the interaction between synthetic particles, and rising temperatures due to climate change may exacerbate the risks to aquatic organisms, indicating the need for further research to understand multiple stressor effects and to inform environmental management strategies.

# Zusammenfassung

Partikel können aquatische Organismen z. B. durch Aufnahme oder Wassertrübung beeinflussen. Veränderungen in der Landnutzung und im Wasserfluss beeinflussen die Trübungswerte in Flüssen und Flussmündungen während Plastikverschmutzung allgegenwärtig ist. Sowohl natürliche als auch synthetische Partikel wirken sich unterschiedlich auf Organismen aus, wobei ihre Effekte durch abiotische Faktoren wie Temperatur moduliert werden. Diese Umweltveränderungen komprimieren Lebensräume, was es notwendig macht, dass sich Organismen anpassen, um zu vermeiden, ihre Nischengrenzen zu überschreiten.

Der Longfin Smelt (*Spirinchus thaleichthys*) im San Francisco Delta erfährt einen raschen Bestandsrückgang, was die Dringlichkeit unterstreicht, ihre Empfindlichkeit und Stressreaktionen zu verstehen. Die Genexpressionsanalysen von Longfin Smelts, die vier Wochen lang zwei Temperaturen und drei Trübungsstufen ausgesetzt waren, zeigte, dass zwar die Temperatur das Wachstum signifikant beeinflusste, die Trübungsstufen bis zu 11 NTU jedoch keinen direkten Einfluss auf die Genexpression oder das Wachstum hatten. Die Ergebnisse deuten darauf hin, dass die getesteten Trübungen die Toleranzgrenzen nicht überschritten haben, die erforderlich wäre, um signifikante physiologische Veränderungen hervorzurufen. Basierend auf diesen Ergebnissen sind die verwendeten Parameter für die Aquakultur als geeignet einzustufen.

Neben einem Mangel an Forschung zu den ökotoxikologischen Auswirkungen natürlicher Partikel in Form von Trübung bestehen weiterhin erhebliche Wissenslücken hinsichtlich der Auswirkungen von Partikeln, insbesondere synthetischen Kunststoffen in Form von Fasern, auf aquatische Organismen. Untersuchungen an *Hyalella azteca* haben gezeigt, dass bei erhöhten Temperaturen eine erhöhte Polystyrol Partikelaufnahme und höhere Sterblichkeitsraten auftraten. Die Studie an Schwebegarnelen (*Americamysis bahia*), die Mikrofasern bei unterschiedlichen Temperaturen ausgesetzt waren, ergab minimale Unterschiede in der Sterblichkeit und im Wachstum, obwohl es Variationen in der Produktion von reaktiven Sauerstoffspezies gab, was auf unterschiedliche Stressreaktionen hinweist. Diese Ergebnisse deuten darauf hin, dass die Wechselwirkung zwischen synthetischen Partikeln und steigenden Temperaturen aufgrund des Klimawandels das Risiko für aquatische Organismen erhöhen kann. Ergebnisse unterstreichen die Notwendigkeit weiterer Forschung, um diese Effekte kombinierter Stressoren zu verstehen und um Strategien für das Umweltmanagement zu entwickeln.

# 1. Introduction

## 1.1 Natural and synthetic particles

The significance of natural and synthetic particles and their effects on aquatic environments has increasingly gained attention on a global scale in recent years. Natural non-food particles, including minerals like clay and sand, as well as organic matter such as cellulose and chitin, are far more prevalent in the environment than synthetic particles of similar size (i.e., microplastics: MPs <5 mm; nanoplastics: NPs <1 µm; both categories: MNPs) (Bernhardt et al., 2010; Lenz et al., 2016). The concentration data collected for the river Elbe are significant, as they reveal that fewer than 10 out of every $10^6$ particles in a surface water sample are composed of plastics (Triebskorn et al., 2019). Aquatic organisms often encounter turbid environments with high concentrations of non-palatable particles in the water column (in the order of g/L). Consequently, studies in ecology and aquaculture have investigated organismal needs for, and responses to turbidity caused by suspended sediments and fine particulates (Wilber & Clarke, 2001). It is crucial to investigate the requirements and potential detrimental effects of both natural and synthetic particles, even though ecologically relevant concentrations of these particles can differ significantly. Understanding the full spectrum of particle impacts is essential because natural and synthetic particles are likely to act differently upon aquatic organisms and ecosystems, leading to varying environmental outcomes.

## 1.2 Turbidity: Definition and impacts on aquatic organisms

Turbidity can be defined as the murkiness or cloudiness changing optical property that causes light scattering and absorption (Sugawara & Nikaido, 2014). Factors affecting turbidity effects are water depth, suspended material, and light intensity (Lee & Rast, 1997). Natural suspended materials, including inorganic sediments like clay, silt, sand, and organic materials such as algae and plankton, contribute to turbidity in water bodies. These materials influence water clarity through complex interactions, affecting light penetration, photosynthetic activity, and the overall health of aquatic ecosystems. Dynamic ecosystems such as estuaries and rivers especially experience varying turbidity levels. Sediment deposition, transport, and resuspension of materials depend on wave actions, river morphology, and tidal flows, which can change periodically (Nichols & Biggs, 1985). Long-term climatic changes (Inman & Jenkins, 1999) and seasonal variations

(Tamura et al., 2010) in river flows can further influence turbidity levels. Human actions such as damming and land use additionally influence sediment transport (Inman & Jenkins, 1999).

Turbidity exerts diverse impacts on aquatic organisms. Turbid conditions can lead to a higher occurrence of fish (Sommer et al., 2011), and zones with high turbidity are considered necessary for estuarine fish (Dodson et al., 1989; North & Houde, 2001) because they can influence primary production and food availability caused by lowered light intensities (Bruton, 1985). In contrast, lower turbidities can lead to reduced primary production, resulting in less food availability (Kimmerer et al., 2005) while being an advantage for larger visual predatory fish (Chesney, 1989; Fiksen et al., 2002). Interspecies relationships, such as a reduction of avian, mammalian, and predation by other fish (Bruton, 1985), might be an advantage for planktivorous fish (Gregory & Northcote, 1993; De Robertis et al., 2003). Through enhancing contrasts between prey and predator (Hinshaw, 1985), turbidity at certain levels can also improve the feeding conditions of fish, providing visual contrast and limiting light penetration (Delta Smelt, *Hypomesus transpacificus*, Baskerville-Brldges et al., 2004). Effects of turbidity are also considered to be life-stage specific. Larval fish, as well as small planktivorous fish, benefit from turbidity, one factor determining the distance between predator and prey; this does not seem to be the case for adult piscivore fish (Miner & Stein, 1996; Utne-Palm, 2002). Turbidity influences interspecies relationships and feeding behavior, and it, in turn, affects the stress levels of aquatic organisms.

Stress levels in sensitive fish species, such as the endangered Delta Smelt, can be critical, as both very low and very high turbidity levels can lead to increased mortality (Hasenbein et al., 2016). It was shown that cortisol levels correspond with mortality and feeding performance and that increased turbidities lead to reduced feeding rates at high turbidity of 250 NTU (Hasenbein et al., 2016). Although Delta Smelt deals with different turbidity levels in their environment, enhanced contrast may lead to limited prey detection, reducing their swim activity and predator perception at high turbidity (Hasenbein, et al., 2013). Low turbidities can lead to higher swimming activities and positioning near the surface in this species (Hasenbein et al., 2013). Because of reduced stress levels in higher turbidities, which protect from predators, higher activities in lower turbidities might also be caused by sensing of handlers in laboratory test scenarios, escape responses, and searching behavior (Gregory & Northcote, 1993; Sirois & Dodson, 2000). While turbidity is a significant and variable factor affecting aquatic organisms in various ways, it remains an understudied area outside aquaculture.

## 1.3 Plastic waste and resulting microplastic pollution

The global annual plastic production increased from 0 tons in the 1950s to over 400 million tons in 2019; plastic waste increased from 156 million tons in 2000 to 353 million tons in 2019 (OECD, 2022). It is projected that by 2050, plastic production will escalate to 2000 million tons (Kershaw Peter, 2016). Despite doubling plastic recycling rates since 2006 (PlasticsEurope, 2019), approximately 10 % of the plastics produced still enter the oceans, accounting for 80-85 % of marine litter (Thompson, 2007; Auta et al., 2017). Their production and conversion from fossil fuels are the main parts of their life cycles, contributing to 3.4 % of global greenhouse gas emissions (OECD, 2022). Of the 391 million tons of worldwide plastic production, 32 % finds place in China, 18 % in North America, and 15 % in Europe. While China increased its production by 3 % since 2017, North America's (Canada, USA and Mexico) production was stable, and Europe's production sank by 4 % (PlasticsEurope, 2022). At the end of the life cycle, plastic waste disposal in landfills is still much more common than recycling it. Approximations suggest that 6300 million tons of plastic waste were produced worldwide between 1950 and 2015; only 9 % were recycled, 12 % incinerated, and 79 % stored in natural environments and landfills (Geyer et al., 2017). If this trend continues, plastic will pollute natural environments on larger scales than we are currently experiencing, with potentially exponential effects on wildlife. Estimates for the year 2019 state that 1.7 million tons out of 6.1 million tons (ca. 28 %) of plastic waste reaching aquatic environments flowed into the ocean (OECD, 2022). Plastic particles found in the environment are of different polymer types, mainly polypropylene (PP) used in food packaging, polyethylene (PE), polystyrene (PS), polyvinylchloride (PVC) used in pipes and insulations, polyethylene terephthalate (PET) used in beverage bottles, and polyamides (PA), which are often made of natural gas, coal and petroleum (Wang et al. 2019). Due to their cost-effectiveness, durability, and versatility, plastics are essential worldwide and in many areas, such as agriculture, construction, packaging, sports, electronic devices, and medical facilities (Plastics-the Facts 2019).

More than 92 % of plastics in oceans are particles smaller than 5 mm (Eriksen et al., 2014). Next to the possibility that MPs reach aquatic environments directly through, for example, cosmetics or textiles ("primary microplastics"), it can also arise through breakdowns of larger plastics ("secondary microplastics") (Cole et al., 2011). The report "Primary Microplastics in the Oceans," published by the World Conservation Union IUCN 2017, estimated that more than 800,000 tons of primary MPs reach the oceans yearly (Boucher & Friot, 2017). The secondary MPs are caused

by, for example, mechanical forces such as erosion. Within the marine environment, plastics undergo degradation through physical, chemical, and biological processes (Andrady, 2011; da Costa et al., 2016; Alimi et al., 2018). The degradation of plastics relies on the presence of oxygen and sunlight (Andrady, 2011; Gewert et al., 2015), with photodegradation being the most effective process (da Costa et al., 2016). Beaches are particularly susceptible to plastic degradation in the marine environment, as they provide favorable conditions for high photodegradation rates (Andrady, 2011). Sources for secondary MPs are synthetic textiles (35 %), tire abrasion (28 %), urban dust (24 %), road markings (7 %), and ship coatings (3.7 %) (Boucher & Friot, 2017). A study by Feng et al. (2020) revealed that tourism emerged as a primary source of MPs in water bodies; in contrast, facility agriculture and previous secondary industries were identified as significant contributors to MPs in soil. Additionally, there was compelling evidence of noticeable levels of MPs associated with human activities, even in remote regions (Feng et al., 2020).

Microplastics are ubiquitous in all environment compartments, spanning from terrestrial to aquatic environments and encompassing atmospheric and geographically remote regions (Dioses-Salinas et al., 2020; Feng et al., 2020). Examining the widespread MP distribution through a physical lens, abiotic processes such as wind, rivers, and oceanic currents are the primary drivers (Barnes et al., 2009; Sherman & Van Sebille, 2016). The presence of MPs exhibits significant variability, with concentrations ranging from 0-7 particles/$m^3$ in air, 1-4712 particles/kg in soil, 1-26 particles/L in aquatic environments, and 0-199 particles/organism globally (Parashar et al., 2023). The greatest overlap between MPs and marine life is expected to occur in coastal regions (Clark et al., 2016). Especially freshwater ecosystems, their sediment-water interfaces (Besseling et al., 2015, 2018; Foley et al., 2018; Hurley et al., 2018), and benthic/hyporheic zones of rivers are proposed to represent MP accumulation hotspots (Frei et al., 2019; Drummond et al., 2020). The San Francisco Estuary Institute (SFEI) published a report about MPs in the San Francisco Bay-Delta, California (SFBD), in 2019. In stormwater, scientists found between 1.3 and 30 microparticles/L, mainly fragments (59 %) and fibers (39 %). Treated wastewater showed 0.063 microparticles/L, primarily polyethylene (31 %). In prey fish, the average number of MPs per fish was between 0.2 and 0.9 for non-fiber MPs and between 0.6 and 4.5 for plastic fibers (Sutton et al., 2019). In a recent investigation of plastic pollution in the SFBD conducted by Zhu et al. 2021, a comprehensive sampling campaign revealed that both urban wastewater effluent and stormwater runoff played significant roles as pathways for MPs to enter the urban bay. That study demonstrated that urban stormwater runoff with concentrations approximately 140 times higher

was a considerably larger pathway for MPs than wastewater, which predominantly consisted of fibers (Zhu et al., 2021).

Challenges in studying MPs include accurately detecting them, understanding their interactions with organisms across multiple trophic levels, and elucidating their effects on animal and human health, all of which require further investigation (Barboza et al., 2018). Various properties and behaviors of MPs in the environment must be considered: Physical (migration, sedimentation, and accumulation), chemical (degradation and adsorption), and biological behaviors (ingestion, translocation, and biodegradation) (Wang et al., 2016). Experiments of this thesis deal with three out of five challenges which were described to be of primary concern in the context of MP research (Krause et al., 2021):

- “Quantify MPs exposure hotspots and mechanisms of uptake pathways into food webs.
- Determine the importance of ecological and behavioral aspects (from color preferences to bioturbation) that affect microplastic abundance and uptake.
- Assess the impacts of freshwater MPs accumulation and associated additives, sorbed contaminants, and pathogens on the behavior and performance of host organisms and critical ecosystem functioning.”

## 1.4 Biological effects of particles

### 1.4.1 Aquatic ecotoxicology of microplastics

The properties of particles that are most relevant for ecotoxicological effects are often unclear. Unlike traditional chemical testing, where effects are tied to the substance's concentration or dose at the target site, particle toxicity may depend more on characteristics such as surface area or size distribution than on concentration alone (Potthoff et al., 2017). Generally, MPs were shown to influence reproduction, growth, immunotoxicity, neurotoxicity, photosynthesis, genotoxicity, locomotion, and feeding ability (Bhutto & You, 2022), as shown in Figure 2.

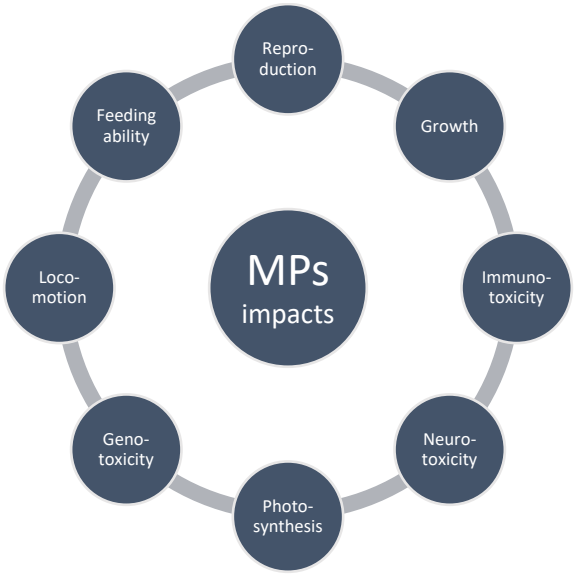


*Figure 2 MPs impacts based on Bhutto & You, 2022.*

The impacts of MPs on the functioning of aquatic ecosystems are still largely unknown (Eerkes-Medrano et al., 2015; Lambert & Wagner, 2016). MPs were described as infiltrating marine food webs via four routes: ingestion, inhalation, entanglement, and trophic transfer (Setälä et al., 2018). Laboratory investigations have confirmed that invertebrates and various fish species actively consume MP particles, which can cause immobilization in these organisms (Oliveira et al., 2013; Rehse et al., 2016; Besseling et al., 2018) and disrupt predator-prey relationships (Rochman et al., 2017). Emerging evidence indicates that MPs can extend their impact to higher levels of biological organization, leading to population and behavior shifts that can ultimately affect the ecological function of keystone species (Galloway et al., 2017). Models can be used to understand and predict the movement and retention of MPs in the environment and, consequently, the likelihood of organism-particle interaction. Often, these models assume that Stokes' settling velocity governs the movement of these particles toward the sediment bed. This velocity is influenced by particle density, size, and shape (Dietrich, 1982; Chubarenko et al., 2016; Kooi et al., 2017).

In the food web, adverse effects have been observed in specific taxa with regard to consumption, growth, reproduction, and survival as a result of MP exposure (Wright, et al., 2013a; Foley et al., 2018). Rather than presenting a collective risk, certain organisms may face a higher risk compared to others. For instance, the uptake and potential harm caused by MPs on freshwater

macroinvertebrates are influenced by their feeding strategies and developmental stages (Bhutto & You, 2022). In choice feeding experiments by Gutow et al. from 2016, periwinkles (*Littorina littorea*) did not exhibit a preference between algae with attached MPs and clean algae, suggesting that the snails do not perceive solid nonfood particles in the submillimeter size range as harmful. MPs in the fecal pellets indicate that the particles do not rapidly accumulate within the animals but are primarily expelled through feces. Therefore, seaweeds may move MPs from the water to benthic herbivores in marine environments (Gutow et al., 2016).

Invertebrates were shown to respond to MNP exposure. PS exposure had an impact on the feeding and swimming behavior of mysid shrimp (*Ninox japonica*; Wang et al., 2020). Furthermore, it has been shown that MP exposure can have age- and size-specific consequences on feeding rates, oxidative stress, and offspring production (*Neomysis awatschensis*; Lee et al., 2021). *Hyalella azteca* exposed to tire wear particles responded in a concentration-dependent manner, resulting in an LC50 of 3426 ± 172 particles/mL, while its leachate did not cause a sigmoidal dose-response curve, suggesting different toxicity mechanisms (Khan et al., 2019). Ten-day acute toxicity studies on *H. azteca* exposed to fluorescent PE MPs ($4.6 \times 10^4$ MPs/mL) and PP fibers (71.4 MPs/mL) showed that fibers are significantly more toxic and that the number of taken-up particles (63-75 µm) was concentration-dependent (Au et al., 2015). In *Daphnia magna*, no effect on survival was observed, even with guts full of PE MPs (Canniff & Hoang, 2018). PS MP exposure, however, was shown to decrease growth, inhibit the cholinergic system, and induce cell and oxidative stress in brine shrimp (*Artemia franciscana*, Eom et al., 2020). Additionally, varied effects were observed in fish. Dietary exposure to common MP types did not lead to stress (biochemistry of blood), altered growth rate, and pathology (*Sparus aurata*; Jovanović et al., 2018), while medaka larvae were shown to ingest MPs, inducing sublethal effects on growth and behavior (*Oryzias latipes*; Pannetier et al., 2020).

### 1.4.2 Particle properties and environmental factors influencing microplastic effects

Particle density is an important factor contributing to the effects of MPs. The distribution of particles in the water column is influenced by their density, which determines their proximity to various life stages or species. Polymer materials with densities greater than that of seawater tend to sink. In contrast, polymers with lower densities, which comprise approximately 46 % of plastics (Barnes & Milner, 2005), exhibit positive buoyancy (Coyle et al., 2020). In benthic zones, where high-density

particles are more prevalent, aquatic organisms come into close contact with them, thereby increasing their risk of exposure and uptake (Andrady, 2011). Sinking tests revealed that aggregates and biofouling are crucial in transporting initially buoyant MPs to the seafloor at accelerated sinking rates and in a shorter timeframe than in their free form (Porter et al., 2018). Consequently, the aggregation process surpasses biofouling's effect in promptly overcoming positive buoyancy (Porter et al., 2018).

The sizes of MPs are anticipated to resemble food particles, enabling active uptake. In contrast, smaller NPs (<1 µm), which constitute the majority in nature, may go unnoticed by fish due to their diminutive size – however, using current monitoring techniques makes it still challenging to detect NPs (Conkle et al., 2018; Roch et al., 2019). NPs raise concerns regarding their potential threat. Studies have demonstrated that NPs can undergo bioaccumulation in zebrafish across generations, as these particles can be transferred from mothers to their offspring (Pitt et al., 2018). Within that study, F0 zebrafish exhibited diminished glutathione reductase activity in their brain, muscles, and testes due to the presence of NPs in their food. Subsequently, in F1 embryos and larvae, PS NPs were detected in the yolk sac, gastrointestinal tract, liver, and pancreas. Notably, the F1 generations experienced adverse effects on bradycardia, glutathione reductase activity, and thiol levels (Pitt et al., 2018). In in vitro experiments with PS and polycarbonate nanoparticles on fathead minnows, the nanoparticles interfered with disease resistance, as, for example, shown in an increase in degranulation of primary granules (Greven et al., 2016).

Besides density and size, MPs can also function as vectors and adherence space for long-distance transfers of, for example, pathogens, bacteria, viruses (Lamb et al., 2018), and contaminants (Hartmann et al., 2017; Lamb et al., 2018). DNA from MPs in the North Adriatic was analyzed to characterize bacterial communities. Researchers identified 28 species, such as *Aeromonas spp.* and hydrocarbon-degrading bacteria (Viršek et al., 2017). Given the discovery of *Aeromonas salmonicida* on these particles, it is crucial to conduct further investigations into the implications for ingestion and the consequences of potential disease development resulting from the presence of bacteria.

Further relevant factors to consider include the influence of time and weather on the properties of MPs, which in turn affect their adsorption characteristics and impacts on the environment. MP toxicity can be altered through physical influences leading to the leaching of additives (Wagner et

al., 2014; Dris et al., 2015; Eerkes-Medrano et al., 2015;) or through, for example, weather, which can modify chemical qualities, leading to different tastes of and biofilms on particles (Savoca et al., 2016, 2017). In this context, hydrophobicity and particle size are critical determinants of the physical behavior of pristine MPs, whereas hydrogen bonding, hydrophilicity, and specific surface properties primarily influence the adsorption behavior of aged MPs (Yu et al., 2019). In addition to biofilms, plastics have the potential to undergo weathering, leading to an increase in surface area and the generation of oxygen groups. This biofilm's formation can potentially alter plastic polymers' properties, such as increasing their mass (Lobelle & Cunliffe, 2011; Zettler et al., 2013; Rummel et al., 2017) and modifying their chemical signals. These changes can contribute to an augmentation in the plastic material's charge, polarity, porosity, and roughness (Fotopoulou & Karapanagioti, 2012). In the initial stages, MPs undergo a process of surface fouling characterized by the accumulation of dissolved organic molecules, algae, bacterial cells, larvae, and spores (Andrady, 2011; Lobelle & Cunliffe, 2011). This accumulation results in the formation of a biofilm, often referred to as a "conditioning film" (Lobelle & Cunliffe, 2011; Kaiser et al., 2017), which facilitates the attachment of colonizing invertebrates and microalgae such as barnacles, tubeworms, hydroids, and mussels (Artham et al., 2009). The biofouling process leads to an overall increase in the density of MPs (Kaiser et al., 2017), and once the density exceeds that of seawater, the particles will sink (Andrady, 2011). These differences in density between MPs and seawater influence cyclic motions, which depend on, for example, collision, mortality, growth, and respiration of attached microorganisms (Kooi et al., 2017). Therefore, the position in the water column can be daytime and light dependent. Particles reach their maximum depth around midday when the combined rates of growth and collision exceed the combined rates of mortality and respiration (Kooi et al., 2017). When mortality and respiration rates are high during nighttime, particles move upward. The sinking velocity of initially buoyant particles was also shown to increase with extended incubation periods, causing more biofouling and less impact on the polymer density (Kaiser et al., 2017). The biofilm growth on plastic debris is also positively correlated with metal accumulation (Richard et al., 2019).

It was shown that there are differences between weathered and non-weathered particles, with weathered particle uptake resulting in liver toxicity and virgin MPs causing less stress in fish (Rochman et al., 2013). As an example, one of the most impactful and recent discoveries in the context of the development of polymer toxicity in the environment was a scientific study regarding 6PPD-quinone effects on Coho Salmon (*Oncorhynchus kisutch*; Tian et al., 2021). The

transformation product ("environmental fate") of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD), a widely distributed tire rubber antioxidant, caused acute toxicity for especially Coho Salmon. Following this publication, research with 6PPD-quinone was intensified to evaluate effects on other aquatic organisms such as juvenile Chinook Salmon (*Oncorhynchus tshawytscha*; Lo et al., 2023), Atlantic Salmon (*Salmo salar*) and Brown Trout (*Salmo trutta*) (Foldvik et al., 2022), and even crustaceans (Hiki et al., 2021).

### 1.4.3 Combined effects of microplastics and temperature

Alterations and combinations of stressors in aquatic ecosystems present significant challenges to organisms inhabiting these environments. Ocean warming, acidification, and deoxygenation exhibit long-lasting effects, implying that once these alterations have taken place, it could take centuries for the ocean to regain its original state (Gruber, 2011). However, our understanding of the combined effects of MNPs and temperature is still in its infancy.

Temperature change can be an additional stressor to pollutants and can influence their toxic effects. For example, the dissolved oxygen levels in water decrease with rising temperatures, meaning that organisms with high oxygen demand, such as cold-water salmonids, are more likely to be affected (Chapra et al., 2021). 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. Furthermore, if particle accumulation clogs gills, oxygen transport into the bloodstream can be additionally affected. Temperature is also a main abiotic factor influencing lipid, protein, and overall energy status (*Neomysis integer*, Verslycke & Janssen, 2002). It has been shown that temperature and PS MP exposure can lead to combined adverse effects in *A. franciscana,* and generally, higher particle concentrations and temperatures lead to higher mortalities (Han et al., 2021).

### 1.4.4 Factors affecting microplastic internalization

Aquatic organisms frequently harbor MPs in their gastrointestinal tracts, which can be acquired through various uptake pathways, such as ingestion with prey or accidental intake during foraging activities (Roch et al., 2020). The exposure levels and subsequent uptake of particles by fish and crustaceans are influenced by their positions in the water column and their feeding strategies. Feeding strategies can change during a lifespan with the additional age-specific MP effects, as

shown for marine mysid *Neomysis awatschensis* with lower tolerance levels to MPs at younger ages (Lee et al., 2021).

Knowledge regarding the sensory system of organisms concerning MNP uptake is limited. It has been suggested that visually driven planktivorous fish take up and ingest MP particles that have a similar position in the water column or resemble their natural prey (de Sá et al., 2015; Ory et al., 2017, 2018). In general, fish are especially susceptible to ingesting MPs because of their attractive coloration, similarities to food (Mizraji et al., 2017; Ory et al., 2017, 2018), and buoyancy (Jovanović et al., 2018). Chemosensory-oriented fish appear capable of discerning between inedible MPs and edible food (Roch et al., 2020). Abiotic factors such as temperature can influence taste preferences, which refer to the likelihood of particles being ingested based on the reactions of taste bud cells (Kasumyan & Doving, 2003). MP particles were spit out if not mixed with food, indicating that fish can discriminate in a gustatory way (Ory et al., 2018). Species such as *D. magna* exhibit the ability to selectively avoid consuming MPs (Aljaibachi & Callaghan, 2018), indicating sensory recognition, too.

Roch et al. (2020) conducted a study on four species (rainbow trout, *Oncorhynchus mykiss;* grayling, *Thymallus thymallus*; common carp, *Cyprinus carpio*; crucian carp, *Carassius carassius*) and found that cultured and wild fish exhibit differences in their MP uptake behavior. Wild fish ingest more food-like particles at higher concentrations, regardless of their feeding status, while particles that differ from their prey are not extensively ingested and do not increase with particle concentration. On the other hand, cultured fish consume both food-like and food-unlike particles, and their intake increases with rising particle concentrations in the water. Another distinction is that cultured fish exhibit less color discrimination and actively ingest more MPs in the absence of food compared to wild fish (Roch et al., 2020). These findings suggest that colors, in addition to foraging behavior and particle size, could be the main parameters for distinguishing between food-like and food-unlike particles, thereby influencing the likelihood of MP uptake. Possible reasons for these differences include selective breeding, which can lead to decreased discernment in food intake and epigenetic effects and adaptations prior to experiments (Marcotte, 1986; Thodesen et al., 1999; Gavery & Roberts, 2017).

The internalization of MNPs can, for example, depend on the individual species' physiology and particle properties. Regurgitation, an evolutionary adaptation mechanism to empty the stomach of

undigestible substances, is a widespread process observed in various invertebrates and crustaceans, serving purposes such as food transfer among individuals, defense behavior and suppression, and extracellular digestion (Saborowski et al., 2019). Atlantic ditch shrimp (*Palaemon varians*) has an intricate fine-meshed filter system in the stomach that can segregate particles based on size. Some of the smallest particles (0.1 µm) pass this filter system, while larger particles were expelled through the hindgut along with fecal matter. These mechanisms of the pyloric filter within the stomach likely contribute to safeguarding the midgut gland (Saborowski et al., 2022). Besides filtering mechanisms, the retention time of particles is a main factor contributing to MNP effects. The retention of MPs was described to be dependent on food availability (Cole et al., 2013; Watts et al., 2014), particle shape (Murray & Cowie, 2011), and especially particle size (Galloway, 2015). So far, research has primarily focused on animal interactions through dietary ingestion, but recent findings reveal that MPs can also adhere to biota via bioadhesion, impacting their abundance and bioavailability in the environment, with potential ecotoxicological effects yet to be fully understood (Kalčíková, 2023).

Once particles are taken up, their ingestion is described to lead to effects such as nutritional depletion, physical damage, suffocation, and gut blockage (Jovanović, 2017). Today, we have limited knowledge about particle size-dependent internalization: Particles <5 µm can pass the gastrointestinal tract wall and accumulate in long-living species (Roch et al., 2020). Brine shrimp larvae consume 10 µm PS spheres, egest 97 % after 3 hours, and show deformed intestinal epithelia (Wang et al., 2019); particles >20 µm were shown to not translocate into tissue (Devriese et al., 2015); short term exposures of 3 hours on grass shrimp (*Palaemonetes pugio*) showed, that spheres and fragments <50 µm are not acutely toxic, while fibers of 93 µm length cause higher mortalities than smaller sizes with a residence time of particles in the gut being 43 ± 14 h, and in the gills 37 ± 5 h (Gray & Weinstein, 2017). These differences in size and shape effects require studies covering more MP properties.

Besides the digestive tract, gills are another tissue influenced by MP exposure. In a study conducted by Watts et al. (2014), it was discovered that the shore crab (*Carcinus maenas*) exhibited the ability to respire PS microbeads that had accumulated on the surface of their gills (Watts et al., 2014). Similarly, blue mussels (*Mytilus trossulus*) and Baltic clams (*Macoma balthica*) demonstrated the capability to accumulate MPs on their gills after 24 hours of incubation, although the concentrations of beads were notably higher in the digestive tracts of the same animals (Setälä

et al., 2016). Consequently, regarding scale effects, the uptake of MNPs into the digestive system is of great interest.

## 1.5 Multiple stressor scenarios

Survival probabilities are reduced if animals cannot adapt or deal with a particular stressor or stressors. Some biotic factors influencing survival include food availability, diseases, predation, and intraspecies relationships. Abiotic factors include temperature, oxygen, salinity, and contaminants. Under these conditions and during exposure to stressors, organisms can improve their performance and tolerance levels (Schulte, 2011; McBryan et al., 2013). The deciding factors for declining or increasing performance are the intensity and duration of stressors while also being dependent on specialization, performance capacity, and acclimation of the organism. Threats like land subsidence, sea level rise, and anthropogenic climate change are challenging organisms worldwide (Scavia et al., 2002; Harley et al., 2006). Published studies describe five major sources of stressors and threats for aquatic environments: Habitat destruction and degradation, overexploitation, flow modification, invasive species, and environmental pollution (Kennish, 2002; Dudgeon et al., 2006; Geist, 2011). If organisms are unable to enhance their performance and tolerance levels in response to rapidly changing environments, this will pose a significant threat to the survival of their species.

Stressors normally do not occur singly in an environment; rather, there is a complex interplay of many stressors leading to multiple stressor scenarios (Breitburg et al., 1998; Schindler, 2001). Organisms can adapt to changing environments or employ phenotypic plasticity, which means that organisms can show various phenotypes in response (Agrawal, 2001), with some of them surviving. From the five major stressor sources, it is known that rising temperatures influence swimming behaviors and predation (Davis et al., 2019); salinity influences embryogenesis, life cycles (Romney et al., 2019), and stress-related gene expressions (Hasenbein et al., 2016); turbidities influence predation (Bruton, 1985), feeding conditions of larvae (Baskerville-Brldges et al., 2004), survival (Hasenbein et al., 2016), and swimming behavior (Hasenbein et al., 2013).

## 1.6 Target species

Generally, in the food web, fish and invertebrates are interconnected through predator-prey relationships. Invertebrates typically occupy the role of primary or secondary consumers. They feed on detritus, algae, and smaller plankton, converting these materials into biomass. Fish, particularly smaller or juvenile species, often prey on invertebrates. This predation transfers the accumulated energy and nutrients up the food web, which shows that invertebrates are an essential link, facilitating energy flow from primary producers and detritus to higher trophic levels, including fish (Dauby et al., 2003). The Longfin Smelt (*S. thaleichthysis*), *H. azteca*, and *A. bahia* represent two levels of the aquatic food web interconnected through scale effects (Figure 3). In laboratory experiments, Longfin Smelt, a locally sensitive and endangered fish species, was studied under environmentally relevant turbidity conditions alongside invertebrate model species exposed to both natural and synthetic particles.

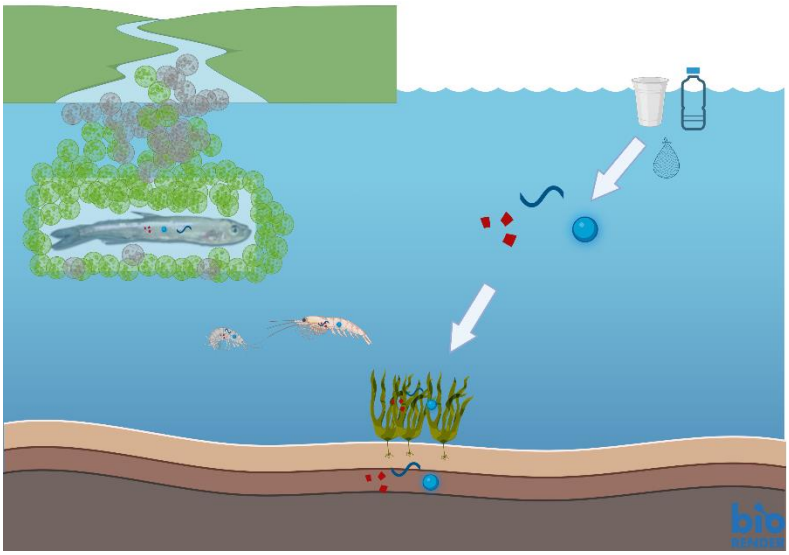

*Figure 3 Synthetic and natural particles impact aquatic ecosystems in distinct ways. Plastic waste breaks down into particles that are ingested by invertebrates and subsequently bioaccumulate in fish. Turbid conditions, caused by factors such as algae and clay, can impair visual perception and alter species interactions. Created with BioRender.com*

Two examples of threatened species, namely the Delta Smelt (*H. transpacificus*) and Longfin Smelt, possess ecological value as indicator species despite their lesser socio-economic significance in the California Delta. Despite efforts by researchers and fisheries management, the declining populations of these species over the past decades have yet to be effectively reversed,

indicating the need for further action to prevent their extinction. Longfin Smelt is one of the most rapidly declining fish species, but it is unknown why (Baxter, 1999; Moyle, 2002; Rosenfield & Baxter, 2007). Conservation efforts for the Longfin Smelt have been based on successful captive rearing of Delta Smelt; however, there are subtle differences in the habitat requirements for both species that require investigation. The pelagic Longfin Smelt can reach 90-110 mm in standard length and has a semelparous 2-year life cycle (Moulton, 1974). This osmerid is a foraging species inhabiting lakes, estuaries, and the Pacific Ocean (Dryfoos, 1965). In 2009, the Longfin Smelt found along the Pacific coast of North America, including the SFBD, was listed as threatened but precluded under the California Endangered Species Act 2009 (CDFG 2009). Understanding their life cycle is crucial to determining the effects of stressors. Larval and juvenile stages have been observed in smaller estuaries in which they likely spawn and rear (Lewis et al., 2020). In summers in the estuary, the juvenile occurrence was recorded as fisheries by-catch or in monitoring surveys, indicating that they moved into higher salinity waters at that time (Rosenfield & Baxter, 2007; Garwood, 2017). Little is known about their oceanic habits, which influence population structure and demography in various ways during their life cycle (Sağlam et al., 2021). After returning to freshwater and low-salinity habitats in the SFBD, they spawn at the end of their life cycle. In the estuary populations, abundance is negatively influenced when freshwater flows are reduced (Moyle, 2002; Feyrer et al., 2007; Sommer et al., 2007), which influences turbidities and carrying capacity for pelagic fishes such as the Longfin Smelt (Kimmerer et al., 2000). The population size in the estuary dropped to approximately 1 % of their historical (pre-1980) abundances with potentially trophic scale effects (Feyrer et al., 2007; Garwood, 2017; Hobbs et al., 2017; Sağlam et al., 2021). Threats to this species include changes in food webs, contaminants, disease, competition, introduced species, predation, anthropogenic influences such as habitat loss and fragmentation due to channelization and levees, loss of genetic diversity, and climate change. Simultaneous effects of physical conditions, such as freshwater inflow or drought, and biological conditions, such as the introduction of the Asian clam (*Potamocorbula amurensis*) in 1986 (Carlton et al., 1990) make the determination of causes for declines challenging.

The SFBD, home of Longfin Smelt, is a heavily altered ecosystem designed to meet California's water supply needs, featuring levees and conveyance structures that extend over 1000 miles. (Lee et al., 2021). Runoff from over 40 % of California’s land area gets introduced into the SFBD, causing 50 % of California’s runoff to be transported in this system (Conomos, 1979; Nichols et al., 1986). The suspended-sediment concentration, which correlates with water turbidity,

decreased from 1991-1998 to 1999-2007, which can occur “when the threshold from transport to supply regulation is crossed as an erodible sediment pool is depleted” (Schoellhamer, 2011). Over the past few decades, within the SFBD, there has been a significant 36 % reduction in turbidity levels, primarily attributed to factors including sediment retention in reservoirs and dams, protective measures for riverbanks, and the reduction of erodible sediment resulting from hydraulic mining activities (Wright & Schoellhamer, 2004; Schoellhamer, 2011). This decline in turbidity is believed to be one of the contributing factors to the Pelagic Organism Decline, which has had significant repercussions on the population of Delta Smelt (Sommer et al., 2007). Mixing zones in estuaries cause high variations of flow, salinity, temperature, and turbidity - all major factors contributing to threatening sensitive fish species such as Longfin Smelt.

Many benthic consumers in aquatic environments, such as gammarids and amphipods, play crucial roles as ecosystem engineers in sediment habitats. These organisms are highly exposed to MNPs, chemical additives, sorbed contaminants, and potential microbial pathogens (McCormick et al., 2014; Frère et al., 2018). Consequently, a significant risk of wide-ranging impacts exists, particularly on the functioning of benthic ecosystems (Izvekova & Lvova-Katchanova, 1972; Ward & Ricciardi, 2007). Benthic fauna may ingest MPs suspended in the water column, particularly filter feeders (e.g., zebra and quagga mussels). This ingestion carries the risk of transferring MPs to the benthic food web through their presence in feces or pseudofaeces (Lederer et al., 2006; Iversen & Poulsen, 2007; Cole et al., 2016; Wieczorek et al., 2019). Biofouling increases MPs' nutritional value, leading to grazing and ingestion (Wright, et al., 2013a,b; Van Cauwenberghe et al., 2015; Vroom et al., 2017).

As bottom feeders, *H. azteca* (Saussure, 1858) are essential players in the food web, contributing to bioaccumulation, scale-, and magnification effects. MPs have an additional pathway to enter freshwater food webs by becoming trapped within benthic or hyporheic biofilms (Sgier et al., 2016). These biofilms serve as sites for MP accumulation, potentially making them more accessible to other organisms, such as biofilm grazers (Mccormick et al., 2016). As a sensitive model organism commonly used by the U.S. Environmental Protection Agency for toxicity tests, exposure studies are carried out at their preferred water temperatures of 23.8 to 27.3 °C (Javidmehr et al., 2015). The freshwater amphipod, known for its ability to thrive in both fresh and brackish water environments, exhibits omnivorous grazing and deposit-feeding behavior (Strong, 1972). These characteristics render them suitable and compelling subjects for MP research, which often focuses

on *H. azteca* and related organisms. Widely distributed in freshwater environments, *H. azteca* is commonly found and has a diverse diet encompassing particles within MPs' size range. Fluorescent particles can be used to investigate bioaccumulation in *H. azteca* in a time-efficient and cost-effective analytical way (Kuehr et al., 2022). This approach was carried out with *H. azteca* exposed to fluorescent PE particles and PP fiber to determine the LC50 over ten days, with the result that fibers (71.43 MPs/mL) were significantly more toxic than spheres ($4.64 \times 10^4$ MPs/mL) (Au et al., 2015). *D. magna* exposed to 63-75 µm fluorescent green PE microbeads ingested particles in high amounts, exposure time- and concentration-dependent; however, this did not affect survival and reproduction (Canniff & Hoang, 2018). It was even described that the beads could have functioned as substrates for algae and, therefore, provide energy (Canniff & Hoang, 2018). *Gammarus roeseli* coexposed to phenanthrene and MP beads caused reduced bioavailability (Bartonitz et al., 2020), indicating that the fate and chemistry of particles determine the binding of cofactors and finally modulate toxic effects. Furthermore, it was shown for *G. roeseli* that particles up to 1000 nm did not affect swimming velocity (Götz et al., 2022). Therefore, non-environmentally relevant concentrations can be more suitable for investigating mechanistic responses.

The third study organism, *A. bahia* (formerly *Mysidopsis bahia*), is commonly used in toxicology studies because of its short life spans, early sexual maturity, ease of culture, and sensitivity (Nimmo, 1977; Lussier et al., 1999; Hirano et al., 2004). Mysids are especially suitable for studying particle effects because they feed on larger prey and passively filter small particles, such as phytoplankton and particulate organic matter (Viitasalo et al., 1998). They were first described in 1969 in Galveston Bay, Texas (Molenock, 1969). Since then, they have been used for toxicity tests by, for example, the U.S. Environmental Protection Agency (Stephen et al., 1985). Also, their high temperature tolerance makes them suitable test organisms in the context of climate change; their brood durations, for example, can take 15.5 days at 16 °C to 4.6 days at 29 °C, and survival and growth depend on temperature (Lussier et al., 1999; Wortham-Neal & Price, 2002). Besides survival, growth and fecundity are described as valuable endpoints in 7-day exposure studies (Lussier et al., 1999). Mysids play an essential role in estuarine plankton and are an important keystone species in estuarine and marine food webs (de Almeida Prado, 1973), making them suitable for MP research.

## 1.7 Goals and objectives

Gaining knowledge about the impacts of natural and synthetic particles on an individual level, including evaluating different polymer types, can provide valuable insights for assessing ecotoxicological effects, preventing threats to species, and implementing effective water protection measures. This knowledge can ultimately benefit species conservation efforts. To fully understand the effects of particle exposure and uptake, their toxicology and environmental chemistry, as well as their impacts on the ecological level, need to be considered. The approaches detailed in this dissertation can establish a foundation for determining the value of various tools, such as endpoint selection, within the context of particle research. Specifically, the following objectives were addressed to test hypotheses:

**Objective 1** sought to elucidate the effects of natural particles, specifically turbidity, on the growth and stress-related gene expression of Longfin Smelt, considering temperature variations as an abiotic factor. Turbidity, indicative of suspended particles in water, can affect light penetration predator-prey relationships and, thus, primary productivity, which in turn influences the growth and physiological responses of aquatic organisms. Temperature, a critical factor in aquatic ecosystems, can modulate organisms' metabolic rates and stress responses. By examining how turbidity and temperature affect growth and stress-related gene expression of juvenile Longfin Smelt, this objective aims to provide insights into the complex relationships between environmental variables and biological responses.

Hypothesis 1.1: Because of larval stage preferences, a temperature of 11 °C would yield accelerated growth rates compared to those at 14 °C for the juvenile stage.

Hypothesis 1.2: Given that the rearing and acclimation occurred closer to 11 °C, less pronounced transcription levels in stress- and growth-related genes would be expected at 11 °C when compared to treatments conducted at 14 °C.

Hypothesis 1.3: Since juvenile Longfin Smelt exhibits a migratory behavior and occurs in clearer ocean waters, turbidity conditions within the range of 1-11 NTU would result in limited impacts on growth and the expression of examined genes for this life stage. This would suggest that tested

turbidities fall within a shared tolerance range. Nonetheless, a probable threshold response to turbidity is anticipated, with an optimal range expected between insufficient and excessive levels.

**Objective 2** focused on the temperature-dependent uptake of fluorescent PS beads, mimicking plastic particles of varying sizes (500 nm and 1000 nm), and its potential associations with behavior and growth endpoints in *H. azteca*. MNP pollution is a growing concern in aquatic environments, and understanding the factors influencing uptake by organisms is crucial. Temperature variations can affect the physiological processes governing particle ingestion and assimilation in aquatic organisms. By characterizing the temperature-dependent uptake of MNPs and investigating their impacts on behavior and growth endpoints, this objective aims to uncover potential correlations between particle exposure and adverse effects on *H. azteca* health.

Hypothesis 2.1: Elevated temperatures influencing metabolic rates are predicted to increase particle uptake, leading to growth reduction and the manifestation of stress-related swimming behaviors.

Hypothesis 2.2: Due to the altered translocation ability of smaller particles, they are anticipated to demonstrate prolonged retention times, thereby exacerbating their detrimental effects.

**Objective 3** aimed to compare the behavioral and oxidative stress-related effects of different types of particles, specifically natural and synthetic fibers, on *A. bahia*. Synthetic fibers, commonly found in the environment, and natural fibers, such as those from organic matter breakdown, may elicit distinct physiological responses in aquatic organisms. By comparing the effects of these particle types on *A. bahia* behavior and oxidative stress levels, this objective seeks to discern potential differences in their toxicity profiles and ecological implications.

Hypothesis 3.1: Based on related studies, cotton, nylon, polyester, and hemp fiber exposures have more pronounced effects on *A. bahia* and investigated endpoints than fragment exposures.

Hypothesis 3.2: Higher temperature treatments will amplify the stress-related effects of the exposure treatments.

Overall, it was hypothesized that temperature acts as a modifier of particle impacts, and that uptake correlates with stress responses. This research endeavors to advance the understanding of how the environmental factor temperature interacts with particle effects to influence aquatic organisms' growth, behavior, and further stress responses. Such insights are essential for devising effective conservation and management strategies aimed at mitigating the impacts of anthropogenic activities on aquatic ecosystems.

# 2. General methods

To address our objectives, various setups and endpoints were utilized, employing a suite of methods ranging from the molecular to the individual level. Further methods are described in more detail in the first authored papers (Biefel et al., 2024a,b,c) in the appendix and in related co-authored papers (Segarra et al., 2021; Pasparakis et al., 2023).

## 2.1 Experimental setups

To test the hypotheses, a range of experimental setups involving fish tanks and beakers, each equipped with precise temperature control systems to ensure environmental consistency, were employed. The experimental protocols included daily feeding regimes tailored to the specific dietary requirements of the test organisms to maintain their health and normal metabolic functions throughout the studies. Additionally, regular mortality checks were conducted to monitor the survival rates and assess any potential impacts of the experimental conditions on the organisms. The fish tanks and beakers (Figure 4) were designed to reduce external influences, with regulated water quality parameters and temperature conditions that matched the organisms' native habitats. Beakers in environmental test chambers were used for controlled experiments, allowing for the precise manipulation of experimental variables and detailed observation of individual responses such as growth.

*Figure 4 Experimental setup for Longfin Smelt at UC Davis, featuring algae transported from external tanks into buckets via pipes to regulate turbidity (left). Beaker arrangement in temperature-controlled chambers to minimize external influences on H. azteca (right).*

Experiments were conducted at both the University of California Davis, and Oregon State University. Both provided state-of-the-art facilities and resources necessary for conducting high-quality, reproducible research. Collaboration between researchers at these institutions facilitated a comprehensive investigation by leveraging their combined expertise and technical capabilities. Experimental designs ensured robust and reliable data collection, enabling the thorough testing of hypotheses and the drawing of meaningful conclusions.

## 2.2 Molecular and biochemical approaches

Sensitive molecular endpoints are valuable to determine the sublethal effects of particles. The methods employed concentrated on gene expression analysis and the measurement of reactive oxygen species (ROS). Quantitative PCR was extensively utilized (e.g., Cole et al., 2016; DeCourten et al., 2019) to evaluate which cellular pathways were impacted by stressors like turbidity and temperature. By quantifying specific functional RNA strands, the potential production of corresponding proteins, providing insights into cellular responses such as cortisol production, were inferred. Cortisol, a primary hormone produced during stress, indicates the activation of the hypothalamic-pituitary-interrenal (HPI) axis. Stress responses also trigger the production of ROS, which are byproducts of cellular oxidative metabolism. ROS play significant roles in cell survival, cell death, differentiation, cellular signaling, and the production of inflammatory factors

(Livingstone, 2001; Bergamini et al., 2004; Bayir, 2005; Lesser, 2006; Krumova & Cosa, 2016). The utilized kit employed a fluorescent dye that reacts with ROS, generating a fluorescent signal detectable at 520 nm using a microplate reader. These molecular endpoints offer a detailed understanding of how organisms respond to environmental stressors at the cellular level, highlighting specific biochemical pathways and processes affected by temperatures and particles.

## 2.3 Swimming behavior

Locomotion is an essential aspect of an organism's overall health and functionality, and changes in behavior can serve as sensitive indicators of environmental stressors. The swimming activity of individual organisms was recorded in well plates and tracked by a camera using a computer-assisted video analysis system (EthoVision from Noldus) (Figure 5). Movement alterations can indicate neurotoxic effects or metabolic disruptions caused by various pollutants, including pesticides, heavy metals, and pharmaceuticals. In particular, there are four key domains where swimming behavior plays a crucial role, as elucidated by Dodson et al., 1997: 1. Swimming behavior assays serve as the foundational mechanism for population-level behavior, including horizontal and vertical migration; 2. Individual behavior holds significance in shaping the outcomes of predator-prey interactions, particularly within the pelagic environment, where prey movement serves both as a cue to predators (Brewer & Coughlin, 1995) and a determinant of encounter rates (Gerritsen & Strickler, 1977); 3. The individual feeding rate may have a connection with swimming behavior; and 4. Toxic substances, whether natural or anthropogenic, that influence swimming behavior can indirectly affect the pelagic (Dodson et al., 1995) and benthic (e.g., Hopkins et al., 2003; Lamberson et al., 2018) communities.

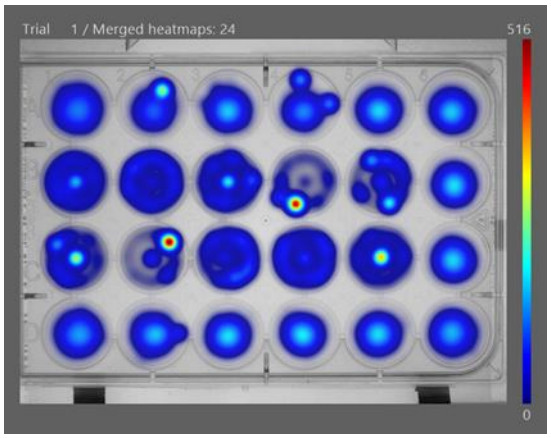


*Figure 5 Heatmap depicting swimming activity in a well plate over time.*

## 2.4 Confocal microscopy

The “Advanced Imaging Facility” at UC Davis has provided a state-of-the-art confocal microscope, a crucial tool for studying particle uptake in biological systems. This microscope utilizes focused laser beams to generate high-resolution, three-dimensional images of organisms' digestive tracts, enabling the observation and analysis of the uptake of labeled particles. Using ImageJ software, confocal images were quantitatively analyzed to measure particle fluorescence intensity and assess uptake efficiency (Figure 6).

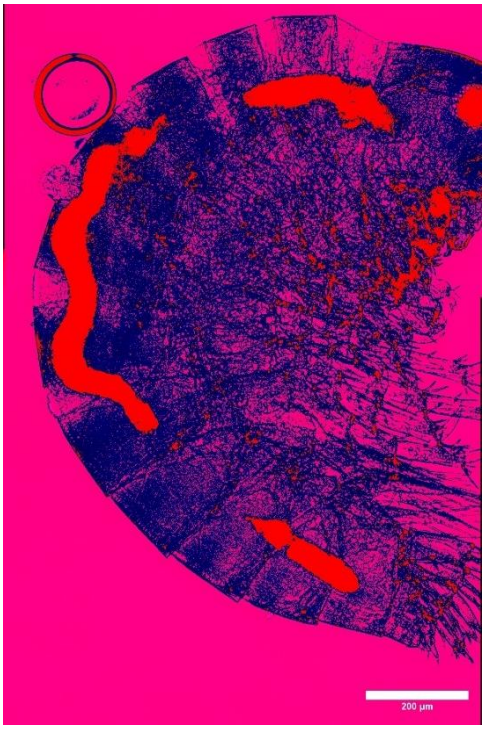


*Figure 6 Fluorescent particles visualized using confocal microscopy.*

## 2.5 Statistics

In the statistical analysis, when comparing groups, typically, ANOVA for parametric data and the Kruskal-Wallis test for non-parametric data were employed if ANOVA assumptions were not met. ANOVA assessed differences in means when assumptions like normality and homogeneity of variances were satisfied. Conversely, Kruskal-Wallis evaluated differences in medians and was robust to violations of ANOVA assumptions, which was especially the case for behavioral data. Both tests provided p-values indicating significant ($p < 0.05$) group differences, with post-hoc tests often utilized for further analysis. This standard approach ensured robust comparisons across groups while considering the distributional properties of the data.

# 3. Results

## 3.1 Study 1: “Turbidity and temperature effects on growth and gene transcription of threatened juvenile Longfin Smelt (*Spirinchus thaleichthys*)”

Investigating how Longfin Smelt physiology and stress responses are affected by different environmental factors like temperature and turbidity is essential for their effective management, cultivation, and conservation. This study evaluated the performance of juvenile Longfin Smelt (181 to 228 days post-hatch) in terms of growth and gene expression after four weeks of exposure to two temperatures (11 °C and 14 °C) and three turbidity levels (1, 4, and 11 NTU). Post-exposure, measurements of fork length, wet weight, condition factor, and the transcription of 12 genes related to osmoregulation, growth, metabolism, and stress response were conducted. Results indicated greater growth and condition factors at the lower temperature (11 °C), with turbidity showing no effect on growth, condition factor, or transcriptomic stress response. However, reduced expression of *Catalase*, *Citrate Synthase*, and *Growth Factor Receptor Bound Protein* 10 at 14 °C suggested temperature-related metabolic and growth changes. These findings imply that rearing juvenile Longfin Smelt at 11 °C and low turbidity (<11 NTU) is suitable, while slightly higher temperatures limit growth and metabolic capacity.

Turbidity levels up to 11 NTU may fall within the same tolerance range for Longfin Smelt, which could explain the lack of observed differences in their genomic responses. This suggests that the turbidity levels tested did not exceed the threshold where significant physiological changes would occur. Alternatively, it is possible that the turbidity variations within this range did not influence the specific biological pathways investigated. Another potential explanation is that the acclimation period of four weeks was adequate for the organisms to adapt to the different turbidity levels, thus minimizing any detectable effects. Consequently, the observed responses may reflect the organisms' capacity to maintain homeostasis across the range of turbidity conditions tested.

*My role in this study* encompassed several key aspects, including the building of fish tanks, daily fish care, sample collection, primer design, qPCR execution, statistical analysis, and manuscript writing.

## 3.2 Study 2: "Polystyrene Plastic Particles Result in Adverse Outcomes for *Hyalella azteca* When Exposed at Elevated Temperatures"

This study investigated the impacts of particle uptake, size, and temperature on the amphipod *H. azteca*. Organisms were exposed to blue-fluorescent PS beads (500 nm and 1000 nm in diameter) at a concentration of 0.43 mg/L for 96 hours at three different temperatures (21, 24, and 27 °C). Survival and growth were assessed, along with particle uptake visualized through confocal microscopy and analyses of swimming behavior post-exposure. Mortality rates increased significantly at 27 °C, with both particle presence and temperature affecting growth. Behavioral responses varied with particle treatments: Reduced activity was observed with 1000 nm particles, while both reduced and increased activity were observed with 500 nm particles. Particle uptake levels varied across individuals of one beaker and increased with temperature in the 500 nm treatments, but no migration beyond the gut was detected. Particle size correlated with uptake and was associated with changes in behavior.

These findings underscore that particle size and temperature interact to influence physiological and behavioral responses in the model species *H. azteca*. The elevated temperatures not only intensified the adverse effects of particle exposure but also highlighted the potential for synergistic impacts on aquatic organisms. This research underscores the urgent need to address plastic pollution within the broader context of climate change, as both factors have the potential to collectively pose significant risks to aquatic health and ecosystem integrity.

*My role in this study* encompassed designing the experiment, daily animal care, running behavioral assays and confocal microscopy, measuring size and weight, statistical analysis, and manuscript writing.

## 3.3 Study 3: “Interactive effects between water temperature, microparticle compositions, and fiber types on the marine keystone species *Americamysis bahia*”

The interplay between rising water temperatures, oxidative stress, and the presence of microfibers presents significant threats to marine ecosystems, potentially compromising the health and stability of aquatic food webs. However, studies on the effects of fibers are rare. This study examined the mysid shrimp *A. bahia*, a key species in estuarine and marine food webs, across four experimental trials involving different fibers (cotton, nylon, polyester, hemp at 3 particles/mL, approximately 200 µm in length) and fragments (low-density polyethylene (LDPE), polylactic acid (PLA), and their leachates at 5, 50, 200, 500 particles/mL, 1-20 µm). Three temperatures (22, 25, and 28 °C) were investigated to assess the effects in a climate change context.

Post-exposure growth and swimming behavior were evaluated, with additional assessments of reactive oxygen species and particle uptake after microfiber exposure. Swimming behavior was also assessed in an oxygen-depleted environment to simulate the physical impact of microfiber exposure, such as gill obstruction. Results indicated minimal growth effects from fragment exposure, with PLA leachate increasing activity at 25 °C and 28 °C and LDPE exposure reducing activity at 28 °C. Cotton exposure resulted in fewer behavioral differences compared to controls and other fibers, while hemp fiber exposure significantly elevated ROS levels at 28 °C. Microfibers were primarily found in the gastric and upper gastrointestinal tract, suggesting prolonged residence and potential obstruction.

The results suggest that fibers, depending on the polymer type, have a more significant impact than fragments and can exacerbate oxidative stress, which is particularly problematic given the increasing water temperatures.

*My role in this study* encompassed designing the experiment, preparing the particle solutions, daily animal care, running behavioral and oxidative stress assays, measuring size and weight, statistical analysis, and manuscript writing.

# 4. General discussion

This thesis aimed to deepen the understanding of particle effects on aquatic organisms amidst rising temperatures globally. As the concentration of greenhouse gases increases, atmospheric temperatures rise, leading to climate change and further elevating water temperatures (Ledley et al., 1999; Bijma et al., 2013; Röck et al., 2020). According to emission forecasts, global average surface temperatures are projected to rise by approximately 1.8 °C to 4.0 °C over the next century (Solomon et al., 2007; Priya et al., 2023). Existing research suggests that global climate change will influence the fate, distribution, and toxicity of contaminants with potential long-term impacts on aquatic environments, yet current regulatory and monitoring efforts based on established guidelines often fail to account for these changes, leaving most aquatic risk assessments and regulations inadequate in addressing climate-induced alterations in pollution dynamics (Hutton et al., 2024). Understanding species responses is crucial for developing effective conservation strategies and management practices aimed at protecting ecosystem stability and services. For instance, identifying species and ecosystems most vulnerable to these combined stressors can inform targeted interventions, such as habitat restoration, pollution control measures, and climate adaptation plans. Moreover, comprehensive research on these combined effects can guide policymakers in revising water quality standards and regulations to address the synergistic impacts of climate change and particle pollution, ensuring the long-term health and resilience of aquatic environments.

Three studies systematically investigated the impacts of natural and synthetic particles, in conjunction with temperature, on two trophic levels: Fish and invertebrates. The investigation involved three laboratory experiments, each focusing on distinct stressor combinations, including natural algal cells as particle sources for turbidity (study 1), synthetic polystyrene MNPs (study 2), and synthetic fragments plus synthetic and natural fibers (study 3). Results revealed that particles and temperature have the potential to interact, leading to adverse outcomes. Specifically, the increased mortality of *H. azteca* exposed to PS MNPs at 27 °C compared to controls and lower temperatures indicates lethal, synergistic effects. However, lower concentrations of MPs tested on *A. bahia* did not lead to lethal but sublethal effects. Findings, especially of study 2, indicate that both temperature and particles can affect various physiological endpoints in aquatic organisms, including survival, growth, swimming behavior, and uptake quantity. Furthermore, natural and synthetic particles can impact species' physiology in quite different ways. While turbidity is known

to play a crucial role in interspecies relationships, MNPs can have more direct and detrimental effects, such as intestinal damage due to uptake. For study 1 on Longfin Smelt, the investigated expression of genes involved in osmoregulation, growth, metabolism, and stress response were not significantly impacted by turbidity levels up to 11 NTU, suggesting these turbidity levels lay within the species' tolerance zone, confirming Hypothesis 1.3. However, the higher temperature of 14 °C led to reduced growth and condition factors, indicating a preference for cooler water at 11 °C, which confirms Hypothesis 1.1. The lower temperature of 11 °C, however, was not conclusively shown to have positive effects measured by gene expression (Hypothesis 1.2). The uptake of fluorescent MNP PS beads by *H. azteca* in study 2 was found to be higher at elevated temperatures. This increased assimilation correlated with higher mortality, reduced growth, and altered swimming behavior, highlighting a temperature-mediated impact on plastic particle assimilation, confirming Hypothesis 2.1. Although the concentrations used were not environmentally relevant, the results demonstrate that a combination of PS beads and higher temperatures can pose a threat to *H. azteca*. Additionally, different particle sizes triggered distinct behavioral responses, indicating that the physiological and behavioral impacts of MNPs vary with particle size. Smaller particles were, however, not ultimately confirmed to translocate more easily and, therefore, would lead to increased detrimental effects (Hypothesis 2.2). Regarding phenotypic plasticity and adaptation strategies, we observed significant variability in the quantities of particles in the guts of *H. azteca*. These differences among individuals within the same beaker could have contributed to the high variability in growth and behavioral responses. Comparing the effects of natural and synthetic fibers on *A. bahia* revealed that cotton fibers, being biodegradable and composed of natural cellulose, caused relatively less oxidative stress and fewer behavioral changes than hemp and synthetic fibers. This reduced stress response to cotton could be attributed to its more inert nature in aquatic environments, leading to minimal disruption of cellular processes. In contrast, hemp fibers induced a higher stress response, likely due to their complex structural composition, which can increase ROS production and subsequent oxidative damage within cells. While fiber exposure was indicated to have more pronounced effects than fragments (Hypothesis 3.1), higher temperature treatments amplified the stress-related effects of exposure treatments (Hypothesis 3.2).

## 4.1 Natural vs. synthetic particles

The three experiments aimed to investigate both natural (e.g., cotton fibers, algae) and synthetic (e.g., PS, PLA) particles, a comprehensive approach that addresses significant gaps in current research. While many studies focus exclusively on MNPs, there is a notable deficiency in research examining the impacts of turbidity. However, elevated turbidity has the potential to decrease fish species richness and diversity while increasing the abundance of benthic species that rely on chemoreception for foraging and predator avoidance, potentially shifting food webs toward predators that utilize non-visual sensory modalities, thereby altering the overall food web structure (Lunt & Smee, 2020). The interaction between natural or synthetic particles with temperature is additionally rarely investigated, despite their potential compounded effects on aquatic ecosystems. This knowledge gap highlights the need for integrative approaches that consider multiple stressors to better reflect natural conditions and provide a more holistic understanding of particles' ecological implications. Although these experiments utilized different setups, species, particle types, and temperatures, results revealed that while low levels of turbidity did not negatively impact fish at either of the tested temperatures, MNPs exhibited discernible effects, specifically on physiological and behavioral changes in invertebrates, such as altered swimming patterns and signs of oxidative stress. These effects were temperature-dependent, with increased temperatures exacerbating the adverse impacts of MNP exposure.

The evaluation of effects resulting from exposure to different particle types needs to take particle concentrations into account. In an experiment by Zink et al. 2024, *D. magna* was exposed to the same concentration of bentonite (natural) and PE MPs. In bentonite treatments, daphnids maintained feeding efficiency and increased digestive activity compared to controls; however, those exposed to MPs showed decreased feeding efficiency and increased peristalsis without increased expulsion, indicating that MPs do not pass through the digestive tract as effectively as bentonite (Zink et al., 2024). This result is confirmed by a meta-analysis showing that MPs can be up to eight times more detrimental than suspended sediments (Ogonowski et al., 2023). These outcomes, however, also depend on factors such as particle quality (e.g., size, concentration) and target species (e.g., position in the food web), as it has been confirmed that at high concentrations of 1,000 mg/L, both natural and plastic materials exhibit a similar capacity to inhibit the growth of unicellular alga *Raphidocelis subcapitata* (Gorokhova et al., 2020).

Analogous to natural particles, MNPs are not classical contaminants influencing distinct molecular and physiological pathways. Particle properties can change dynamically over time due to interactions in the environment. Biotic interactions, including biofouling (Fazey & Ryan, 2016; Kaiser et al., 2017), egestion (Cole et al., 2013, 2016), and bioturbation (Näkki et al., 2017), as well as physical processes like fragmentation (Andrady, 2017), were shown to influence characteristics and movement of plastics. Studies indicate that *H. azteca* can fragment particles through its digestive system, which includes a gastric mill for food crushing (Schmitz & Scherrey, 1983; Rani-Borges et al., 2023). Increased metabolic rates and feeding demands at the highest temperature in studies 2 and 3 possibly accelerated particle breakdown, leading to altering effects over time due to changes in particle dimensions. Addressing critical aspects of MNPs, including their physicochemical properties, toxicological concerns, and bioavailability, is imperative, given the challenges in interpreting organisms' biological responses to particles (Chouchene et al., 2023).

## 4.2 Organismal responses to multiple stressors in the context of climate change

In dynamic ecosystems like the SFBD, characterized by fast and significant modifications in its physical, chemical, and biological components (Kimmerer, 2002; Cloern & Jassby, 2012), aquatic organisms face great challenges due to swiftly shifting conditions that challenge the adaptability of organisms to multiple stressors. The effects of multiple stressors are intricate and capable of interacting synergistically, additively, or antagonistically, thereby resulting in cumulative impacts on species habitats, exceeding the sum of individual stressor effects. Stress can, for example, influence metabolic and growth rates due to resource investment into immunity and defense mechanisms (Schreck & Tort, 2016). Stressors influence species performance and contribution, resulting in adaptations and restrictions to a particular range of environmental conditions (Hooper et al., 2008). As it relates to turbidity and organismal requirements, through applying the conceptual model of the law of tolerance (Shelford, 1931), it becomes evident that turbidity ranges below 11 NTU represent the same tolerance zone of juvenile Longfin Smelt (study 1). Integrating further physiological responses at sublethal levels with whole organism endpoints across a wider range of turbidities is essential to accurately delineate distinct tolerance zones and to understand the stress responses of Longfin Smelt, which is important for conservation and aquaculture management.

As highlighted by Segner et al. (2014), the critical concern lies in understanding how biological or ecological receptors react to the concurrent presence of multiple stressors. In data across all three experiments, observed responses often did not change linearly to the experimental stressors. These outcomes are explainable because the effects of stressors can be non-monotonic and ultimately lead to differences from single-stressor effects (Segner et al., 2014). A review of 88 papers by Jackson et al., 2016 on freshwater ecosystems calculated 41 % antagonistic, 28 % synergistic, 16 % additive, and 15 % reversed effects; the overall average net effect of warming combined with a second stressor being antagonistic, which contradicts the greater prevalence of reported synergies in marine systems. The synergy of temperature and PS particles observed in study 2 was also described for *A. franciscana*, demonstrating that higher concentrations of PS and elevated water temperatures both contributed to reduced survival and growth (Han et al., 2021). Outcomes of study 2 speak for synergistic effects of 0.43 mg/L PS MNPs at 27 °C as mortality rates increased significantly for both particle sizes, 500 nm and 1000 nm. In a broader context, if water temperatures and concentrations of MNPs continue to rise, this could pose a significant threat to the keystone species *H. azteca*.

The stability of aquatic communities depends on species sensitivity to stressors and the population dynamics of dominant species (Flöder & Hillebrand, 2012). The effects of multiple stressors can have far-reaching consequences for species, their niches, and ecosystems (Figure 7). The “fundamental” niche of organisms, describing all abiotic conditions a species can live in (Hutchinson 1957) can be influenced by particles and temperature. Additionally, biotic factors, such as species interactions and food availability, contribute to, and delineate the actual habitat in which organisms exist, known as the "realized" niche (Chase & Leibold, 2003). Direct effects of turbidity, for example, can play a significant role in the realized niche of a species. The presence of natural particles and their associated turbidity can influence light and resource availability due to, for example, algal growth and predator-prey relationships. It also affects the competitive balance among species, driving changes in niche utilization and occupancy. In contrast, synthetic particles can introduce toxic chemicals through leaching, persist in the environment for extended periods, and physically obstruct feeding and respiration in aquatic organisms.

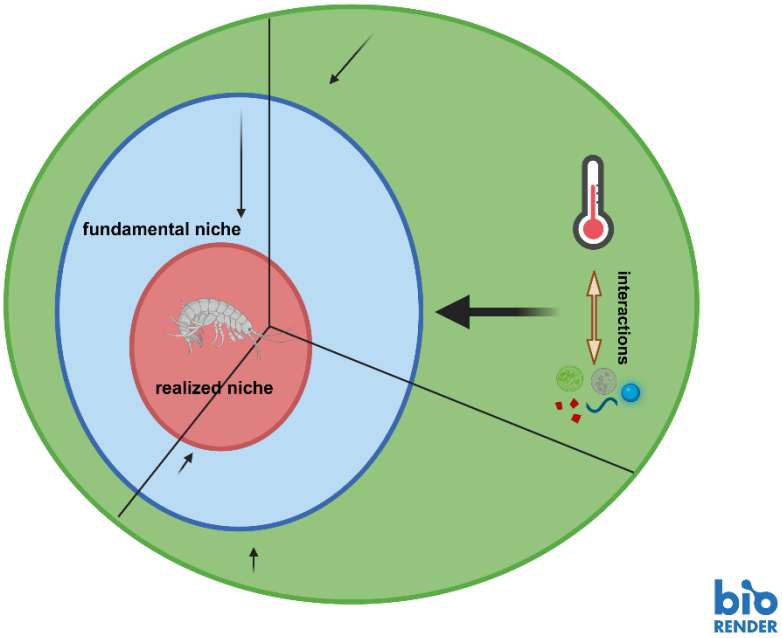


*Figure 7 The interactive effects of particles and temperature can lead to complex and often detrimental impacts on the fundamental (blue circle) and realized (red circle) niches of aquatic organisms, influencing their distribution, behavior, physiology, and ecosystem interactions (green circle). These combined effects can act synergistically, resulting in more significant impacts than the additive effects of each stressor individually. Created with BioRender.com.*

Organisms' responses to stressors can provide insights into whether the organism's niche may be shifting (Kassahn et al., 2009; Pörtner et al., 2010). A significant obstacle in developing a predictive comprehension of organismal responses to multiple stressors lies in elucidating the mechanisms through which a first stressor influences physiological responses to a second stressor. At the organismal level, the first stressor can either enhance tolerance to the second stressor (cross-tolerance) or render the organisms more vulnerable to the second stressor (cross-susceptibility) (Todgham & Stillman, 2013). Study 2 suggests that temperature may exert an influence on the effects of MNPs through, for example, increasing the metabolic rate (Rist et al., 2016; Wen et al., 2018) or indirectly by, for instance, promoting the growth of bacteria (Carpenter et al., 1972). Consequently, temperature and MNPs possibly led to cross-susceptibility. This influence of temperature on pollutant effects can further be categorized as shown by Hooper et al. (2013): Global climate change, e.g. temperature, and pollution can interact as "toxicant-induced climate susceptibility", where exposure to pollutants affects how organisms respond and adapt to temperature or "climate-induced toxicant sensitivity", where temperature influences an organism's

capacity to endure pollution (Hooper et al., 2013). Ultimately, investigating ecotoxicology in the context of climate change inherently involves examining multiple stressors.

Furthermore, it needs to be considered that many variables can influence the extent of multiple stressor effects. Factors such as the age of organisms, recovery intervals, and the sequence of stressor exposure can greatly affect research findings. Stressor effects can, for example, vary depending on chronic exposures, such as consistently elevated mean annual temperatures, or acute, short-term events, such as heatwaves with extreme temperatures. This presents a major challenge in ecotoxicology, as researchers must assess an extensive range of stressor combinations on various species.

## 4.3 Mechanisms of particle internalization and the role of temperature

The high variability of particle uptake across individuals of one beaker in study 2 indicates that there are different levels of avoidance or clearance behavior. If the experiment had been conducted over a longer duration, it is possible that more individuals would have adapted by avoiding particle uptake, thereby experiencing reduced adverse effects. The avoidance behavior observed in Atlantic ditch shrimp (*P. varians*), manifested through their gastric filters, serves as an efficient means to intercept larger microparticles, thereby impeding their passage into the midgut gland (Saborowski et al., 2022). This mechanism, involving filtration followed by expulsion, presents plausible avenues for learning and adaptation during an experiment. This would be a good sign for the adaptive capacities in the environment. However, this avoidance is likely concentration-dependent and incurs a trade-off, as reduced particle uptake may have resulted in lower nutrient intake.

Elevated temperatures place severe constraints on the metabolic and cellular processes of ectothermic organisms (Gillooly et al., 2001; Ohlberger, 2013), causing metabolic rates to surge towards the thermal optimum for a given species. Representative to other species, *D. magna* increases metabolic rates at rising temperatures (Filho et al., 2011). This surge not only alters individual feeding rates but also fundamentally reshapes the interactions between consumers and resources (Brown et al., 2004; Rall et al., 2012; Ohlberger, 2013). In response to elevated temperatures and body mass, the metabolic and feeding rates of *G. pulex* undergo significant increases, starkly aligning with predictions derived from the Metabolic Theory of Ecology (Gillooly

et al., 2001; Brown et al., 2004). The interaction between rising water temperatures and MPs demonstrated an antagonistic effect at the lowest MP concentration, whereas it exhibited a synergistic effect at higher MP concentrations (*D. magna*, Guilhermino et al., 2021). These synergistic effects can be attributed to increased metabolic rates and subsequent feeding during periods of high particle availability, aligning with the results of study 2.

## 4.4 The importance of endpoint choice

Evaluating sublethal effects in ecotoxicology is challenging due to the need to select sensitive organisms and appropriate endpoints. Sensitive species, like certain invertebrates or early life stages of fish, can reveal subtle biological changes at lower contaminant concentrations than those causing mortality. The choice of endpoints, such as enzyme activities, reproductive success, growth rates, and behavioral patterns like swimming activity, is critical as they must effectively reflect underlying physiological or behavioral stress. This approach enables early detection of environmental risks and informs timely mitigation efforts to safeguard aquatic ecosystems.

Swimming behavior in aquatic ecotoxicology is an excellent endpoint for studying stressor effects because it is a sensitive indicator of sublethal toxicity and overall health (e.g., Little & Finger, 1990; Segarra et al., 2021; Siddiqui et al., 2022). Behavior serves as a crucial link between levels of biological organization, connecting laboratory-measured subcellular processes with organismal and species responses, which would infer effects at the ecosystem level. Changes in swimming patterns can reflect neurological, muscular, and metabolic disruptions caused by particles. Additionally, swimming behavior is relatively easy to observe and quantify, providing a practical and effective way to assess various stressors' impact on aquatic organisms. Studies 2 and 3 showed that both target organisms' swimming behavior is a valuable endpoint for evaluating particle effects.

Particle treatments and temperature have significantly impacted various behavioral endpoints in *H. azteca* of study 2. Out of the eight endpoints investigated, six (thigmotaxis, cruising, moving time, acceleration, meander, zone alternations) were significantly influenced by particle treatment, and four (cruising, moving time, acceleration, meander) by temperature leading to hypo- and hyperactivity depending on particle size. In the 500 nm at 27 °C treatment group, which had the highest mortality, total distance moved and velocity were significantly higher than in the

corresponding controls, indicating hyperactivity. Hyperactivity is an escape response, acting as an adaptive mechanism that allows organisms to avoid and cope with stressful conditions (Araújo et al., 2016). Based on assessed locomotion responses, the mode of action (e.g., the clogging of gills), however, might be different between the two particle sizes. Specifically, exposure to 1000 nm particles resulted in reduced activity, while 500 nm particles caused both decreased and increased activity levels depending on the specific behavioral metric. Physical stress and the effort to digest and microscale abrasions are the main uptake consequences that can lead to behavioral changes (Wright et al. 2013; Karami et al. 2016). Additionally, MNPs may impair metabolic rates by obstructing oxygen uptake (Rist et al., 2016), which can significantly affect swimming behavior. These factors collectively explain the particle size and temperature-dependent variations in locomotion observed in study 2. In study 3, total distance moved and thigmotaxis varied in response to the light-dark cycle and temperature but remained unaffected by fiber type or fragment concentration. Interestingly, deviations in swimming behavior from controls did not exhibit a concentration-dependent trend. At 28 °C, *A. bahia* exposed to LDPE at concentrations of 5, 50, and 200 particles/mL exhibited significantly reduced activity levels, characterized as hypoactivity, compared to their activity levels at lower temperatures. However, aside from this hypoactive response, increasing the temperature did not induce significantly different behavioral effects compared to lower temperatures.

Oxidative stress responses were shown to be influenced by temperature and oxygen content in the water (Birnie-Gauvin et al., 2017). Measuring ROS levels and responses to oxidative stress is crucial in the context of particle exposure at different temperatures. Since the 1950s, ocean warming has reduced the availability of dissolved oxygen, which decreases by about 6 % for each 1 °C increase in temperature between 0 and 15 °C. By the end of the century, low oxygen zones may expand by over 50 % due to continued warming and rising atmospheric $CO_2$ (Oschlies et al., 2008). When combined with particles clogging gills, this reduction in dissolved oxygen can pose a significant threat to aquatic organisms. The uptake of particles can lead to the generation of ROS, the triggering of inflammatory responses, and the accumulation in tissues causing localized oxidative stress, disrupting cellular processes, and leading to chronic oxidative damage (Lushchak, 2011; Von Moos & Slaveykova, 2014; Horie & Tabei, 2021). Elevated ROS levels can signify oxidative damage to proteins, lipids, and DNA which compromises cell function and viability. Examining these responses helps elucidate the mechanisms of toxicity, assess the severity of the stress response, and understand how temperature variations may exacerbate or

mitigate the harmful effects of particle exposure on aquatic organisms. Understanding oxidative stress responses to particle exposures is additionally essential because a significant rise in hypoxic zones (characterized by reduced dissolved oxygen) in both freshwater and marine environments is expected (Meier et al., 2011).

In study 1, the tested turbidity levels did not affect the expression of *citrate synthase*, an indicator of metabolic and aerobic capacity in fish (Pelletier et al., 1993; Majed et al., 2002). This indicates that the tested turbidities lay within the same zone of tolerance. However, lower temperature led to an upregulation of *citrate synthase*, suggesting a preference for 11 °C over 14 °C. Possibly, the chosen environmental parameters were too close to each other, or the investigated pathways were not affected. Measuring lamellae width and hematocrit can be future endpoints to investigate. For example, three darter species exhibit limited plasticity in gill morphology, as there were no compensatory changes observed in hematocrit levels or Na+/K+ ATPase activity to maintain homeostasis under varying environmental conditions (10 and 25 °C; 8 and 94 NTU) while temperature and/or turbidity significantly affected the number of ionocytes, lamellae width, and hematocrit (Firth et al., 2024).

Study 3 also included the swimming behavior analysis in an oxygen-depleted environment. Animals exposed to nylon, polyester, and hemp did not show statistically significant differences in oxygen levels between 25 °C and 28 °C upon cessation of swimming activity. The difference in this loss of equilibrium was significant between the two temperatures in cotton and control treatments, indicating that other fibers impair gill function, which is necessary to maintain oxygen supply. Due to particles' ability to hinder oxygen uptake, metabolic rates may have been adversely affected by the fibers, potentially leading to changes in swimming. These locomotion results confirm ROS analysis, which showed that hemp at the highest tested temperature led to increased ROS levels, indicating that a threshold was surpassed due to temperature and polymer type.

## 4.5 Further influences and risks of particle-induced toxicity

The phenomenon attributed to "Trojan horse vector-effects" of particles can cause the uptake of toxic additives (Oliveira et al., 2013; Luís et al., 2015; de Sá et al., 2018). Future pollutants may have their effects and contributions altered by interactions with particulate matter. Different polymer types possess distinct physical properties, and unlike naturally suspended organic matter,

MNPs exhibit greater hydrophobicity and have a higher capacity for sorbing hydrophobic pollutants. Organic pollutants can, for example, diffuse into PE polymers but not into PP because of greater gaps between PE polymer chains (Teuten et al., 2007; Karapanagioti & Klontza, 2008). Consequently, the vector effect of MNPs can significantly impact the fate of hydrophobic persistent organic pollutants in natural environments (Rios et al., 2007; Liu et al., 2019). Besides organic compounds such as macronutrients and nucleic acids, inorganic molecules, like minerals and ionic substances, can interact with the surfaces of plastic particles, forming what is known as an "eco-bio-corona" (Canesi & Corsi, 2016). Antibiotics like sulfadiazine, amoxicillin, tetracycline, trimethoprim, and ciprofloxacin hydrochloride also bind to MPs (Li et al., 2018; Shen et al., 2018). Metals like aluminum, chromium, manganese, iron, cobalt, nickel, zinc, cadmium, and lead accumulated in similar concentrations to MPs (e.g., PET, LDPE, PP), and aging in the environment led to increased metal binding to MPs (Rochman et al., 2014).

Environmental parameters, such as salinity (Velzeboer et al., 2014; Wang et al., 2015), can influence the sorption capacities of MNPs. PS, PE, and lubrication oil sorption capacity were shown to increase with higher salinity (Hu et al., 2017). In contrast, salt addition showed decreased adsorption ability of DDT and ciprofloxacin - most likely because of cation competition (Lei et al., 2018; Li et al., 2018). Adsorption capacities of the metals Cd, Co, and Ni decreased with higher salinity, while Cr increased (Holmes et al., 2014). In the context of ocean acidification, studying the effects of pH on MNPs sorption capacities is of high interest, too. Due to competition and activity reduction of ions, e.g. metals can pH-dependently sorb to MNPs (Holmes et al., 2014). Electrostatic attraction can significantly influence sorption processes, and previous research has demonstrated that the maximum sorption capacities of tylosin on PS and PVC (Guo et al., 2018) and of perfluorooctanesulfonate on PE and PS occur at lower pH levels (Wang et al., 2015).

Ultimately, the adsorption characteristics that influence the movement of particles in the environment pose a threat to ecosystem health, as they are anticipated to change rapidly in the future due to factors such as rising water temperatures and ocean acidification. A model by Kooi et al. (2017) incorporates variables such as settling rates, biofilm development, ocean depth profiles, light penetration, water and particle density, temperature, salinity, and viscosity; results show that the maximum particle concentration can be found at intermediate ocean depths (Kooi et al., 2017). As a result, certain organisms may experience heightened exposure to particles,

potentially exacerbating toxicological effects and influencing ecological dynamics through altered feeding, reproduction, and survival rates. This should be considered in future studies.

Importantly, studies have demonstrated that additives such as Bisphenol A (BPA), known as an endocrine-disrupting substance, and certain flame retardants exhibit increased toxicity effects on organisms when associated with MPs (Planelló et al., 2008; Wardrop et al., 2016). This has direct implications for human health. The entry of MNPs into the human food web (Lu et al., 2016; Tang, 2017; Wright & Kelly, 2017; Zhang et al., 2018) and the potential of NPs to penetrate cell walls of organisms (Kashiwada, 2006; Rosenkranz et al., 2009) have raised concerns among regulators and industries worldwide regarding impacts of BPA leaching from MPs (Wagner et al., 2014; Dris et al., 2015; Eerkes-Medrano et al., 2015). This can ultimately affect human health, so political and regulatory actions are necessary. For example: On 7 September 2022, California achieved a groundbreaking achievement by becoming the first government worldwide to enforce mandatory testing of MPs in drinking water. The State Water Resources Control Board approved a comprehensive policy handbook outlining a four-year testing strategy, including logistics and criteria for selecting the public agencies responsible for conducting the tests (California Safe Drinking Water Act. Health and Safety Code 116376., 2018; California Water Boards, 2022).

## 4.6 Conclusion

Effects of particles and temperature can significantly affect individual organisms and, consequently, population dynamics, necessitating their combined inclusion in risk assessments and management strategies. The environmental relevance of these findings is crucial as pollution and climate change increasingly threaten aquatic ecosystems, making informed conservation and sustainable management decisions imperative. Understanding the intricate interactions between particles and temperature enables predictions and mitigations of cumulative effects on biodiversity and ecosystem health in our rapidly changing world. In terms of implications, it is worth noting that while these experiments and results are typically specific to certain conditions with a narrow range of parameters, their significance can extend to encompass further value, not only through providing a toolset for future investigations. Regarding the positioning of these studies within the research community, it is important to consider their groundwork and potential extension:

a) *Behavior as a crucial endpoint in ecological studies:* This research has demonstrated that swimming behavior can serve as a robust endpoint for assessing the ecological impacts of particles and temperature. Behavioral endpoints offer immediate and quantifiable indicators of organismal health, capturing the integrated responses of species to changes in their environment. Unlike physiological or biochemical markers, which may require invasive techniques and extensive analysis, behavioral observations can be conducted in real time and with minimal disruption to the organisms. This makes behavioral studies particularly valuable for ongoing environmental monitoring and management. Given the growing challenges posed by climate change, pollution, and habitat destruction, prioritizing the development and application of behavioral metrics in ecological research will provide critical insights into the adaptive capacities and vulnerabilities of various species.
b) *Expanding concepts and tools across species and particle types:* The methodologies and findings offer valuable concepts and tools that can be broadly applicable across different species and particle types. This is particularly important due to the existing knowledge gaps regarding the ecological roles and responses of various key species, especially under conditions of mixed environmental stressors like turbidity and MNP pollution. By employing a combination of behavioral assays and particle exposure experiments, researchers can gain a more comprehensive understanding of how different species interact with and are affected by particulate matter in their habitats. This approach is essential for addressing the current imbalance in research efforts, which often focus on either turbidity or MNPs in isolation rather than investigating them together. More holistic and effective conservation and management strategies can be developed by applying these concepts and tools to a wider range of species and environmental contexts.
c) *Environmental implications and the need for climate-change responsive research:* Results have significant implications for environmental health and management, particularly concerning the threatened Longfin Smelt and the particle uptake mechanisms of invertebrates. The disruptions observed in these species highlight the broader ecological consequences of particulate pollution. As climate change continues to exacerbate environmental stressors, it is crucial to understand how rising temperatures may interact with turbidity and particle pollution to influence species behavior and ecosystem dynamics. Future studies should integrate temperature as a key experimental parameter to reflect the changing climate conditions accurately. Ultimately, this research underscores the importance of comprehensive and climate-responsive approaches in ecological studies to

safeguard biodiversity and protect ecosystem function in the face of accelerating environmental change.

## 4.7 Outlook

Emerging challenges in particle pollution encompass a range of issues that are becoming increasingly significant due to advances in technology, urbanization, and changing environmental conditions:

- Rapid changes in turbidity (due to e. g. human-induced erosion from agriculture, urban runoff, improper land management, industrial discharges, dredging, construction, and excessive nutrient inputs (Lee et al., 2015; Ehlman et al., 2020; Masson-Delmotte et al., 2021)) will further disrupt feeding, respiration, reproduction, and habitat quality for aquatic organisms, induce physiological stress, influence predator-prey relationships and larval development, alter ecosystem dynamics, enhance pollutant bioaccumulation, and influence light penetration, thereby threatening the structure and stability of populations and communities.
- Nanoparticle proliferation: The widespread use of nanomaterials in various industries leads to increased concentrations in the environment. Their small size and unique properties pose unknown risks to ecosystems, while their detection and characterization techniques are yet limited.
- MNP accumulation: The prevalence of MNPs in aquatic and terrestrial environments is rising, with unknown long-term effects on wildlife and human health as these particles enter food webs and accumulate in organisms. Because MNPs can have significant implications for energy fluxes, with potential consequences that extend throughout the entire food web, pollution can result in alterations in aquatic productivity (Nava & Leoni, 2021). The widespread use of plastic equipment has also led to the pervasive presence of MNPs in aquaculture environments, possibly leading to food safety issues.
- Multiple stressor effects: The interaction of particle pollution with other environmental stressors, such as temperature changes, chemical pollutants, and habitat destruction, creates complex and often unpredictable effects on ecosystems. Given the numerous variables that must be considered in ecotoxicological studies, researchers face significant challenges in examining all stressors and translating laboratory findings to real-world

environmental outcomes. Consequently, utilizing model organisms and simulation models can be beneficial in addressing these complexities.

- Human health impacts: Increasing evidence that MNPs which can be transported in the atmosphere, lead to respiratory and cardiovascular diseases (Verma et al., 2016), necessitates improved monitoring and regulation to protect public health. NPs can further be absorbed by human cells, posing various risks, including cytotoxicity, inflammation, oxidative stress, and potentially contributing to cancer and diabetes (Fan et al., 2022; Wang et al., 2023).
- Regulatory challenges: The rapid development of new materials outpaces the ability of regulatory frameworks to assess and manage the risks associated with emerging pollutants effectively.
- Detection and measurement: Advancements in detection and measurement technologies are needed to accurately quantify and characterize the diversity of particles in the environment, especially MNPs.
- Environmental transport and fate: Understanding the pathways and transformations of particles in the environment is crucial for predicting their distribution, persistence, and impacts.
- Ecological impacts: More research is needed to elucidate particle pollution's sublethal and chronic effects on a wide range of species and ecological processes. Advancements in, for example, genomics and proteomics have expanded the range of available molecular biomarkers while still being underrepresented in ecotoxicology.
- Public awareness and education: Increasing public awareness and understanding of particle pollution and its impacts can drive better management practices and policy decisions.

Addressing these challenges requires a multidisciplinary approach, combining research, policy development, technological innovation, and public engagement to mitigate the risks associated with particle pollution. Future measures to reduce MNP pollution include reducing plastic use and waste, especially that of single-use plastics, promoting recycling practices, implementing bans on microbeads used in products, and enhancing wastewater treatment infrastructure. To strengthen the groundwork for risk assessment, forthcoming experimental approaches must not only discern between the effects of food scarcity and particle toxicity but also determine whether MNPs provoke distinct responses compared to naturally occurring particles (Ogonowski et al., 2018). Expanded

research is necessary to encompass a wider array of particles with multiple properties and within different environmental contexts in order to enhance the holistic comprehension of the intricate interplays between MNPs and aquatic ecosystems (Figure 8).

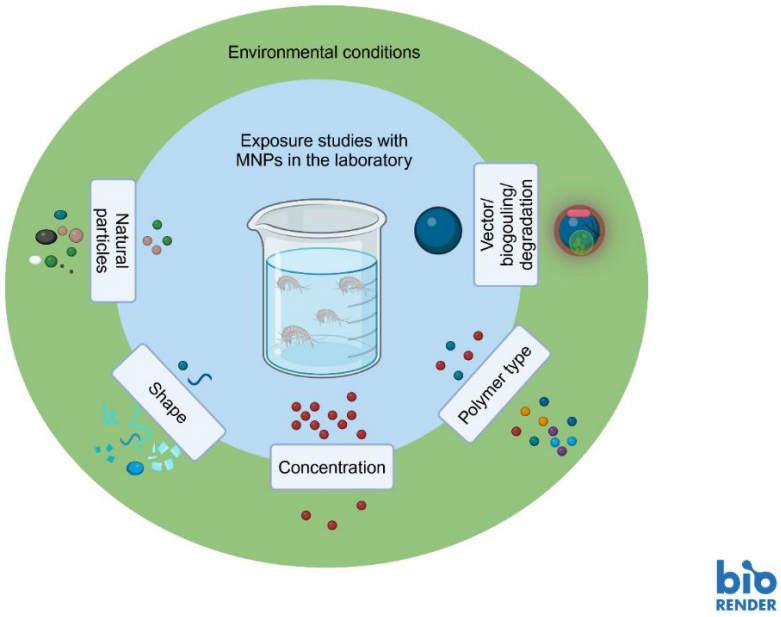


*Figure 8 Conditions used in most laboratory MNP exposure studies (blue) differ significantly from those in natural aquatic environments (green), especially regarding the presence of natural particles, particle shapes, concentrations, polymer types, and vector effects, biofouling, and degradation. Based on Rist 2019. Created with BioRender.com*

Translating findings from laboratory studies on individual organisms to broader population and community-level field studies has consistently been a challenge in ecotoxicology. Using both environmentally realistic systems, such as mesocosms, and highly controlled, standardized systems in experimental studies on particles is valuable. While the former offers site-specific data and contextual relevance, and the latter provides detailed mechanistic insights, each approach addresses different research questions and should be chosen based on the study's objectives, making them complementary. Studies on MNP effects vary widely in conditions, including particle types, concentrations, exposure durations, and test organisms, with incomplete reporting complicating result comparison and reproducibility, so standardizing test systems could improve consistency. For this work in controlled and standardized systems, findings underscore the substantial impact of exposure on behavioral endpoints, thereby enhancing the capacity to assess risk by identifying indicators. A multiple-endpoint toolkit has the potential to aid in assessing the

risks associated with particle exposure and uptake, as well as their roles in acting as vectors for contaminants. Further investigation is warranted to assess co-exposure to particles and per- and poly-fluoroalkyl substances (PFAS), especially in the context of water temperature as an additional factor, to elucidate the role of particles as vectors for emerging contaminants in the environment (Parashar et al., 2023).

# 5. Acknowledgements

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# 6. Publications and presentations

## 6.1 Peer-reviewed publications included in this thesis and author contributions

1. Biefel, F., Pasparakis, C., Cocherell, D. E., Hung, T. C., Carson, E. W., Fangue, N. A., ... & Connon, R. E. (2024). Turbidity and temperature effects on growth and gene transcription of threatened juvenile Longfin Smelt (*Spirinchus thaleichthys*). Aquaculture, 741296.

*Author contribution:* Conceptualization, AET, CP, FB, JG, NAF, REC; methodology, investigation, formal analysis, data curation, writing AET, CP, DEC, FB, JG, REC; review, editing and investigation AET, CP, EWC, FB, JG, NAF, REC, T-CH; supervision, project administration, funding acquisition, AET, EWC, NAF, REC, T-CH.

2. Biefel, F., Brander, S. M., Connon, R. E., & Geist, J. (2024). Polystyrene Plastic Particles Result in Adverse Outcomes for *Hyalella azteca* When Exposed at Elevated Temperatures. Water, 16(10), 1360.

*Author contribution:* Conceptualization, FB, SB, RC, JG; methodology, investigation, formal analysis, data curation, writing, FB; review, editing and investigation, FB, SB, RC, JG; supervision, project administration, funding acquisition, RC (PI).

3. Biefel, F., Geist, J., Connon, R. E., Harper, B., & Brander, S. M. (2024). Interactive effects between water temperature, microparticle compositions, and fiber types on the marine keystone species *Americamysis bahia*. Environmental Pollution, 348, 123906.

*Author contribution:* Conceptualization, FB, JG, REC, SMB; methodology, investigation, formal analysis, data curation, writing FB, JG, REC, SMB; review, editing and investigation FB, BH, JG, REC, SMB; supervision, project administration, funding acquisition, SMB (PI).

## 6.2 Peer-reviewed publications not included in this thesis

Segarra, A., Mauduit, F., Amer, N. R., Biefel, F., Hladik, M. L., Connon, R. E., & Brander, S. M. (2021). Salinity changes the dynamics of pyrethroid toxicity in terms of behavioral effects on newly hatched delta smelt larvae. *Toxics*, 9(2), 40.

Pasparakis, C., Lohroff, T., Biefel, F., Cocherell, D. E., Carson, E. W., Hung, T. C., ... & Todgham, A. E. (2023). Effects of turbidity, temperature and predation cue on the stress response of juvenile delta smelt. *Conservation Physiology*, 11(1), coad036.

## 6.3 Oral and poster contributions related to this thesis

Segarra A., Biefel F., Amer N., Hladik M., Connon R. E., Brander S. M.: Evaluation of Sublethal Pyrethroid Toxicity Across a Salinity Gradient in Early-Life Stage of the Endangered Delta Smelt (*Hypomesus transpacificus*); *SETAC North America 41st Annual Meeting*, virtual, USA, Nov 15, 2020.

Biefel F., Pasparakis C., Wampler A., Cocherell D. E., Connon R. E., Fangue N. A., Hung T., Todgham A. E.: Vulnerability of Longfin Smelt to changes in turbidity and warming: a physiological perspective; *Delta Stewardship Council - 11th Biennial Bay-Delta Science Conference*, virtual, CA, USA, Apr 6, 2021.

Biefel F.: Microplastic monitoring - a caging study with juvenile Chinook Salmon; *2nd Annual NorCal SETAC Science Showcase*, virtual, CA, USA, May 26, 2021.

Biefel F., Brander S. M., Connon R. E., Geist J., Harper B.: Effects of Microplastics (LDPE, PLA; natural and synthetic fibers) in combination with temperature on Mysid Shrimp (*Americamysis bahia*): A physiological perspective; *NorCal SETAC's 30th Annual Meeting*, Davis, CA, USA, Sept 15, 2022. (2nd place of student presentations)

Biefel F., Connon R. E., Geist J., Harper B., Brander S. M.: Effects of Microplastics (LDPE, PLA) on Mysid Shrimp (*Americamysis bahia*) in combination with temperature: A physiological

perspective; *SETAC North America 43rd Annual Meeting*, Pittsburgh, PA, USA, November 14, 2022.

Biefel F., Connon R. E., Geist J., Harper B., Brander S. M.: Climate change: Do effects of microplastics and fibers on mysid shrimp interact with temperature; *Pacific Northwest Consortium on Plastics* - Hatfield Marine Science Center (OSU), Newport, OR, USA, Dec 13, 2022.

Biefel F., Brander S. M., Connon R. E., Geist J.: Effects of nano-, microplastics, and temperature on Crustaceans; *SETAC Europe*, Dublin, Ireland, May 2nd, 2023.

# 7. Literature


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# 8. Appendix

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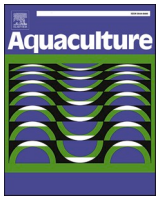

# Turbidity and temperature effects on growth and gene transcription of threatened juvenile Longfin Smelt (*Spirinchus thaleichthys*)

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ABSTRACT

The Longfin Smelt (LFS, *Spirinchus thaleichthys*) population within the San Francisco Estuary, California, has experienced a substantial reduction, diminishing to <1% of their historical abundance. This decline has culminated in their classification as a threatened species under the purview of the California Endangered Species Act. Understanding their physiology and stress response in relation to varying environmental conditions, such as temperature and turbidity, is crucial for LFS culturing, management, and conservation. In this study, we assessed juvenile LFS (age range during exposure: 181 to 228 days post hatch, dph) performance as measured by growth and gene expression following four weeks at two temperatures (11 °C and 14 °C) and three turbidity levels (1, 4, and 11 nephelometric turbidity units (NTU)). At the end of the 4-week exposure period, we conducted assessments encompassing fork length, wet weight, condition factor, and examined alterations in the transcription of 12 genes. The selection of these genes aimed at determining responses associated with osmoregulation, growth, metabolism, and general stress, as all of which are potentially influenced by temperature and/or turbidity. Weight and condition factor was significantly higher at lower temperature, whereas turbidity had no effect on growth, condition factor, and transcriptomic stress-response. Instead, the lower expression levels of *Catalase*, *Citrate Synthase* and *Growth Factor Receptor Bound Protein 10* at 14 °C were indicative of metabolic and growth-related changes governed by temperature. This suggests that rearing of LFS at 11 °C and low turbidity (<11 NTU) is suitable for the juvenile stage, whereas growth as well as metabolic capacity is limited at slightly warmer temperatures.

## 1. Introduction

Across the globe, human influences have led to the depletion of >90% of species that were once ecologically significant from previously diverse and productive estuaries and coastal seas (Lotze et al., 2006). Such impacts in the San Francisco Estuary, California, have resulted in numerous fish species being listed as threatened or endangered (Moyle et al., 2012; Brown et al., 2016; Brennan et al., 2022). Of particular interest for fish conservation in the estuary are Longfin Smelt (LFS, *Spirinchus thaleichthys*) and Delta Smelt (DSM, *Hypomesus transpacificus*), for which the roles of multiple environmental stressors in population declines have been studied (Nally Mac et al., 2010; Glibert et al., 2011; Brooks et al., 2012; Moyle et al., 2016; Nobriga and Rosenfield, 2016; Hobbs et al., 2017). Both species face similar threats and have overlapping habitats, making insights from DSM studies invaluable for LFS conservation efforts. DSM was listed as threatened in 1993 under the U. S. Endangered Species Act (USFWS, 1993) and as endangered in 2010 by the State of California (CDFG, 2010). The southernmost populations of LFS are listed as threatened by the State of California in 2009 (CDFG, 2009). Due to the occurrence of both species in the estuarine mixing zone, which is where pronounced changes in turbidity, salinity, and temperature co-occur (Moyle et al., 2016), an understanding of the

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impact of such changes on the physiological performance of smelt is of crucial importance. Turbidity is suspected to be a key factor influencing the biotic resources for fishes, such as the abundance and diversity of prey items as well as predator-prey relationships (Baskerville and Lindberg, 2004; Nelson et al., 2015; Kurobe et al., 2022).

Longfin Smelt is an anadromous fish species that inhabits waters from the Aleutian Islands to the San Francisco Estuary (Moyle, 2002; Sommer et al., 2007). Recent efforts to rear LFS in captivity have provided novel opportunities for hypothesis-driven research regarding the ecological niche of this species. In particular, life-stage specific information is required for culturing of LFS in aquaculture facilities, which aim at providing adequate water quality conditions to promote fish health and optimal growth and performance of captive-bred specimens that can be used for population augmentation (Jeffries et al., 2016; Hobbs et al., 2017; Lewis et al., 2019, 2020; Tempel et al., 2021; Yanagitsuru et al., 2021, 2022; Mulvaney et al., 2022; Hung et al., 2023). Currently, the capacity to culture LFS is limited to early life stages; however, if LFS can successfully undergo its entire life cycle in a controlled culture environment, this development will not only help with population augmentation, but it would also facilitate LFS utilization in fundamental and applied research, offering valuable insights into the species that can inform conservation, management decisions, and practical research efforts aimed at refining their breeding methods.

While substantial research has been conducted on the impact of altered environments on DSM, facilitated by the availability of captive culture populations (e.g., Baskerville and Lindberg, 2004; Lindberg et al., 2013), relatively little empirical research has been conducted to determine the habitat requirements of LFS. In this study, we focus on understanding temperature and turbidity requirements for juvenile LFS, both of which have already demonstrated their importance in the cultivation of DSM (Hasenbein et al., 2016; Tigan et al., 2020; Pasparakis et al., 2022, 2023) and the embryonic/larval stage of LFS (Yanagitsuru et al., 2021, 2022). Elevated temperatures and the growing frequency of heatwaves pose a substantial threat to the future survival of smelt species (Moyle et al., 2016), especially because of impacts to pivotal life history events such as spawning and migration (Basevkin et al., 2023). Temperature also directly impacts the solubility of oxygen, which has implications for all gill-breathing species with high oxygen demands (Chapra et al., 2021). Understanding life-stage specific physiological responses to elevated water temperature is crucial to predict species declines and inform management.

Turbidity results from the scattering and absorption of light by suspended particles and is frequently described as the 'cloudiness' of water (Kirk, 1985; Henley et al., 2000). The level of turbidity depends on suspended organic and/or inorganic materials, while factors such as light intensity and water depth further contribute to effects on organisms (Lee and Rast, 1997). Light reflected off these particles is used as a means of measuring turbidity, which is expressed as nephelometric turbidity units (NTU). Turbidity is an understudied, yet significant driver of ecosystem function that influences trophic interactions and species compositions (Lunt and Smee, 2014). It is also a crucial factor in determining optimal rearing conditions for fish in aquaculture production systems (Becke et al., 2018, 2020). Turbidity requirements can vary significantly across fish species (Utne-Palm, 2002), affecting for example larval and juvenile DSM feeding ability and resulting growth rates, as well as impacting predator prey interactions, and levels of stress (Hasenbein et al., 2013; Tigan et al., 2020; Pasparakis et al., 2023).

It has been postulated that visual feeders benefit from certain levels of turbidity that facilitate contrast to see their prey. Turbidity levels that are too high, however, may result in clogged and damaged gills, diminished visibility and thus reduced feeding (Gardner, 1981; Grimaldo et al., 2009). For DSM late-larvae (60 dph), Hasenbein et al. (2016) identified turbidity levels ranging from 25 to 80 NTU as beneficial in the context of feeding rates; diminished cortisol concentrations were observed within the range of 35 to 80 NTU, while heightened levels were noted at 5, 12, and 25 NTU. Additionally, turbidity was shown to influence the feeding conditions of DSM larvae, which are visual predators, by limiting light penetration (Baskerville and Lindberg, 2004; Tigan et al., 2020). This also accounts for initial studies on the larval stage of LFS, which have indicated the necessity for low turbidity levels in captivity (Yanagitsuru et al., 2021, 2022). Turbidity requirements for DSM have been described as life-stage dependent, and play an essential role in feeding behavior and potential predator evasion (Feyrer et al., 2007; Nobriga et al., 2008; Grimaldo et al., 2009; Sommer et al., 2011; Brown et al., 2013; Hasenbein et al., 2013; Sommer and Mejia, 2013; Ferrari et al., 2014; Bennett and Burau, 2015; Pasparakis et al., 2023).

Considering the heightened sensitivity and the current conservation status of LFS in the natural estuary habitat, it is imperative to gain a comprehensive understanding of the impacts of changes in water quality parameters, notably temperature and turbidity, on the physiological performance of LFS. Moreover, rearing the juvenile stage of LFS presents specific challenges, and establishing suitable conditions for the juvenile life stage necessitates a deep comprehension of physiological thresholds in performance. While there is established information regarding the importance of turbidity for culturing DSM, there is a dearth of data on the turbidity needs for LFS. When considering aquaculture, one might anticipate that LFS and DSM would share similar requirements due to their comparable distribution patterns in the estuary during certain life stages. This suggests that their juvenile stages are likely to thrive under similar environmental conditions characterized by specific temperature ranges and levels of water turbidity. However, the tendency of LFS to concentrate in San Francisco Bay during the late juvenile and adult stages, as part of their anadromous life cycle, likely accounts for the shift in the population center from landward to seaward (Moyle, 2002). It is important to note that their life cycle differs from DSM in that the juvenile to adult stages of LFS, depending on water temperature and if it is a low or high flow year, are typically found in oceanic environments, suggesting that clear waters may be more suitable for LFS at this life stage (Grimaldo et al., 2020).

This study had two main objectives: 1) to contribute to the fundamental knowledge of LFS early life stage physiology and 2) to better understand water quality requirements of juvenile LFS to be practically applied to enhance LFS culture. Therefore, we assessed growth and stress-related gene expression under holding conditions of different turbidity (1–11 NTU) and temperature levels (11 °C and 14 °C). Many of the selected genes are connected to the hypothalamus–pituitary–interrenal (HPI) axis and its end product cortisol. Cortisol is a bioindicator of stress, which combines glucocorticoid and mineralocorticoid actions and is therefore essential in the context of hydromineral homeostasis (Wendelaar Bonga, 1997) as well as osmoregulation, growth, and reproduction (Mommsen et al., 1999). Specifically, we hypothesized that:

(i) For the larval stage preferences, a temperature of 11 °C would yield accelerated growth rates in comparison to those at 14 °C.
(ii) Given that the rearing and acclimation occurred closer to 11 °C, less pronounced transcription levels in stress- and growth-related genes would be expected at 11 °C when compared to treatments conducted at 14 °C.
(iii) Since juvenile LFS exhibit a migratory behavior and occur in clearer ocean waters, turbidity conditions within the range of 1–11 NTU would result in limited impacts on growth and the expression of examined genes for this life stage. This would suggest that tested turbidities fall within a shared tolerance range. Nonetheless, a probable threshold response to turbidity is anticipated, with an optimal range expected between insufficient and excessive levels.

Consequently, lower temperature and low turbidity may be beneficial while also implementable for the rearing of LFS.

## 2. Methods

### 2.1. Fish source, experimental setup, and maintenance

Longfin Smelt were spawned from wild-caught broodstock and reared at the UC Davis Fish Conservation and Culture Lab (FCCL). The experimental setup was similar to that described in Pasparakis et al. (2022). At 164–183 dph, fish were transferred from the FCCL to the UC Davis Putah Creek Facility (PCF) research lab. Upon arrival, groups of 30 fish (720 total) were distributed into 24, 57-L black polyethylene tubs (referred to herein as sub tanks) fitted with standpipes, allowing for ca. 57 L. Sub tanks were distributed in groups of three within four 400-L tanks (referred to hereafter as holding tanks, eight in total) which were contained across two separate recirculating aquaculture systems (Fig. S1). The holding tanks served as experimental water baths with each system having separate temperature control units. Water was continuously pumped from external reservoirs that maintained experimental algal concentrations used to attain the selected turbidity levels (Table S2). Fish were fed twice daily and directly via filtering a standardized volume of live brine shrimp (*Artemia franciscana*) culture with a brine shrimp net. External reservoirs were maintained to 7.0–7.4 ppt salinity with Instant Ocean (Aquarium Systems, Mentor, OH) to guarantee optimal conditions in all treatments for brine shrimp. Each sub tank was fitted with independent, external biofiltration units through which water was treated with seeded k1 biomedia, returning to the tanks using an airlift mechanism via airstones. These external biofiltration units also served to maintain the oxygen levels in the sub tanks as well as help maintain the turbid mixture in suspension. Sub tanks were covered with circular lids made from shade cloth, and styrofoam lids were used to cover each holding tank.

Longfin Smelt were subjected to a daily 12-h light/12-h dark cycle, which was maintained using a timer connected to the lighting system. Lights reached 7 to 120 lx as measured above the water surface by a portable digital light meter (LX1330B, Dr. Meter) and were placed within each holding tank lid to investigate turbidity-light interactions and their effects on feeding efficiency and stress responses. Water quality parameters were measured during the acclimation and exposure period (Table S2). Temperature (°C), dissolved oxygen (mg/L), and salinity (ppt) were measured daily with a hand-held YSI 556 MPS meter (YSI Inc., Yellow Springs, OH). Ammonia, nitrite, and nitrate concentrations, as well as pH, were measured biweekly. Ammonia, nitrite, and nitrate measurements were conducted using a marine care multi test kit (Red Sea, Houston, TX), with additional ammonia measurements taken using a Hach pocket colorimeter (Hach Company, Loveland, CO), whereas pH was measured using a pinpoint pH monitor (American Marine Inc., Ridgefield, CT). Mortality was checked daily, and any dead fish were removed. Fish care and use protocols were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee (IACUC Protocol #16591).

Since temperature is directly related to performance traits such as growth and development (Claireaux et al., 2006; Eliason et al., 2011), we tested two sublethal thermal regimes (11 °C and 14 °C) based on recommendations for rearing early life stages (9–12 °C) and one degree lower than the temperature threshold (15 °C) above which has been shown to be harmful to LFS performance, hatch success, growth, and mortality (Grimaldo et al., 2017; Yanagitsuru et al., 2021). Following a two-week laboratory acclimation period and reaching an age of 181–200 dph, acclimation at two different temperatures (11 °C and 14 °C) and turbidities (1, 4, and 11 NTU) was conducted over a period of four weeks. Sampling was conducted following this 4-week acclimation, at an age ranging from 209 to 228 dph. This was the first study to use juvenile LFS in a prolonged experimental setup. Decreased turbidity levels relative to environmental conditions were chosen based on our limited understanding of tolerances and to facilitate a thorough mortality assessment. This approach was chosen to minimize mortality risks and enhance the feasibility of accurately monitoring survival rates over four weeks. The study was conducted using four replicates of 30 fish for each treatment. During the two-week laboratory acclimation, fish were initially acclimated to the system for 11 days, at 11 °C in clear water, and after which they were transitioned to treatment conditions over a period of three days. The temperature for the higher temperature treatment was gradually increased from 11 °C to 14 °C at a rate of 1.3 °C per day, while the 11 °C treatment was kept constant over the same three-day period. Turbidity was adjusted using predetermined volumes of *Nannochloropsis* spp. algae (Nanno 3600 – High yield grow out feed; Reed Mariculture Inc., USA). No algae was added in the 1 NTU treatments and turbidity levels were increased from 1 to 4 and 11 NTU at a rate of 3 NTU per day. Algae were added daily to reservoir tanks connected to each sub tank based on the FCCL protocol for DSM. Temperature was measured using eight HOBO data loggers, set at 15 min intervals, randomly distributed in one sub tank per holding tank set to 11 °C or 14 °C. Turbidity was measured daily using a Hach pocket colorimeter (Hach 2100q portable turbidity meter, Hach Company, Loveland, CO).

Longfin Smelt were not fed during the 24 h prior to the sampling. At the end of the exposure, juvenile LFS were sampled under low luminosity after switching off the lights to minimize handling stress. All fish were euthanized using an overdose of buffered tricaine methanesulfonate (MS-222; 50 mg/L; buffered to a neutral pH with sodium bicarbonate; Western Chemical, Ferndale, WA), weighed (to the nearest 0.001 g) and measured (fork length; to the nearest 0.1 cm) to calculate Fulton's fish condition factor (K). Fulton's condition factor was calculated using the following eq. $K = 100\ W/\ L^3$, where W is the weight (g), and L is the fork length (cm) (Fulton, 1904). After body measurements, the whole fish was immediately snap-frozen in liquid nitrogen, with 12 fish per treatment archived at −80 °C for gene expression analysis.

### 2.2. Water physicochemistry

Pretreated clean water from outdoor reservoir tanks and biofiltration in each sub-tank maintained stable water quality throughout the system. In conjunction with daily temperature assessments via YSI, each tank was equipped with a designated HOBO temperature logger (Onset). The logger was systematically rotated among treatment sub-tanks every two days, capturing temperature readings (°C) at 30-min intervals. The average water temperatures during the exposure period were 11.2 ± 0.0 (Low) and 13.8 ± 0.0 (High) °C, and average turbidity levels were 1.0 ± 0.1, 3.6 ± 0.1, and 10.9 ± 0.5 NTU, respectively, closely matching nominal values. More detailed water parameters are shown in Table S2.

### 2.3. Gene transcription analysis

To aid RNA extraction, whole fish were cryogenically ground using a mortar and pestle. Mortars were placed on dry ice, into which the samples were placed, and grinding was aided using liquid nitrogen to break down the tissue into fine, homogeneous powder. Immediately after grinding, the powder was transferred into cryogenic tubes, set on dry ice, and stored at −80 °C until further processing.

Total RNA extraction was performed using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) in an automated QIAcube (Qiagen). Extraction efficiency and RNA quantity were verified using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Total RNA integrity was visually verified by non-denaturing gel electrophoresis using a 1% (*w/v*) agarose gel. QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA from 1 μg of total RNA, following the manufacturer's protocol, to a final volume of 40 μl. Quantitative PCR (qPCR) was conducted using a 2× PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, USA). Cycling conditions were 2 min at 50 °C; 2 min at 95 °C; 40 cycles: of 15 s at 95 °C, 15 s at 55 °C, 1 min at 72 °C; and 15 s at 95 °C. Mean cycle threshold (Ct) values of triplicate technical replicates were used relative to reference genes. Gene expression data were normalized to the geometric mean of 3 reference genes (*GAPDH, RPL7,* and *ACTB*),

which showed the highest consistency across treatments (Table S1). Expression of target genes were then calibrated to fish maintained at a turbidity of 1 NTU and at 11 °C (i.e., data is presented relative to this condition, which corresponds to current rearing practices). The genes of interest for this study and reference genes are listed in Table 1, their predicted expression in response to experimental parameters in Table 2.

Primers used in this study were designed in house using whole transcriptomic data available from a prior LFS study (Jeffries et al., 2016). Primers were designed using PrimerQuest™ (Integrated DNA Technologies) or Primer-BLAST (NCBI), and their efficiencies were determined using a five-point standard curve in three replicates established from a 10-fold serial dilution of cDNA. The amplification efficiency (E) of primers was calculated as $E = 10^{-1/slope}$ on a calibration curve.

### 2.4. Statistical analyses

Analyses were conducted and graphs generated with GraphPad Prism (Version 10.1.2, © 1992–2021 Graphpad Software, LLC). Statistical assumptions were tested using jamovi (Version 2.3.16). Normal distribution was verified via Shapiro-Wilk tests and homogeneity of variances via Levene's tests. Weight, length, and Fulton's condition factor data did not meet assumptions for normal distribution and homogeneity of variances; therefore, a non-parametric test (Kruskal-Wallis) was used followed by Dunn's multiple comparison test.

Relative expression of target genes was determined using the method described by Pfaffl (2004) and presented as log2-fold change. A two-way ANOVA, followed by Tukey multiple comparison tests with a single pooled variance, was used to test the main effects of temperature and turbidity as well as their interaction on gene expression of 12 genes of interest. *POMCB* was not normally distributed ($p = 0.003$), and *HSD11b2* did not show homogenous variances ($F = 2.79$; $p = 0.024$) so non-parametric tests (Kruskal Wallis test followed by Dunn's multiple comparison test) were applied. Because there were no significant differences across turbidity treatments, we also were able to consider them graphically as ad hoc replicates within each temperature. Quantitative PCR data are presented as average ± standard error (SEM); significance was set at $p < 0.05$.

## 3. Results

### 3.1. Survival, length, weight, and condition factor

Average survival per treatment was high and ranged between 79.2% - 90.6%, with no significant differences between treatments (Table S3). Fork length, weight, and K were not significantly different between turbidity treatments ($p = 0.07$, $p = 0.08$, and $p = 0.36$). However, weight and K were affected by temperature: At 11 °C, weight ($p = 0.01$) and K ($p < 0.001$) were significantly higher than at 14 °C (Fig. 1). Over the 4-week exposure, this resulted in a difference of the final weight by 77.4 mg (771.3 ± 23.7 mg at 11 °C versus 693.9 ± 24.2 mg at 14 °C) and of K by 0.054 (0.539 ± 0.011 at 11 °C versus 0.486 ± 0.006 at 14 °C). Examined via ANOVA, no statistically significant interactions were identified between temperature and turbidity concerning survival, length, weight, or Fulton's condition factor.

### 3.2. Gene transcription

The impact of turbidity and temperature-turbidity interactions on gene transcription was non-significant for all 12 genes of interest (Fig. S2). This suggests that, at the time of sampling, the metabolic, growth, oxidative, and physiological stress associated with these

**Table 1**
Selected longfin smelt (*Spirinchus thaleichthys*) genes. Primer sequences and efficiencies for quantitative PCR analysis.

| Gene name | Gene code | General function | Primer forward | Primer reverse | Efficiency (%) |
|---|---|---|---|---|---|
| Catalase | *CAT* | Defense against oxidative stress | GGTTCGCTGTCTGGTGCTCTA | TCGAGGTGGTCGTCATAGCG | 96.3 |
| Citrate Synthase | *CS* | Synthesis of citrate from oxaloacetate and acetyl coenzyme A, capable of oxidative metabolism | GCATTCCGGAGTGTCAGCA | CCTTCAGGGAGAGGCTCTTGA | 106.8 |
| Growth Factor Receptor Bound Protein 10 | *GRB10* | Encodes growth factor receptor-binding protein that interacts with insulin receptors and insulin-like growth-factor receptors | TGCATCAAGCCTAGCAAGGTG | CATCTTCCGCGCACATCAT | 98.4 |
| Potassium sodium hyperpolarization-activated cyclic nucleotide-gated channel 2-like | *HCN2* | Voltage-gated potassium channel activity | ACCTTGTGGAGGAGGAGGGA | TGATGAGGCGAGCCTTCGAG | 101.3 |
| Heat Shock Protein Family A | *HSP70* | Thermal Stress/General Stress – protein chaperone | GACCGTGCGATTGGTCGTCA | ATCAGGGCGACGATGCAGTT | 107.1 |
| Heat Shock Protein 30 | *HSP30* | Thermal Stress/General Stress – protein chaperone | TCGCTTCTCCAGAACGACTTC | GCCTTGCTGGAGTTCCTTCAT | 97.0 |
| Cholesterol 7-alpha-monooxygenase-like; full = cytochrome *p*450 7a1 | *CYP7a1* | Metabolism and synthesis of cholesterol, steroids, and other lipids. This endoplasmic reticulum membrane protein converts cholesterol | GGTGTCGGACGTGAGCATGA | GGCCACACCACAGAGAACCT | 96.5 |
| Proopiomelanocortin b precursor | *POMCb* | Precursor of adrenocorticotrophic hormone; HPI axis | GTGTGTCGTGGCTGTTGACC | TATCTTTGCAGTAGGGGCGGT | 99.2 |
| Corticosteroid 11-beta-dehydrogenase isozyme 2-like | *HSD11b2* | Cortisol conversion; HPI axis | CACACATCCACTGCTCCCAG | CTCTGGGGCGTGGTGAACAAT | 104.4 |
| Transferrin | *TFR* (HIF1A mediated) | Transport of iron to cells, response to hypoxia | TGCTCTCCTCAAAGGCCTCG | GCTGATACAGCACCAACGCA | 109.2 |
| Insulin-Like Growth Factor 1 Receptor | *IGF1r* | Tyrosine kinase activity, transformation events. | AGCTGGAGACCTTCATGGGC | CCAGCGTGTGGGAGTGTCTT | 106.9 |
| Glucose Transporter 5 | *GLUT5* (= SLC2A5) | Glucose transportation | TCATCGCCCAGCCTGATCTC | TGGCTGGTGCTACCAGAGAC | 101.3 |
| Glyceraldehyde-3-Phosphate Dehydrogenase | *GAPDH* | Reference | GACCTGACCTGCCGTTTGAC | TCCGTGAGCAGCTTTCTTGA | 94.9 |
| Ribosomal Protein L7 | *RPL7* | Reference | GCATCGCCCTCACTGACAA | CTCATGGATCAGGTCCTCAACA | 94.5 |
| Actin Beta | *ACTB* | Reference | CCATCGGCAACGAGAGGTT | GCAGGACTCCATACCGAGGAA | 101.5 |
| Ribosomal Protein S9 | *RPS9* | Reference | TGGAACTGGAGGAGCTGATGA | CAGCCAATACTTCCAGGACGAC | 92.2 |

**Table 2**
Predictions and observations related to the expression of genes of interest (A) and the condition factor (B) in Longfin Smelt (*Spirinchus thaleichthys*, 209–228 dph) in response to experimental parameters turbidity of 1–11 NTU, and temperature of 11–14 °C (+, increased expression; −, decreased expression; nee, no expected effect; ns, non-significant).

| Gene code | Described function/pathway | Related literature | Prediction in response to increasing turbidity (NTU) | | | Prediction in response to increasing temperature (°C) | | Observation in response to increasing turbidity (NTU) | | | Observation in response to increasing temperature (°C) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 4 | 11 | 11 | 14 | 1 | 4 | 11 | 11 | 14 |
| **A)** | | | | | | | | | | | | |
| *CAT* | maintenance of pro- and antioxidant balance | Di Giulio et al. (1989); Ahmad et al. (2000); Bagnyukova et al. (2005a, 2005b); Lesser (2006); Shin et al., 2006; Vinagre et al. (2012) | + | | | + | | ns | | | – | |
| *CS* | indicator for the metabolic and aerobic capacity and growth rates of fish | Pelletier et al. (1993, 1994); Majed et al. (2002) | nee | | | – | | ns | | | – | |
| *GRB10* | growth suppressor, overexpression can inhibit tyrosine kinase activity | Charalambous et al. (2003); Plasschaert and Bartolomei (2015) | nee | | | + | | ns | | | – | |
| *HCN2* | cardiac activity, acute stress events | Jackson et al., 2007; Hassinen et al. (2017); Kolesnikova (2021) | nee | | | + | | ns | | | ns | |
| *HSP70* | influences the activin/nodal/transforming growth factor-β and bone morphogenetic protein receptors | Yamashita et al. (2010); Hasenbein et al. (2016) | + | | | + | | ns | | | ns | |
| *HSP30* | can regulate the H + -ATPase induced by various kinds of stress, ubiquitinated proteins degraded via proteasomes or lysosomes can cause upregulation | Piper et al., 1997; Young and Heikkila (2010) | nee | | | + | | ns | | | ns | |
| *CYP7a1* | involved in the cholesterol catabolic process and growth, as well as the bile acid synthesis; bile acid was described to be beneficial for energy metabolism and health condition of farmed fish | Yun et al. (2012); Chiang (2017); Kong et al. (2021); Wang et al. (2023) | nee | | | – | | ns | | | ns | |
| *POMCb* | precursor of adrenocorticotropic hormone (ACTH) which is involved in cortisol production | Alsop and Aluru (2011); Hasenbein et al. (2013); Pasparakis et al., 2023 | – | | | + | | ns | | | ns | |
| *HSD11b2* | regulation of glucocorticoids converting cortisol into inactive cortisone | Krozowski et al. (1999); Tomlinson and Stewart (2001); Hasenbein et al. (2016); Pasparakis et al. (2023) | + | | | – | | ns | | | ns | |
| *TFR* | immune response of macrophages to, for example, bacterial infections | Pxytycz and Józkowicz (1994); Stafford and Belosevic (2003); Neves et al. (2009) | nee | | | + | | ns | | | ns | |
| *IGF1r* | stimulates cartilage sulfation of growth hormones, can result in reduced growth but increased longevity and stress resistance | Jones and Clemmons (1995); Gabillard et al. (2003); Holzenberger et al. (2003); Muñoz (2003); Cypser et al. (2006); Zhang et al. (2018) | nee | | | – | | ns | | | ns | |
| *GLUT5* | indicator of nutritional status and transports fructose, in fish liver and muscle, glucose transporters react to fasting and hypoxia | Polakof et al. (2012), Hasenbein et al. (2013), Wang et al. (2019); Koepsell (2020); Pasparakis et al. (2023) | + | | | – | | ns | | | ns | |
| **B)** | | | | | | | | | | | | |
| | Condition Factor | Yanagitsuru et al. (2021); Pasparakis et al. (2023) | + | | | – | | ns | | | – | |

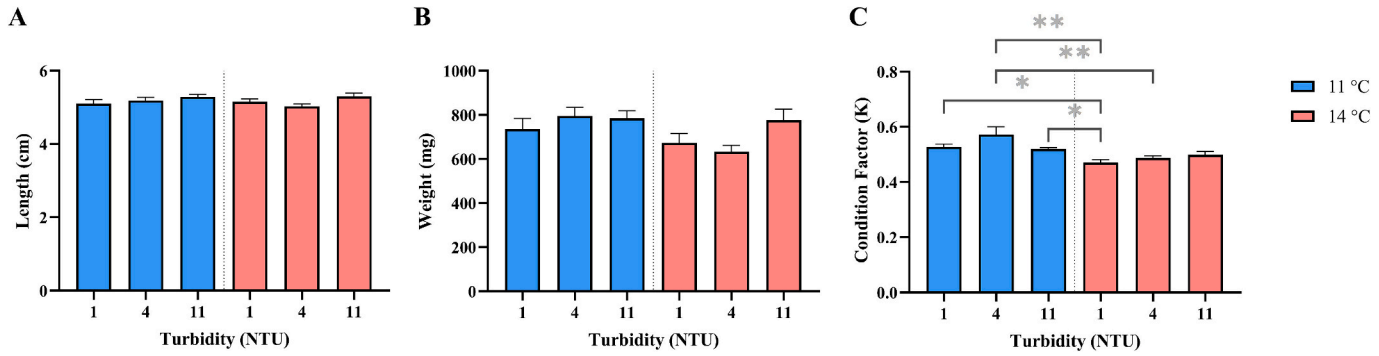


**Fig. 1.** Longfin Smelt (*Spirinchus thaleichthys*) growth: Length (A), weight (B) and condition factor K (C) of Longfin Smelt (209–228 dph) after 4 weeks at rearing conditions of turbidity (1 NTU, 4 NTU, and 11 NTU) and temperature (11 °C in blue, 14 °C in red) treatments. Data ($n = 4$) are presented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathways were not affected or that these biological processes might be more strongly regulated by other genes not analyzed. In contrast, temperature had a significant effect on transcription of *Catalase* (*CAT*; temperature F = 4.73, $p = 0.033$; turbidity F = 0.46, $p = 0.63$; interaction F = 1.60, $p = 0.21$), *Citrate synthase* (*CS*; temperature F = 6.19, $p = 0.015$; turbidity F = 0.82, $p = 0.44$; interaction F = 0.029, $p = 0.97$), and *Growth Factor Receptor Bound Protein 10* (*GRB10*; temperature F = 4.45, $p = 0.039$; turbidity F = 0.55, $p = 0.58$; interaction F = 0.76, $p = 0.47$) [Fig. 2].

Non-significant linear trends of gene expression with increasing

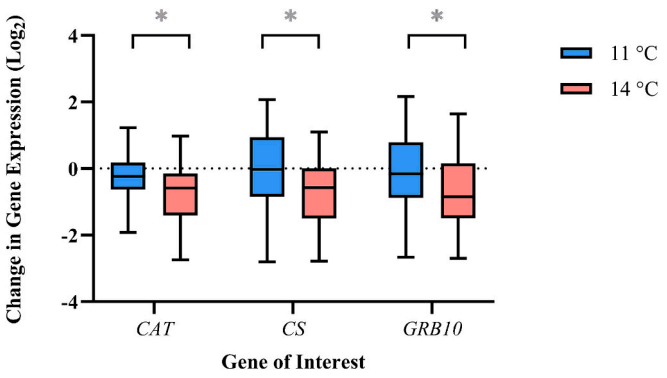


**Fig. 2.** Longfin Smelt (*Spirinchus thaleichthys,* 209–228 dph) gene expression levels: *Catalase*, *Citrate Synthase*, and *Growth Factor Receptor Bound Protein 10* at 11 °C (blue) vs. 14 °C (red) treatments at the end of the 4-week exposure period. Data ($n = 12$) are presented as mean (Box and whiskers, min to max), (* $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

turbidity were observed: Increasing expression means of *HSP30* (general stress) and *GLUT5* (nutritional status) at 11 °C, and *Cyp7a1* (cholesterol catabolic process and growth) at 14 °C; decreasing expression means for *POMCb* (HPI axis, precursor of adrenocorticotropic hormone, cortisol production) at 11 °C, and *IGF1r* (influencing growth hormones and transformation events) at 14 °C.

## 4. Discussion

Survival rates of juvenile LFS within the evaluated temperature range of 11 °C to 14 °C and turbidity levels ranging from 1 NTU to 11 NTU over a 4-week duration imply that these environmental conditions are suitable for their successful rearing at this life stage. At lower temperature, both weight and condition factor exhibited significant increases, while turbidity did not exert any discernible effect on growth, condition factor, or transcriptomic stress-response. The significant outcomes over the 4-week period represent crucial findings for aquaculture, indicating that 11 °C is likely more favorable than 14 °C, aligning with our initial hypothesis (i). Our findings are supported by those reported by Baxter et al. (2010) and Yanagitsuru et al. (2021), where temperatures of 9 °C and 12 °C were found to be more suitable than 15 °C for rearing larval stages of LFS. At 11 NTU, juveniles exhibited greater length compared to lower turbidity conditions; however, this was non-significant. This was also observed in similar studies for juvenile DSM weights and condition factors at 14 °C, where increased growth was determined at 10–11 NTU compared to 1–2 NTU (Pasparakis et al., 2023), and at up to 9 NTU for late-stage DSM larvae (Tigan et al., 2020); this could suggest the presence of a visual element in the feeding process, with turbidity potentially affecting prey contrast and, consequently, influencing feeding ability. Consequently, increased weight and condition factor, an indicator of overall health and well-being at 11 °C, suggest that this condition is beneficial, particularly since growth-related endpoints have been highly connected to survival on the basis that "bigger is better" (Sogard, 1997; Brander, 2003).

The transcription of genes associated with stress and growth does not conclusively establish that a lower temperature of 11 °C is closer to optimum for the cultivation of juvenile LFS. Three genes were significantly influenced by temperature: *Citrate Synthase* (*CS*), *Catalase* (*CAT*), and *Growth Factor Receptor Bound Protein 10* (*GRB10*). *CS* catalyzes the synthesis of citrate from oxaloacetate and acetyl coenzyme A and plays an important role in oxidative metabolism. It can be an indicator for the metabolic and aerobic capacity of fish (Pelletier et al., 1993; Majed et al., 2002) as well as growth rates (Pelletier et al., 1994). The higher expression level at 11 °C compared to 14 °C, supports our hypothesis that 11 °C can be beneficial for aerobic metabolism and growth. *CAT* and *GRB10*, however, showed lower expression at 14 °C than at 11 °C. *CAT* is a key enzyme in the bodies defense against oxidative stress and is a main driver in the maintenance of pro/antioxidant balance (Di Giulio et al., 1989; Ahmad et al., 2000; Bagnyukova et al., 2005a, 2005b; Lesser, 2006). Differences in *CAT* expression suggests potential modulation of cellular defense mechanisms and responses to oxidative stress. The lower expression levels of *CAT* at 14 °C compared to 11 °C could potentially indicate less oxidative stress at 14 °C. *GRB10*, a suppressor of growth and cellular proliferation interacts with insulin and insulin-like growth-factor receptors. It is known that overexpression can inhibit tyrosine kinase activity and result in growth suppression (Charalambous et al., 2003; Plasschaert and Bartolomei, 2015). At a temperature of 14 °C, the expression levels of *GRB10* were lower compared to those at 11 °C, making it improbable, based on the temperatures examined, that its expression was sufficiently high to impact growth but potentially affecting tyrosine kinase activity. We hypothesized (ii), that stress- and growth-related gene expression (e.g., *Proopiomelanocortin b precursor* (*POMCb*), *Heat shock protein 30* (*HSP30*), *Glucose Transporter 5* (*GLUT5*)) would indicate a preference for treatments at 11 °C. However, given that there were no notable expression alterations in any other genes of interest, hypothesis (ii) could not be confirmed. It remains plausible that temperatures tested may not impact the pathways associated with investigated genes. Based on our findings and those of other researchers (e.g. Yanagitsuru et al., 2021), temperatures below 14 °C can be considered to be within the thermal tolerance of LFS which explains the absence of cellular thermal stress responses (e.g., HSP expression changes; Jeffries et al., 2016).

Turbidity can elicit both favorable and unfavorable outcomes in fish, contingent on the species (Utne-Palm, 2002). Prior research has utilized *CAT* (e.g., Shin et al., 2006), *Heat shock protein 70* (e.g., Hasenbein et al., 2016), *Proopiomelanocortin* (e.g., Hasenbein et al., 2013), and *11-beta-hydroxyteroid-dehydrogenase* (e.g., Hasenbein et al., 2016) as candidate genes to evaluate responses to turbidity. Nevertheless, there were no significant differences in gene expression in LFS at tested turbidities up to 11 NTU, supporting hypothesis (iii), indicating that the migratory behavior of juvenile LFS to clearer ocean waters would lead to diminished dependence on turbid conditions. However, particularly at turbidity levels higher than those examined in this study, it is possible that turbidity could influence the HPI axis and consequently cortisol levels. This turbidity effect on stress is suggested by e.g., the observed reduction in the means of *POMCb* expression with increasing turbidity at 11 °C. The outcomes of this investigation indicate that, for culturing of the juvenile stage, turbidity might not be a crucial water criterion to uphold, as long as it remains at low levels. This result might not be surprising given that this developmental stage migrates toward ocean waters characterized by low turbidity and lower temperatures (Rosenfield and Baxter, 2007). In contrast to analogous investigations on DSM with different experimental designs (such as those conducted by Hasenbein et al. (2013, 2016)), we examined the impact of turbidity at relatively lower NTU levels and over a 4-week exposure duration, allowing adequate time for potential acclimation of LFS. This design employed herein may explain the observed lack of significance in gene expression outcomes due to e.g., significant plasticity and acclimation capacity to various turbidity levels over time, which was shown for guppy, *Poecilia reticulata* (Ehlman et al., 2015) and zebrafish, *Danio rerio* (Suriyampola et al., 2018). Overall, the absence of significant gene expression changes could be due to the environmental conditions being within the optimal range, the four-week acclimation period allowing animals to normalize any acute genomic alterations, or the influence of different pathways not detected by the candidate gene-targeted examination.

The relationship between growth and gene expression is intricate and dynamic. Under conditions of stress, organisms divert energy toward the restoration of homeostasis, consequently diminishing the energy allocation available for developmental and growth processes (Schreck and Tort, 2016). During development and differentiation,

however, there is a tendency for oxidative metabolism to be elevated, and concurrently, for body size to impact gene expression (Kocmarek et al., 2014). Findings by Kocmarek et al. (2014) indicate heightened expression of general stress and immune response genes in larger juvenile fish, relative to earlier larval life stages, indicative of enhanced adaptability to environmental changes. Despite the comparable size and age of LFS within the study, the modulated expression of *CAT*, *CS*, and *GRB10* might have also been influenced by size. Under hypothesis (ii), our prediction was that growth-related gene expression (e.g., *GRB10*, *GLUT5*) would indicate a preference for 11 °C. We could not support this hypothesis. Despite the growth data favoring 11 °C, gene expression data was inconclusive, not discarding 14 °C as a suitable rearing temperature. Multiple physiological endpoints need to be investigated in future to determine optimal conditions for LFS. Particularly, further research is warranted for various life stages, given that physiology can be influenced by the size of the fish and the distinct developmental stages occurring in diverse environments.

## 5. Conclusion

In an ecosystem such as the San Francisco Estuary, which has experienced substantial alterations in its physical, chemical, and biological aspects (Kimmerer, 2002; Cloern and Jassby, 2012), rapidly changing conditions challenge aquatic organisms. Rearing LFS in captivity is becoming increasingly important for population conservation and potential augmentation in the wild, as well as for the provision of research organisms toward better understanding specific requirements of this species. Taken together, temperature ranges of 11–14 °C and turbidity levels <11 NTU were found to be generally suitable for rearing juvenile LFS at an age range of 181 to 228 dph. Our results indicate that aquaculture practices could potentially forego adding algae into rearing tanks, toward increasing turbidity, leading to reduced costs and efforts (Hung et al., 2023). Reporting our non-significant gene expression results in response to turbidity is crucial as they often go unreported, leading to biased meta-analyses, despite the ethical obligation to document all findings from well-designed studies, which may help minimize publication bias and improve scientific dissemination (Visentin et al., 2020). Knowledge of the life-stage-specific impacts of culturing temperature and turbidity levels on LFS, as documented in this study, is an essential prerequisite for minimizing stress, optimizing growth, and supporting animal health. Future research and conservation efforts should integrate additional environmental factors (e.g., salinity), husbandry parameters (e.g., stocking density and life history), or life-related aspects (e.g., food type and habitat) to elucidate their interactions comprehensively. Understanding the tolerance thresholds and ecological needs for each developmental stage of LFS is crucial for advancing both basic science and its application in derived conservation initiatives.

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## CRediT authorship contribution statement

**Felix Biefel:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christina Pasparakis:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dennis E. Cocherell:** Resources, Methodology. **Tien-Chieh Hung:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Evan W. Carson:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Nann A. Fangue:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Juergen P. Geist:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Anne E. Todgham:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Richard E. Connon:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, 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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# Polystyrene Plastic Particles Result in Adverse Outcomes for *Hyalella azteca* When Exposed at Elevated Temperatures

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**Abstract:** Micro- and nano-plastics are pervasive pollutants in global ecosystems, yet their interactions with aquatic wildlife and abiotic factors are poorly understood. These particles are recognized to cause subtle detrimental effects, underscoring the necessity for sensitive endpoints in ecotoxicological exposure studies. We investigated the effects of particle uptake, size, and temperature on *Hyalella azteca*. Organisms were exposed to blue fluorescent polystyrene beads (500 nm and 1000 nm in diameter) at 0.43 mg/L for 96 h at temperatures mirroring climate predictions (21 °C, 24 °C, 27 °C). Besides survival and growth, particle uptake, visualized via confocal microscopy, and swimming behavior were analyzed. Mortality rates increased at 27 °C, and particle presence and temperature affected organism growth. Particle treatments influenced various behaviors (thigmotaxis, cruising, movement, acceleration, meander, zone alternation, and turn angle), with hypoactivity observed with 1000 nm particles and hypo- as well as hyper-activity responses with 500 nm particles. Particle uptake quantities were variable and increased with temperature in 500 nm treatments, but no migration beyond the gut was observed. Particle size correlated with uptake, and relationships with behavior were evident. Elevated temperatures exacerbated particle effects, highlighting the urgency of addressing plastic pollution in light of climate change for aquatic organism welfare and ecosystem health.

**Keywords:** locomotion; fluorescent microplastics; uptake; confocal microscopy



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## 1. Introduction

The first actual synthetic, mass-produced plastic called "Bakelite" was developed by Leo Baekeland in 1907 [1]. According to the OECD outlook for 2060, plastic leakage to the environment is projected to double to 44 million tonnes per year, while the build-up of plastics in aquatic environments will more than triple, exacerbating environmental and health impacts [2]. This waste can undergo further degradation into smaller particles (microplastics: MPs < 5 mm; nanoplastics: NPs < 1 µm; both categories: MNPs). Plastic particles of various sizes are considered a global pollution problem, though their environmental effects are often unknown. Primary MPs (e.g., those designed for commercial use) and secondary MPs (those resulting from degradation) are present ubiquitously across various environmental compartments, encompassing aquatic and terrestrial ecosystems and atmospheric and geographically isolated areas [3,4]. NPs represent a category of debris that remains relatively understudied and poorly characterized. However, paradoxically, they may pose the highest risk compared to other types of aquatic litter. This heightened risk stems not only from their capacity to penetrate biological barriers, but also from their extensive surface area, which could significantly influence the mechanisms of bioaccumulation and bioamplification of other pollutants [5]. Plants, for example, can function as

sinks for anthropogenic litter [6], and aggregation of plastic particles increases with rising temperatures and particle concentrations, deeming them either more or less accessible to certain taxa [7].

One of the earliest publications identifying plastic pollution as a "worldwide oceanic problem" was published in 1984 [8], depicting the detection of plastic materials during the 1970s in several aquatic, benthic, and planktonic samples, as well as describing impacts on seabirds and seals. Seasons and weather shifts influence litter accumulation [9], while plastic breakdown is driven by environmental conditions such as temperature, pH levels, ultraviolet ray exposure, and the effects of friction with rocks and sediment, wind action, and animal interactions; on being ingested by animals, enzymatic activity can result in further fragmentation into smaller particles [10–12]. Besides size, plastic particles can be categorized by shape, such as fiber, rod, ellipse, oval, sphere, quadrilateral, triangle, free-form, and unidentifiable [13]. In aquatic environments, the concentration, size, and shape of MNPs are the main properties influencing the uptake and consequential effects on organisms [14–16].

Synthetic MPs can exhibit pronounced adverse impacts relative to natural particles, with varying sensitivities observed across taxonomic groups [17]. Exceptions may occur, as evidenced by natural fiber types like hemp, which have been demonstrated to exert greater oxidative stress on mysid shrimp *Americamysis bahia* compared to synthetic fibers [18]. Among those effects, mortality is not a commonly observed effect of MP ingestion, presuming low risk at environmentally realistic concentrations [19]. However, sublethal effects have been described to include altered swimming behavior (*Daphnia magna*, [20]), development (*Xenopus laevis*, [21]), innate immune function (*Pimephales promelas*, [22]), liver inflammation (*Danio rerio*, [23]), effects on reproduction (*D. magna*, [24]), and growth (*Chironomus tepperi*, [25]). Thus, there can be indirect and potentially high risks at the population level. The factors mentioned above can further degrade MPs into NPs. NPs carry elevated ecological implications due to their augmented surface-area-to-volume ratio, leading to amplified vector effects for pollutants and bacteria, with smaller particles capable of passive membrane permeation and larger ones requiring active transport mechanisms [26,27]. MNPs can accumulate in the digestive tract, with particle size and shape influencing retention time and distribution within tissues [28,29]. Prior research indicates that larger particles, even those in the low-micron size range, can translocate the gastrointestinal tract, fillet, and livers of wild fish [30], bioconcentrate, and remain in tissues such as the gastrointestinal tracts, even after an extended period of depuration [31].

Fish and benthic macroinvertebrates exhibit complex ecological interactions within aquatic ecosystems, influencing nutrient cycling, trophic dynamics, and habitat structure [32]. Benthic macroinvertebrates are a group of organisms highly susceptible to the presence of MNPs in aquatic ecosystems. They are particularly vulnerable because they can readily ingest plastic particles in the sediment, an environmental compartment with high MP levels [33]. Amphipods and isopods are especially effective models for evaluating the potential toxicity of contaminants and pollutants because they serve as intermediaries between primary producers and higher-level consumers [34,35]. Consequently, their role as prey for fish introduces the potential for scale and magnification effects in the transfer of MP through the food web. *Hyalella azteca* (HA; Saussure, 1858) is widely distributed in aquatic ecosystems and can be easily cultured in laboratories [36,37]. They are epibenthic detritivores and a model test species recognized by the United States Environment Protection Agency (USEPA). HA are approximately 1 mm long and 0.04 mg in weight upon hatching, eventually attaining a maximum length of around 7 mm and 8 mg at maturity [38]. Given its ecological niche within the food web, HA is inherently predisposed to actively uptake and consume MNPs when present in its natural habitat. Studies using HA have demonstrated a gut retention of 24 to 28 h (tire wear particles, [39]).

Diverse plastics originating from industrial activities are introduced into natural ecosystems, notably aquatic environments. Polystyrene (PS), a thermoplastic polymer, was the first synthetic polymer shown to occur in coastal waters in 1970 [40], and is

now commonly found in aquatic environments, alongside polyethylene, polypropylene, and polyvinylchloride [41,42]. PS is a high molecular weight synthetic aromatic polymer derived from the monomer known as styrene, and commonly used for producing foam, cups, and containers [43]. Studies using HA have confirmed that PS fragmentation during ingestion and egestion is significant, while its ingestion also led to changes in enzymatic oxidative stress biomarkers [44,45].

Rising temperatures associated with global warming have significant implications for aquatic organisms and ecosystems. Temperature fluctuations can affect crustaceans' lipid, protein, and overall energy status [46,47]. Temperature and MNP effects can interact, thereby modulating their toxicity through diverse mechanisms. Studies have demonstrated that exposure to PS at varying temperatures can produce compounded adverse effects on *Artemia franciscana*, with higher temperatures resulting in reduced growth and increased mortalities [48]. Furthermore, an elevated temperature was shown to intensify the bioconcentration, immobilization, and oxidative stress effects of polyethylene MPs on *D. magna* [49].

In addition to traditional endpoints such as survival and growth, which are extensively utilized in ecotoxicological studies, recent publications have highlighted the use of photomotor assays measuring parameters involved in behavior (e.g., [50,51]). The behavioral assessment holds significant promise as a powerful tool in the field of aquatic toxicology and water quality monitoring [52,53]. Behavior, shaped by biotic and abiotic factors, enables organisms to respond to environmental changes, including contaminant exposure [54]. Stressors, whether acute or chronic, can adversely affect various behavioral aspects, such as feeding, which can ultimately influence survival and population dynamics [55,56]. Jacob et al. [57] reviewed differences in behavioral, sensory, and neuromuscular function indicators between control and fish exposed to virgin (not artificially aged or loaded) MNPs and revealed that the majority of endpoints demonstrated significant effects; boldness, exploration, activity, and locomotion were especially affected.

The objective of this study was to determine whether the experimental parameters of particle size (500 nm vs. 1000 nm) and water temperature (21 °C, 24 °C, 27 °C) influence survival, growth, and swimming behavior (video-based tracking), and to which extent MNPs uptake (fluorescence) might contribute to adverse effects. We primarily aimed to evaluate whether the quantity of PS uptake would affect locomotion, as PS exposure was previously shown to influence the feeding and swimming behavior of mysid shrimp *Neomysis japonica* [58]. The selection of the two bead sizes was based on their representation of both micro- and nano-categories, with limited existing literature on studies involving these particular sizes. Because temperature can change the uptake, elimination, and resulting effects on an organism, there is a need to study particle effects at different temperatures, especially in the context of global warming. We use HA, a species recognized for its sensitivity to environmental changes, and a model organism for assessing the risks associated with pollution, thereby indicating ecosystem health.

We hypothesized that (i): elevated temperatures influencing metabolic rates are predicted to increase particle uptake, leading to growth reduction and the manifestation of stress-related swimming behaviors; and (ii): due to the altered translocation ability of smaller particles, they are anticipated to demonstrate prolonged retention times, thereby exacerbating their detrimental effects.

Echoing proposals that knowledge derived from engineered nanoparticle toxicity research can inform risk assessments of PS particles [59], PS studies could prove invaluable in identifying knowledge gaps and research needs by providing a baseline for future toxicity investigations [60].

## 2. Materials and Methods

Animal husbandry and toxicity assessments were conducted following USEPA guidelines [61,62], with some adaptations.

### 2.1. Animal Source and Acclimation

*Hyalella azteca* were obtained from Aquatic BioSystems Inc. (Fort Collins, CO, USA) at age 4–5 days and placed, on arrival, in a 2 L beaker containing 1 L of the water they were shipped in, which was mixed with 1 L culture water (with 0.22 micron filtered well water diluted by 50% using MilliQ). They were then maintained at 24 °C in temperature-controlled chambers (Thermo Scientific Precision Model 818 and VWR, Thermo Fisher Scientific, Marietta, OH, USA) under a 16:8 h light/dark cycle. Following an initial 24 h habituation, individuals were separated into three 1 L beakers (120 individuals in each) for a further 24 h to increase the culture water content. Then, they were transferred to 500 mL of 100% culture water and brought to treatment temperatures (21, 24, and 27 °C) at a rate of 1 °C/day over a period of three days. Animals were maintained in water (culture water) so as to adjust water hardness to 180 mg/L $CaCO_3$. The resulting physicochemical parameters measured using a multi-meter (MultiLab 4010-3W, YSI Inc., Yellow Springs, OH, USA) were: pH 8.83, conductivity 946 µs/cm, dissolved oxygen 98.0% DO, salinity 0.4%, Total Dissolved Solids TDS 944 mg/L, Oxidation-Reduction Potential U −105.6 mV. Beakers were aerated using glass pipettes and covered with parafilm to avoid atmospheric contamination. Any dead organisms were removed daily. During acclimation, animals were fed daily with 3 mL/L YCT (Aquatic BioSystems Inc., Yeast, Cereal Leaves, Tetramin, produced in accordance with EPA recommendations [63], 1850 mg/L average total solids).

### 2.2. Particle Source, Particle–Food Preparation, and Concentration Determination

Polystyrene beads with a density of about 1.03 g/$cm^3$ were purchased from Applied Microspheres GmbH (formerly BS-Partikel GmbH, Mainz, Germany) at a concentration of 5% m/m (see spectral absorption and emission graph of blue fluorescence in Figure S1). The surface composition of these unmodified PS beads consists of unaltered polystyrene without surface functionalization, potentially featuring negatively charged sulfonic acid end groups. These particle solutions were also used by Götz et al. [64,65]. Particle mean diameters were 519 nm (NPs) and 1294 nm (MPs) with blue fluorescence. Particle stock solutions consisted of 50 mL MiliQ water, 50 mL YCT food, and 100 µL of PS particles; gentle shaking for 10 min and vortexing were used to homogenize the solution before adding the calculated volume to each beaker of 100 mL culture water (Table S1) and either 500 nm or 1000 nm PS beads at a single concentration of 0.43 mg/L. In order to prioritize the assessment of uptake quantities, our experimental design excluded leachate devoid of particles. This decision was additionally informed by the lack of observed fluorescence translocation from the gut to surrounding tissue during our range-finding study.

At present, there is a lack of adequate quantitative analytical methods to evaluate NP concentrations in environmental settings. However, it is theorized that secondary NP concentrations are likely to escalate due to their release via fragmentation and degradation processes of larger particles in marine and freshwater environments. Based on mass conservation principles, estimates suggest that NP concentrations could reach levels $10^{14}$ times higher than those currently measured for MPs [66]. Relative to total plastic weight instead of PS particle counts, worst-case scenarios showed higher concentrations in the environment: in playa wetlands, USA, 5.51 mg/L [67]; Southwest Europe and East Asia, 0.32 mg/L to 1.89 mg/L [68–70]; and Taihu Lake, China, 30–50 mg/L [71,72]. Studies indicate that the transport and distribution of plastic particles are governed by solution chemistry, particle size, and mineral surfaces [73]. Consequently, variations in particle sizes potentially influence the formation of PS conglomerates, resulting in localized yet elevated PS concentrations. As suggested by Lee et al. [7], NP aggregation should increase, correlating with both particle concentration and temperature.

### 2.3. Exposure and Water Physicochemical Parameters

After acclimation, ten individuals were transferred into each 100 mL exposure beaker (3 beakers/treatment/temperature, N = 30 individuals/temperature/treatment). All surviving animals were used for growth measurements and uptake quantification. For locomotor

assays, a total of 9 × 24-well plates were run sequentially on a single day to evaluate behavioral responses of 6 individuals/beaker, N = 18 individuals/temperature/treatment. During exposure, the three environmental chambers were used to regulate water temperature. The means ± SEM for the exposure duration were 20.7 ± 0.1 °C, 23.9 ± 0.1 °C, and 27.1 ± 0.1 °C, respectively, measured using HOBO loggers (Onset, MA, USA) placed in additional beakers in each chamber (Figure S2).

Conductivity, dissolved oxygen, salinity, and pH were tested daily (Table S2); ammonia (RedSea, Houston, TX, USA) reached a maximum of 0.8 µM, nitrite 0 ppm, and nitrate 40 ppm (API, Philadelphia, PA, USA) by the end of the exposure. Air samples did not show relevant MP contamination across the exposure time in the chambers. HA exposed to a single particle dose of 0.43 mg/L (500 nm: 64,000,000 p/mL; 1000 nm: 8,000,000 p/mL of the micro size 1000 nm) at the beginning of the 96 h exposure, i.e., there was no water renewal during the exposure, and continuous aeration contributed to oxygen supply and particle suspension homogeneity. The exposure concentration was determined with a prior range-finding study starting with the environmental relevance with the tested endpoints survival, growth, swimming behavior, and uptake at medium temperature (Table S1). Our objective was to identify a concentration capable of eliciting behavioral responses and observable particle uptake. Although the chosen concentration does not reflect ecological conditions directly, it allowed for a detailed examination of the mechanistic relationships among endpoints and revealed differences from controls compared to lower concentrations. Additionally, food particles were introduced in the primary investigation to enhance uptake further. Reported PS concentrations are nominal due to particle–food mixture interference with fluorometric and light microscopy evaluation.

During exposure, mortality, along with water physicochemical parameters, was recorded daily. At the conclusion of the exposure, specific groups of organisms were transferred using a transfer pipette into individual wells of a 24-well plate, ensuring careful handling, with three biological replicates (i.e., organisms were taken from three replicate beakers of each treatment) and six replicates (animals) from each beaker to (A) run behavior trials or (B) run behavior trials with subsequent individual confocal imaging for fluorescent particle uptake quantification via fluorescent pixel counting (three biological, three technical replicates). After the behavior trials, animals were euthanized on ice and stored in 3% paraformaldehyde for subsequent analysis.

### *2.4. Growth: Total Length, Capsule Length, and Dry Weight*

Total length, defined as the length along the dorsal edge from the tip of the rostrum to the telson tip, and capsule length, defined as the distance from the tip of the rostrum to the posterior margin of the cephalon [74], were measured via image analysis using Fiji version ImageJ 1.53t [75,76]. Images were taken on a ruler using a Leica S8APO stereomicroscope (Leica Microsystems, Chicago, IL, USA) and Canon EOS Rebel T6 SLR camera (Canon, Tokyo, Japan).

For dry-weight measurements, empty and opened 1.5 mL microcentrifuge tubes were dried at 60 °C for a minimum of 2 h and were subsequently allowed to cool for at least 30 min within a desiccator at room temperature. After measuring the tube weights (Sartorius Quintix, Goettingen, Germany, with a readability of 0.01 mg), all organisms that were not utilized for individual confocal microscopy (later called Group (B)) were pooled from each respective beaker and relocated into the tubes to dry them for 24 h at 60 °C with the caps removed. After cooling down within the desiccator, the total weight was measured and divided by the number of individuals.

### *2.5. Locomotor Behavior Assay*

Behavioral studies were conducted using a DanioVision Observation chamber (Wageningen, The Netherlands) and integrated steady flow of water set to treatment temperature via chiller (TECO-US, Terrell, TX, USA). A 35 min LD (Light:Dark) cycle test was performed with alternating light and dark cycles of 5 min, following protocols described

in Siddiqui et al. [77,78]. After the exposure, HA was pipetted into randomized wells of 24-well plates (Corning Costar, Corning, NY, USA, 24-well Clear, Product Number 3524). Organisms were habituated for 30 min in the climate-controlled exposure chambers of their treatment temperature before being transferred into the Noldus system for evaluation. The LD cycle protocol consisted of an acclimation time of 5 min, following three alternating light and dark cycles of 5 min durations. During behavioral trials, a recirculating water system was used to keep HA at the treatment temperature. It was observed that some individuals remained in the center close to the water surface without moving. These individuals were still analyzed, as no mortality was detected, and were active following mechanical stimulus. EthoVision XT 14 software (version 14, Noldus, Wageningen, The Netherlands) was used for video tracking. For correlation analysis, standard endpoints TDM (total distance moved) and thigmotaxis ("wall hugging", avoidance indicator), which describes the ratio of staying in the outer ("hiding") to inner ("boldness") behavior as described before by Segarra et al. [51], and velocity were used. The further behavioral endpoints used were defined before [50,77,78].

### *2.6. Internalization*

After 96 h of exposure, animals were euthanized on ice and stored in paraformaldehyde until whole bodies were transferred on a microscope slide with a cover slip. Fluorescent visualization was used to quantify the uptake of the PS beads. This approach is commonly used to make fluorescent particles visible and screen their location in a non-invasive way [79–85]. For this study, we used a Leica TCS SP8 STED 3X microscope (Wetzlar, Germany) and the LAS X software (Advanced Imaging Facility UC Davis, version 3.5.7.23225). Internalization of particles was measured using Image J 1.53t. However, particle uptake was evaluated through two different approaches.

#### 2.6.1. Group (A): Imaging in 2D

To obtain preliminary insights into the overall PS uptake patterns and variability among individuals, specimens from each beaker were recorded. These recordings were conducted using the Leica microscope set to specific parameters (excitation wavelength: 405 nm, emission wavelength: 500 nm), capturing layered merged tiff images. This approach allowed for a comprehensive visualization of PS uptake across the sampled population (500 nm: 21 °C: N = 18, 24 °C: N = 11, 27 °C: N = 13; 1000 nm: 21 °C: N = 15, 24 °C: N = 10, 27 °C: N = 14). Using the ROI (region of interest) Manager, areas of interest were drawn around the gut region of each individual to measure positive pixels after setting up a color threshold (threshold color settings: Red 0 and 255, Green 0 and 255, Blue 0 and 254; method: Default, Threshold color: Black, Color space: RGB, with Dark background) and transforming the pictures to binary images.

#### 2.6.2. Group (B): Imaging in 3D

A subset of samples, distinct from those utilized in the previous analysis (A), underwent in-depth analysis to deepen the understanding of uptake dynamics. We aimed to uncover potential associations between the quantity of particles internalized by a single organism and its respective locomotor activities. Therefore, high-resolution images recorded with the Leica microscope were used to assess the quantity of ingested particles in the gut region following locomotion assays. This allows us to assign effect results to one individual and analyze their correlation. With this subsample, which had already gone through the behavioral assays, three individuals from each beaker (N = 9) were investigated via confocal microscopy. An excitation wavelength of 405 nm and an emission wavelength of 500 nm were used to make blue, fluorescent PS beads visible, producing 8-layered z-stack images through their body. In ImageJ, pixels above a threshold were counted with the following settings/procedure: (1) image-zstack-maxprojection, (2) threshold-otsu-red and 81 to 255, (3) transformation to binary, otsu dark, tick black background and make new stack, (4) draw ROI around the gut region, (5) ctr + h to create a histogram, and (6) note 255 value from the

list. These images were also used to measure individuals' total and capsule length for the correlation analysis.

### *2.7. Statistics*

To assess whether there were any significant differences across various treatments, we employed two-way ANOVA analyses along with subsequent Tukey multiple comparison tests. The normality of the endpoint data was examined using Shapiro–Wilk tests, while Levene's tests were utilized to assess the equality of variances. In cases where these assumptions were not met, particularly in instances involving mortality, behavior, and fluorescence data, non-parametric KW (Kruskal–Wallis tests) paired with Dunn's multiple comparison tests were employed. To evaluate survival outcomes comprehensively, we utilized Kaplan–Meier survival curves in conjunction with the Logrank (Mantel–Cox) test, which are widely recognized statistical tools for analyzing time-to-event data, particularly for survival times. The Kaplan–Meier method enables the estimation of survival probabilities over time while accounting for censored observations, providing a clear visualization of survival trends among experimental groups. Additionally, the Logrank test used assesses the equality of survival distributions between groups, allowing for the detection of significant differences in survival rates [86]. These statistical approaches were selected due to their robustness in handling survival data and their ability to yield meaningful comparisons and trend assessments within our experimental context. To investigate associations between nonparametric data endpoints, we employed a Spearman correlation analysis. Unless otherwise indicated, 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) and Jamovi (Version 2.3.16.0).

## 3. Results

### *3.1. Survival and Growth*

Survival of control organisms not exposed to particles at 96 h and at 21, 24, and 27 °C was above 95%. While temperature significantly influenced overall survival (KW, $\chi^2 = 6.68$, $p = 0.035$), it was not affected by particle treatment (KW, $\chi^2 = 5.06$, $p = 0.08$; Figure 1a,b). However, at 27 °C, both the 500 nm treatment ($p = 0.033$) and the 1000 nm treatment ($p = 0.043$) exhibited significantly lower survival rates than their respective controls, indicating some combination effect of temperature and particle. Additionally, at 500 nm, there was a significantly lower survival at 27 °C relative to 500 nm at 21 °C ($p = 0.033$). Similarly, the 1000 nm treatments at 24 °C showed significantly higher survival than at 27 °C ($p = 0.0430$). The combination of the highest temperature of 27 °C plus the exposure to PS particles was consequently shown to have adverse effects on survival. This adverse effect was supported by Kaplan–Meier survival curves, which were shown to be significantly different (Logrank Mantel–Cox test: $\chi^2 = 19.44$; $p = 0.0127$) with a significant trend (Logrank test $\chi^2 = 10.11$; $p = 0.0015$). Especially the survival curve of 500 nm treatments at 27 °C differed in comparison to controls ($\chi^2 = 5.008$; $p = 0.0252$).

Total length was significantly influenced by temperature (ANOVA, F = 6.24, $p = 0.003$) and particle treatment (ANOVA, F = F = 6.93, $p = 0.001$) (Figure 2a). Specifically, at 21 °C ($p = 0.008$), 24 °C ($p = 0.008$), and 27 °C ($p = 0.008$), the animals exposed to 1000 nm particles were shorter in total length compared to the respective controls. In contrast, 500 nm treatments were not different in total length compared to respective controls. In all treatments, however, animals were significantly ($p < 0.05$) longer at 24 °C compared to 21 °C. There were no differences in dry weight and capsule length (Figure 2b,c).

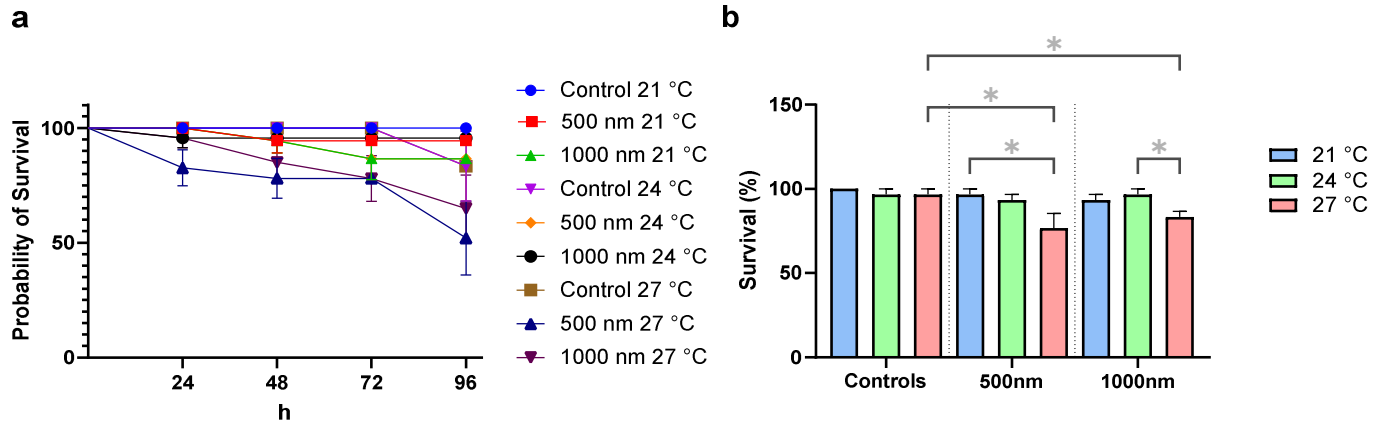


**Figure 1.** Survival during and after the 96 h exposure of *H. azteca* across treatments control, 500 nm PS particles, and 1000 nm PS particles at three temperatures (21 °C, 24 °C, and 27 °C). (**a**): Kaplan–Meier survival curve: probability of survival in percent (*y*-axis) over 96 h (*x*-axis). Survival curves are significantly different, with a significant trend. Survival curves differed at 27 °C between controls and 500 nm particle treatments ($\chi^2$ = 5.008; $p$ = 0.0252). (**b**): Final survival rate at test termination in percent (*y*-axis) * $p < 0.05$, N = 30. (Mean ± SEM).

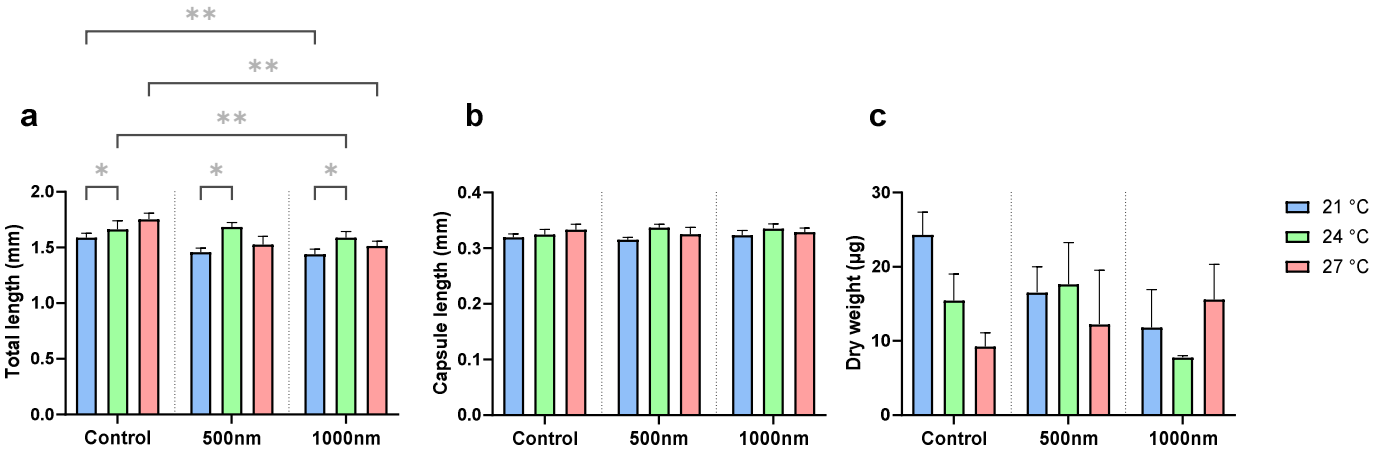


**Figure 2.** Growth *of H. azteca:* Total length (**a**), capsule length (**b**), and dry weight (**c**) (*y*-axis) ($N_{dry\ weight}$ = 2–3) following 96 h treatments control, 500 nm PS particles, and 1000 nm PS particles (*x*-axis) and three temperatures 21 °C, 24 °C, and 27 °C. * $p < 0.05$, ** $p < 0.005$; $N_{lengths}$ = 11–21. Total length was affected by particle treatment (F = 6.93, $p$ = 0.001) and temperature (F = 6.24, $p$ = 0.003). (Mean ± SEM).

### *3.2. Locomotor Behavior Assay*

The particle treatment significantly affected thigmotaxis, cruising, moving time, acceleration, meander, zone alternation, and turn angle ($p < 0.001$ for all endpoints; Table 1). Temperature influenced cruising ($p < 0.001$), moving ($p$ = 0.02), acceleration ($p$ = 0.03), and meander ($p$ = 0.04) significantly. Interestingly, 1000 nm exposures led to hypoactivity, while 500 nm exposures led to decreased and increased behavioral activity endpoints. A Spearman correlation revealed that temperature was significantly correlated with TDM ($p$ = 0.03 in dark, $p$ = 0.01 in light) and velocity ($p$ = 0.03 in dark, $p$ = 0.01 in light) in controls, but not in treatments of animals exposed to particles (Table S3). Changes from dark to light cycles did not result in significant alterations to the behavioral parameters tested.

**Table 1.** Locomotor behavior assay of eight endpoints across PS particle treatment (control, 500 nm, 1000 nm), temperature (21 °C, 24 °C, 27 °C), and cycle (dark, light) after a 96 h exposure on *H. azteca*. A significant increase (↑) or decrease (↓) in comparison to the corresponding control treatment is marked in bold (Kruskal–Wallis, $p < 0.05$; N = 18). (Mean ± SEM).

| Treatment | Temperature | Cycle | | Thigmotaxis | TDM (mm) | Velocity (mm/s) | Cruising Mean | Moving Mean | Acceleration Mean (mm/s$^2$) | Meander Min (deg/mm) | Zone Alternation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Controls | 21 °C | Dark | Mean | 0.17 | 648.17 | 16,200.01 | 0.16 | 0.09 | 32.48 | 548,707.01 | 41.74 |
| | | | SEM | 0.04 | 77.73 | 1942.64 | 0.01 | 0.00 | 14.92 | 154,155.45 | 6.40 |
| | | Light | Mean | 0.14 | 665.36 | 16,629.49 | 0.19 | 0.09 | 62.44 | 447,422.19 | 35.65 |
| | | | SEM | 0.03 | 80.66 | 2016.02 | 0.02 | 0.00 | 22.39 | 72,998.12 | 5.83 |
| | 24 °C | Dark | Mean | 0.20 | 501.83 | 12,526.44 | 0.15 | 0.09 | 71.83 | 41,3407.62 | 56.02 |
| | | | SEM | 0.04 | 61.83 | 1543.29 | 0.01 | 0.00 | 18.13 | 67,852.27 | 7.44 |
| | | Light | Mean | 0.20 | 528.67 | 13,196.59 | 0.15 | 0.08 | 26.67 | 568,502.63 | 58.46 |
| | | | SEM | 0.04 | 55.91 | 1395.66 | 0.01 | 0.00 | 9.78 | 130,905.13 | 7.16 |
| | 27 °C | Dark | Mean | 0.33 | 447.96 | 11,172.90 | 0.17 | 0.10 | 56.47 | 510,822.34 | 77.50 |
| | | | SEM | 0.05 | 84.81 | 2115.36 | 0.01 | 0.01 | 20.02 | 269,753.60 | 10.82 |
| | | Light | Mean | 0.28 | 495.26 | 12,352.93 | 0.19 | 0.09 | 75.39 | 775,993.28 | 74.13 |
| | | | SEM | 0.05 | 81.29 | 2027.59 | 0.01 | 0.01 | 30.74 | 263,831.07 | 10.38 |
| 500 nm | 21 °C | Dark | Mean | **↑ 0.32** | **↓ 394.47** | **↓ 9859.24** | 0.14 | 0.10 | **↑ 81.01** | **↓ 321,832.86** | **↑ 75.52** |
| | | | SEM | 0.05 | 44.41 | 1109.99 | 0.01 | 0.01 | 21.92 | 91,097.54 | 8.93 |
| | | Light | Mean | **↑ 0.33** | 420.62 | 10,512.77 | 0.14 | **↑ 0.10** | 91.31 | **↓ 337,481.7** | **↓ 81.69** |
| | | | SEM | 0.05 | 54.43 | 1360.34 | 0.01 | 0.00 | 24.56 | 92,749.78 | 8.74 |
| | 24 °C | Dark | Mean | **↑ 0.40** | 548.34 | 13,687.72 | 0.15 | 0.09 | 85.23 | **↓ 131,648.31** | **↑ 94.84** |
| | | | SEM | 0.05 | 88.12 | 2199.78 | 0.01 | 0.01 | 26.31 | 16,606.04 | 8.44 |
| | | Light | Mean | **↑ 0.40** | 606.83 | 15,147.97 | 0.16 | **↓ 0.09** | **↑ 162.32** | **↓ 222,524.87** | **↑ 94.63** |
| | | | SEM | 0.05 | 101.73 | 2539.74 | 0.01 | 0.00 | 46.82 | 58,640.33 | 9.38 |
| | 27 °C | Dark | Mean | 0.27 | **↑ 645.79** | **↑ 16,107.80** | 0.21 | 0.08 | 79.04 | 78,552,518.10 | 59.93 |
| | | | SEM | 0.04 | 84.73 | 2113.20 | 0.02 | 0.00 | 27.67 | 50,329,114.99 | 6.37 |
| | | Light | Mean | 0.22 | 663.80 | 16,557.51 | 0.20 | 0.08 | 38.31 | 52,073,199.65 | 53.63 |
| | | | SEM | 0.04 | 85.59 | 2134.75 | 0.02 | 0.00 | 11.29 | 38,954,708.05 | 6.06 |
| 1000 nm | 21 °C | Dark | Mean | 0.26 | **↓ 460.38** | **↓ 11,506.56** | 0.15 | 0.09 | 90.86 | 288,840.77 | 56.74 |
| | | | SEM | 0.05 | 62.18 | 1554.11 | 0.01 | 0.01 | 27.82 | 46,682.69 | 8.55 |
| | | Light | Mean | 0.27 | **↓ 475.73** | **↓ 11,890.01** | 0.14 | 0.09 | 77.79 | **↓ 307,032.33** | 61.56 |
| | | | SEM | 0.05 | 64.43 | 1610.24 | 0.01 | 0.01 | 24.39 | 48,446.12 | 8.92 |
| | 24 °C | Dark | Mean | 0.21 | 642.22 | 16,030.16 | 0.13 | **↓ 0.08** | 55.44 | 368,102.43 | 47.63 |
| | | | SEM | 0.04 | 79.95 | 1995.47 | 0.01 | 0.00 | 14.53 | 68,938.96 | 7.91 |
| | | Light | Mean | 0.21 | 697.79 | 17,417.42 | 0.14 | 0.08 | 53.18 | **↓ 316,204.54** | 51.54 |
| | | | SEM | 0.04 | 88.89 | 2218.80 | 0.01 | 0.00 | 14.26 | 65,979.64 | 7.84 |
| | 27 °C | Dark | Mean | 0.28 | 487.30 | 12,156.83 | **↓ 0.14** | **↓ 0.08** | 54.77 | 566,070.12 | 58.57 |
| | | | SEM | 0.05 | 69.85 | 1742.71 | 0.01 | 0.00 | 17.89 | 257,837.42 | 7.38 |
| | | Light | Mean | 0.28 | 508.36 | 12,682.07 | **↓ 0.13** | 0.09 | 57.39 | 332,440.14 | 61.65 |
| | | | SEM | 0.05 | 77.09 | 1923.03 | 0.01 | 0.01 | 18.50 | 67,763.56 | 8.43 |
| Sign. category effect | Particle treatment | | | **<0.001** | 0.82 | 0.82 | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| | Temperature | | | 0.47 | 0.34 | 0.34 | **<0.001** | **0.02** | **0.03** | **0.04** | **0.28** |
| | Cycle | | | 0.49 | 0.47 | 0.47 | 0.81 | 0.60 | 0.48 | 0.59 | 0.96 |

### *3.3. Internalization*

#### 3.3.1. Group (A)

There was a notable variability in uptake across individuals within a single beaker. The guts of some individuals of the same replicate were filled entirely with PS beads, while others barely showed any fluorescence. Despite high uptake variability, treatments were shown to influence the measured fluorescence in the gut. Temperature ($\chi^2 = 7.47$, $p = 0.024$) and particle size ($\chi^2 = 17.7$, $p < 0.001$) had significant effects on uptake (Figure 3a), and

uptake at 21 °C was significantly higher in the 1000 nm particle treatments compared to 500 nm ($p$ = 0.0128). Generally, it was observed that fluorescence in 500 nm treatments was lower compared to 1000 nm treatments, and that uptake variability was higher in 1000 nm treatments. The average range in 500 nm across all temperatures was 7.1%, and in 1000 nm treatments it was 26.3%. Linear regression revealed that the overall slopes of 500 nm and 1000 nm treatments, however, were not different (F = 0.83, $p$ = 0.46), and the slope of 500 nm treatments was non-zero (F = 162.8; $p$ = 0.0498), indicating increased uptake with temperature. Observationally, animals treated with 1000 nm particles also appeared to exhibit enhanced uptake with rising temperatures (Figure S3).

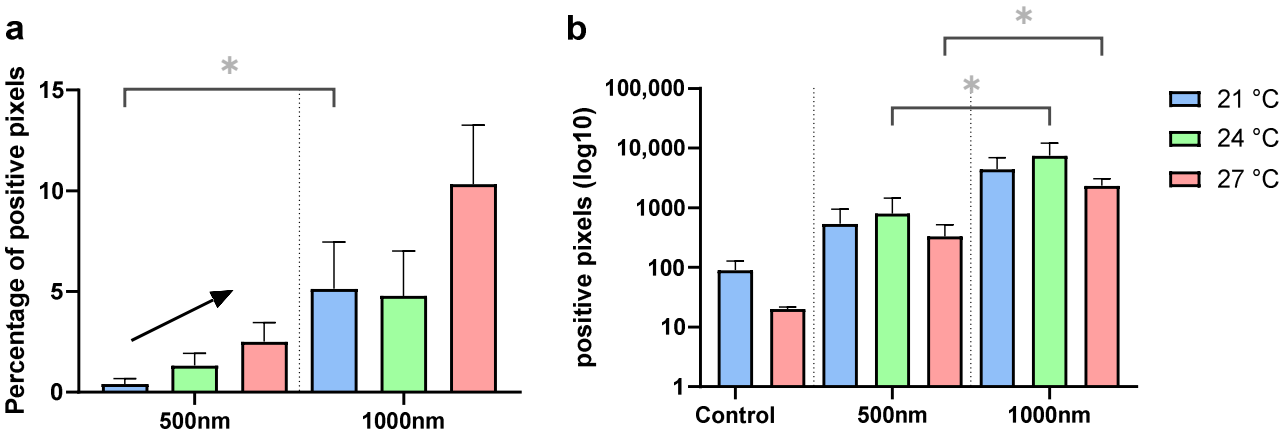


**Figure 3.** (**a**) Group (A): PS uptake comparison between the two investigated particle sizes of *H. azteca* by the percentage of positive pixels ($y$-axis) in relation to total pixel number in the ROI around the gut region. $n$ = 3, * $p$ < 0.05; 500 nm (N = 11–18), 1000 nm (N = 10–15). The increasing slope of 500 nm (arrow, Y = 0.35 × X − 6.99) across temperature treatments is non-zero (F = 162.8; $p$ = 0.0498). (**b**) Group (B): Logarithmic PS uptake of *H. azteca*: Pixels above threshold data ($y$-axis) across treatments control, 500 nm particles, and 1000 nm particles ($x$-axis) and three temperatures 21 °C, 24 °C, and 27 °C. * $p$ < 0.05. Control (N = 3–6), 500 nm (N = 8–9), 1000 nm (N = 9). (Mean ± SEM).

#### 3.3.2. Group (B)

For animals that were maintained in clean water for 30 min acclimation and the duration of the behavioral assays, no significant differences in particle uptake quantity were observed in terms of temperature (KW, $\chi^2$ = 2.38, $p$ = 0.31); in contrast, uptake quantity differed significantly between the particle sizes (KW, $\chi^2$ = 34.56, $p$ < 0.001; Figure 3b). Consistent with the findings from (A), there were higher fluorescence signals in the 1000 nm particle treatments compared to 500 nm particle treatments. In total, 500 nm particle treatments at 24 °C ($p$ = 0.011) and 27 °C ($p$ = 0.025) exhibited significantly lower signals than the 1000 nm treatments at the same temperature. Examples of exposed animals are shown in Figure S4. Particle translocation from the gut was not observed in any surrounding tissues in any individual (Figure 4).

### *3.4. Correlations between Uptake, Particle Size, and Tested Endpoints*

The strongest correlation was found for particle size and uptake (Figure S5). A Spearman correlation analysis was performed specifically for individuals which underwent the locomotion assay and the confocal analysis according to (B). Particle size and uptake demonstrated a rs-value of 0.67 ($p$ < 0.01). Furthermore, uptake correlated with TDM (dark) (rs = 0.35; $p$ = 0.01), thigmotaxis (dark) (rs = −0.28; $p$ = 0.04), velocity (dark) (rs = 0.35; $p$ = 0.01), TDM (light) (rs = 0.30; $p$ = 0.03), and velocity (light) (rs = 0.30; $p$ = 0.03). Total length was related to particle size (rs = 0.28; $p$ = 0.049) and temperature (rs = 0.02; $p$ < 0.01). The other endpoints were not correlated with the uptake quantity.

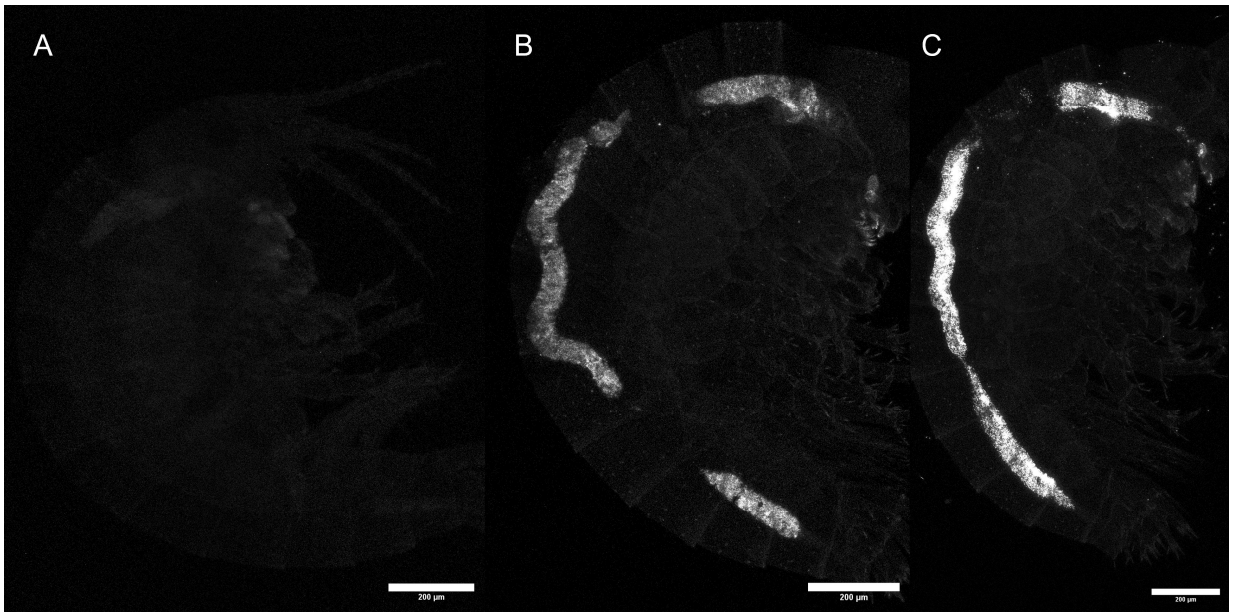


**Figure 4.** Internalization of fluorescent PS beads: control (**A**) and after exposing *H. azteca* to fluorescent PS beads with a diameter of 500 nm (**B**) and 1000 nm (**C**) for a duration of 96 h. Observations revealed a notable uptake in these beads distributed throughout the gastrointestinal tract of the organism.

## 4. Discussion

This study investigated impacts from exposure to micro- and nano-sized PS beads on uptake, locomotion, growth, and survival of the amphipod model species *H. azteca*. It also determined whether temperature would influence the measured effects. We hypothesized that elevated temperatures would lead to increased particle uptake, in turn leading to reduced growth and altered swimming behavior. This holds particular significance for smaller PS beads characterized by prolonged retention times, potentially attributed to factors such as increased probability of biodistribution and accumulation. Results confirmed our hypothesis (i) that elevated temperatures would increase particle uptake, resulting in decreased growth and survival. PS beads of 500 nm were not conclusively demonstrated to be more detrimental than 1000 nm beads in the context of survival and growth (hypothesis ii). The highest tested temperature of 27 °C, combined with 500 nm and 1000 nm PS beads at a concentration of 0.43 mg/L, led to harmful effects on HA. Besides survival curves exhibiting discernible temperature-dependent trends, total length exhibited significant alterations due to temperature and particle size. HA exposed to 1000 nm particles across all temperatures was shorter in total length compared to respective control groups. These results confirm findings in *A. franciscana*, which showed that besides higher PS concentrations, warmer water temperatures led to decreased survival and growth [48]. However, no differences in total length were determined between the 500 nm treatments and controls. The reasons might be that the larger-sized particles resulted in higher uptake due to a higher likelihood of confusion with food sources of similar size, such as yeast of 5 µm [87], which is part of the YCT food combination on which they were fed. It is interesting that although an apparent decline in dry weight was observed with increasing temperatures in the control group, this trend appeared to be absent in the exposure treatments involving 500 nm and 1000 nm PS beads. This discrepancy might indicate that the presence of these MNPs could potentially mitigate the effects of temperature on dry weight. Results also indicated that uptake increased with temperature and that behavioral effects differed depending on particle size.

Bead uptake could have reduced energy supply, leading to reduced total body length in 1000 nm treatments. Studies have demonstrated that *D. magna* reduces feeding rates when exposed to MPs [88]. The inhibition of metabolic rates due to exposure to MPs has been documented in various aquatic organisms [89,90], underscoring the capacity of

these minute pollutants to hinder physiological functioning. It also has been shown that microbiomes can be influenced by PS size in shrimp (*Litopenaeus vannamei*; [91]), which might further explain differences in growth and even in survival. However, it is worth mentioning that feeding rates have been reported to remain unaltered or even increase in freshwater amphipod *Gammarus fossarum* exposed to MPs [92] and that feeding rates can also vary over time [93]. Consequently, species' differences and adaptation mechanisms to food sources contribute to case-specific outcomes and can differ between our static exposure with no regular feeding and studies with regular feeding.

Both factors, temperature, and particle size, significantly influenced uptake quantities. Considering the correlation analysis of particle size, temperature, uptake, and total length, our results indicate that higher temperatures caused higher uptake and, ultimately, reduced growth and increased mortality. This result was confirmed by elevated temperatures demonstrating augmentation of the buildup of MPs within fish, consequently influencing the activity of metabolic enzymes [90]. Results are also supported by findings in the detritivores *Gammarus pulex* metabolic and feeding rates rising with larger body mass and higher temperatures, while MPs negatively impacted metabolic rates but not feeding rates [94]. As we observed high variability of particle quantities in the guts of HA, these differences between individuals of the same beaker could have led to high variability of growth and behavior responses. Per the manufacturer's specifications, an equivalent mass ratio of fluorescent dye to styrol was employed for both particle sizes. However, while the polymer weight was equal between 500 nm and 1000 nm treatments, the mean fluorescence intensity of PS beads was observed to scale approximately linear with the bead surface area, indicating that the brightness of spots declines as the bead size decreases [95]. Additionally, the ratio of volume to surface area might have influenced the signal, which could have further been affected by the number of particles (64,000,000 vs. 8,000,000 p/mL) and possible breakdowns in the organisms.

Elevated temperatures impose biological limitations on ectothermic organisms' metabolic and cellular processes [96,97] and increase metabolic rate, reaching the thermal optimum for a given organism, which can subsequently change individual feeding rates and induce modifications in interactions between consumers and resources [97–99]. Metabolic and feeding rates of *G. pulex* both demonstrated an increase in response to elevated temperature and body mass, in alignment with predictions from the Metabolic Theory of Ecology [96,98]. Based on the temperature coefficient or Q10 effect, biological reactions are influenced by temperature and, therefore, the energy status of organisms [46,47], leading to higher feeding demands at 27 °C, which could have caused higher uptake of particles. Increased mortality at 27 °C could have ultimately resulted from higher energy demand and PS beads, providing no nutritional value.

Studies suggest that HA has the capability to induce particle fragmentation attributed to its digestive system, characterized by a gastric mill for food crushing [45,100]. Higher metabolic rates and feeding demands at 27 °C might have caused particles to break down faster, which was not investigated in this study. However, this breakdown of PS beads could have led to a modification of effects over time due to particle dimension changes. For other organisms, smaller particle uptake has been shown to cause higher bioconcentrations and longer retention times [101], which could indicate higher stress-related effects of smaller PS particles. Similar to results on zebrafish showing that 250 and 700 nm particles barely passed the intestinal tract and outer epidermis [102], we did not observe apparent translocation into adjacent tissue leading to physical obstruction. Previous studies describe the necessity to add dye/leachate controls to the experimental design to determine if particles themselves, or the unrestricted movement of the dye molecule through cellular barriers, lead to fluorescence (e.g., [103,104]). However, we did not see fluorescence penetrating the gut wall, indicating that free leachate was absent.

Due to distinctive properties, such as hydrophobicity, plastics can adsorb contaminants (commonly called "vector" effect) of specific chemical properties, including persistent organic pollutants, amplifying the toxic effects on organisms [105]. Modified size-dependent

vector effects can explain growth and behavior differences between 500 nm and 1000 nm treatments. In this context, the volume-to-surface area ratio must be considered. Larger beads have a reduced surface area relative to their volume, and vice versa. As such, the larger surface of smaller particles would increase the likelihood of metabolites, bacteria, or other molecules conglomerating with 500 nm particles over 1000 nm. While the mass of PS was equal between the two size fractions, the particle count of 500 nm beads was also eight times higher than the 1000 nm beads. This would implicate an increased vector effect in 500 nm treatments, which could explain particle size dependent differences such as hyperactivity. Elevated temperatures may have facilitated bacterial growth, which could have further influenced vector effects. When PS was first detected in coastal waters, researchers found that the sampled particles had a high abundance of bacteria on their surface [40]. Growth rates of *Escherichia coli*, for example, exhibit a continuous increment from approximately 5 °C to its optimal temperature of around 37 °C [106], suggesting that bacterial growth or the metabolic byproducts thereof may have played a role in shaping the temperature-influenced outcomes.

Avoidance behavior might have been a reason for the high variability in particle uptake. For example, gastric filters of Atlantic ditch shrimp (*Palaemon varians*) can prevent significantly larger microparticles from reaching the midgut gland because they can filter and finally egest them [107]. The extent to which HA can accomplish this remains unclear. Still, some filter mechanisms and avoidance behavior are likely, which could explain individuals with little particle uptake supported by considerable variability in individual feeding rates, which has been described before [108]. Measured uptake quantities after locomotion assays did not increase with temperature. Locomotion assays, which took place in clear water, could have contributed to egestion, which can happen within two hours [79]. The process of removing animals from the exposure beaker, acclimation time, behavior trials, and euthanization spanned approximately 90 min, during which handling and light stimuli potentially heightened levels of activity and clearance.

In the context of behavioral characteristics, particle treatments, and temperature have demonstrated significant effects on various behavioral endpoints. Of the eight investigated behavioral endpoints, six were significantly influenced by particle treatment, four by temperature, and none by light:dark cycle change. Interestingly, exposure to 1000 nm particles elicited only hypoactivity-related locomotion, while exposure to 500 nm particles yielded an intricate interplay of hypo- and hyper-activity. The 500 nm treatments additionally led to 10 more significant behavioral effect differences to corresponding controls than 1000 nm treatments. This result can lead to the conclusion that the mode of toxic action is particle size dependent. Behavioral differences might furthermore be connected to non-significant but lower average survival rates of 500 nm treatments (76.7 ± 8.8%) compared to 1000 nm (83.3 ± 3.3%) treatments at 27 °C (across all temperatures 89 ± 4.2% and 91 ± 2.6%, respectively). In the treatment with the highest mortality (500 nm, 27 °C), TDM and velocity were significantly higher than in the corresponding controls, indicating hyperactivity. Hyperactivity represents an escape response, functioning as a form of avoidance that serves as an adaptive reaction to evade stressful circumstances [109]. In contrast, in 1000 nm treatments at 27 °C, cruising (fraction spent at a speed of >0.5 mm/s and <20 mm/s) and moving time (fraction spent actively swimming) were reduced in darkness, indicating hypoactivity. This result, combined with reduced total length in 1000 nm treatments, corresponds with a reduction in body length (and oxidative stress), standing out as the primary factor contributing to hypoactivity in zebrafish [110]. Links between growth and reduced swimming activity in response to 15 µm PS beads have also been described for jacopever (*Sebastes schlegelii*, [111]). The reasons for particle-size and temperature-dependent locomotion differences can be various. According to several studies [112,113], physical stress is the primary factor contributing to MP toxicity if the plastics do not contain additives or adhere to contaminants on their surface. This stress arises from the additional effort required to digest inert material and maintain physiological homeostasis [114,115]. Another hypothesis proposes that MPs may cause microscale abrasions in the internal tissues of organisms, rendering them more

vulnerable to other contaminants in the aquatic environment [115,116]. Metabolic rates can also be impacted by adverse effects from MPs, arising from their ability to hinder oxygen uptake [89], which can ultimately affect swimming behavior.

The interplays of experimental parameters and measured endpoints, uptake quantity, and particle size showed the strongest correlation. Although the strength of the relationship between locomotion, growth, and uptake was mainly weak, statistically significant *p*-values indicated that the association between the variables was unlikely to be coincidental. Consequently, uptake may have led to TDM, thigmotaxis, and velocity alterations. Given that behavior acts as a pivotal connector between biological scales, bridging subcellular processes measurable in laboratories with ecological reactions to contaminants in natural settings, the interaction of MNPs with temperature has the potential to influence behaviors such as feeding, predator avoidance, and reproductive success. Additional investigations are necessary to explore the indirect consequences of MNPs exposure, especially in the context of climate change and further abiotic factors.

It is important to recognize that our study possesses certain limitations. One notable limitation pertains to the size, shape, and polymer of the particles used, as well as their concentrations. We recognize that the plastics employed may not reflect the diverse array of materials found in natural aquatic systems, where various types and sizes of plastics coexist at varying concentrations. Detecting NPs in the field remains challenging; however, future studies could explore strategies to do so and evaluate their impact on aquatic systems. In order to establish a robust foundation for risk assessment, future experimental methodologies should not only consider the ability to differentiate between the impacts of food scarcity and particle toxicity, but also ascertain whether MNPs elicit effects distinct from those induced by naturally occurring particles [117]. Knowledge gaps underscore the need for further research encompassing a broader spectrum of plastic characteristics and environmental conditions to provide a more comprehensive understanding of the complex interactions between plastics and aquatic ecosystems. It is imperative to conduct additional investigations on the role of the food chain as a conduit for the distribution of plastic debris, particularly MPs, among aquatic organisms spanning from the primary to higher trophic levels [118]. The investigation of excretion and uptake dynamics over time was not within the scope of this study; however, these aspects represent significant knowledge gaps warranting exploration in future research endeavors. Future studies should also investigate the combined effects of MNPs and temperature on oxidative stress. Exposures of HA to polyethylene terephthalate were shown to influence oxidative stress indicators (Superoxide dismutase, Malondialdehyde, and Glutathione S-transferase), enzymes, which are crucial components of the primary antioxidant defense system against reactive oxygen species [119]. Despite these limitations, our study offers valuable insights into the potential impacts of rising temperatures on plastic pollution dynamics and highlights avenues for future investigations to bridge the gap between laboratory experimentation and real-world ecological contexts. Building upon our findings, future investigations should explore the interactive effects of temperature and plastic pollution on broader ecological processes, such as species interactions, nutrient cycling, and ecosystem functioning. Furthermore, the insights gleaned from our study could inform the development of innovative mitigation strategies and policy interventions geared towards curbing plastic pollution and safeguarding vulnerable aquatic ecosystems. Interdisciplinary approaches, and advances in technology and analytical techniques could be used to find solutions for mitigating the impacts of plastic pollution while following sustainable management practices.

## 5. Conclusions

This study illustrates how variations in environmental temperature can modify the effects of micro- and nano-sized PS beads. Our results show that the combination of PS particles and higher temperatures can cause a risk to HA. Elevated temperatures augmented particle uptake in treatments involving 500 nm particles, whereas growth was diminished in treatments with 1000 nm particles. Swimming behavior exhibited disparities across particle

sizes, while survival rates decreased for both particle sizes at 27 °C. However, understanding how plastic particle effects intertwine with abiotic factors like temperature remains limited to this day. Employing model organisms such as HA, prominent amphipods within aquatic food webs can facilitate in assessing these impacts and identifying potential risks of MNPs such as polystyrene. We also aimed to explore the impact of particle uptake on the behavior of HA, presently lacking in the MNPs literature. Our results highlight the significant influence of exposure on behavioral endpoints, thus improving our ability to assess risk through the identification of indicators. This toolkit can aid in determining the risks associated with plastics by employing a USEPA epibenthic model.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16101360/s1, Figure S1. Spectral absorption and emission graph of polystyrene beads. Blue fluorescent beads with a mean diameter of 519 and 1294 nm were ordered from Applied Microspheres GmbH (formerly BS-Partikel GmbH, Mainz, Germany) at a concentration of 5% m/m (Surface: not-modified polystyrol, no surface functionalization, sulfonic acid end group). Table S1. Tested polystyrene concentrations in the range finder study (*). "Conc. 5" was used in the main exposure study. Figure S2. Temperature logger data from each climate chamber set to 21, 24, and 27 °C during the 96 h exposure starting on 10/21. Table S2. Water parameters were measured via YSI during the 96 h exposure of *H. azteca* to polystyrene particles at three temperatures (21 °C, 24 °C, 27 °C). The three temperatures were regulated in climate chambers (Ch1-3). Table S3. Correlation between *H. azteca* behavioral parameters (either TDM or thigmotaxis) and the polystyrene particle treatment (control, 500 nm, 1000 nm), or the temperature (21 °C, 24 °C, 27 °C) in the dark and light period. Spearman correlation matrix: * $p < 0.05$ are marked in red. Figure S3. Uptake across particle (500 nm or 1000 nm sized polystyrene particles) and temperature treatments of pooled *H. azteca* (N = 14–19) by ranking: 1 = minor fluorescence (only stomach and mouth parts), 2 = fluorescence additionally in parts of the guts, 3 = fluorescence in the majority of the gut. Figure S4. Examples of confocal images for quantifying fluorescent 500 nm or 1000 nm polystyrene particle uptake by *H. azteca* at 24 °C: Maximal intensity of z-stack layers. Figure S5. Correlation between PS uptake and endpoints: Spearman correlation (rs values) between *H. azteca* uptake and further endpoints. Strongest correlation: Uptake vs. particle treatment (rs = 0.67; $p < 0.001$). The r values of 0–0.19 are commonly regarded as very weak, 0.2–0.39 as weak, 0.40–0.59 as moderate, 0.6–0.79 as strong and 0.8–1 as very strong.

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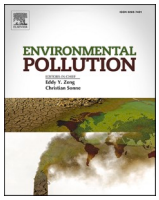

# Interactive effects between water temperature, microparticle compositions, and fiber types on the marine keystone species *Americamysis bahia*☆

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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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(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 ($4.64 \times 10^4$ 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 ($p = 0.81$).

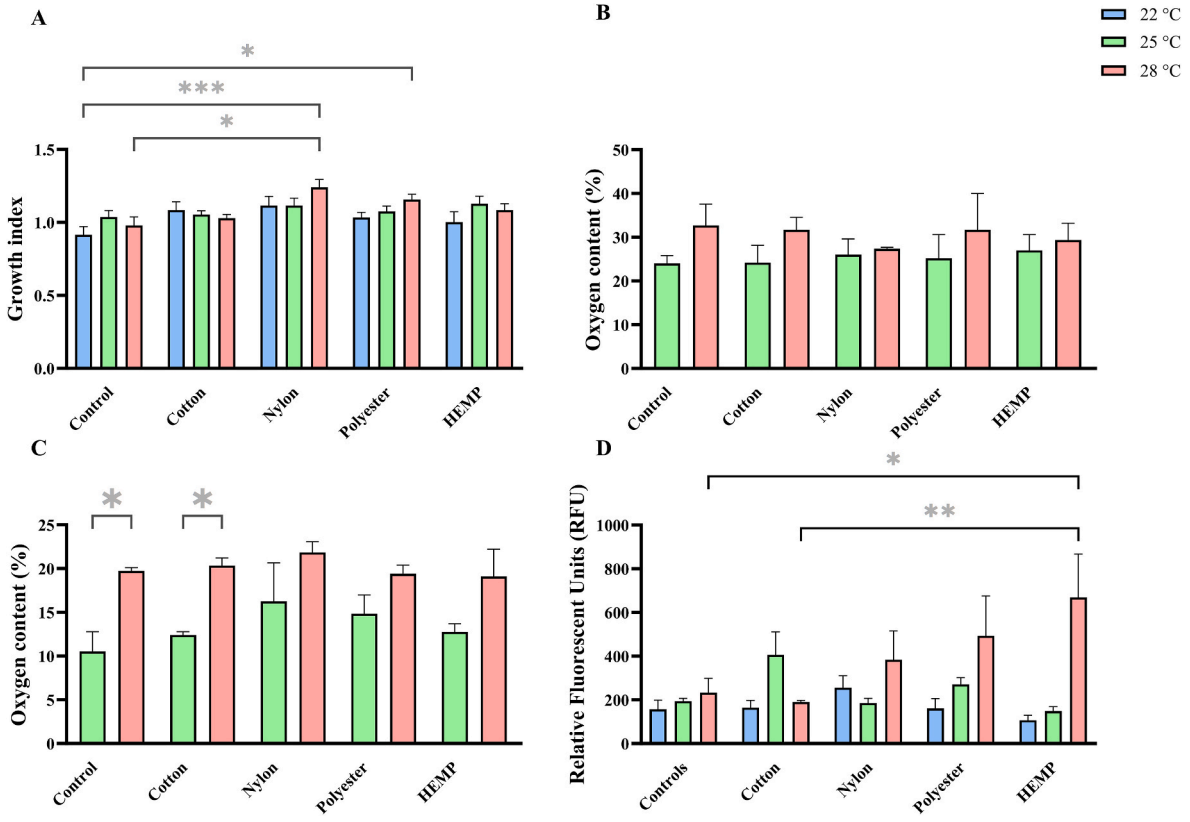


**Fig. 1. Growth index (A), swimming behavior during dissolved oxygen reduction at the onset of bursting behavior (B) and termination of swimming (C), and ROS (D) of *Americamysis bahia*** following a 7-day exposure to fibers (cotton, nylon, polyester, hemp) at three temperatures (22 °C in blue, 25 °C 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

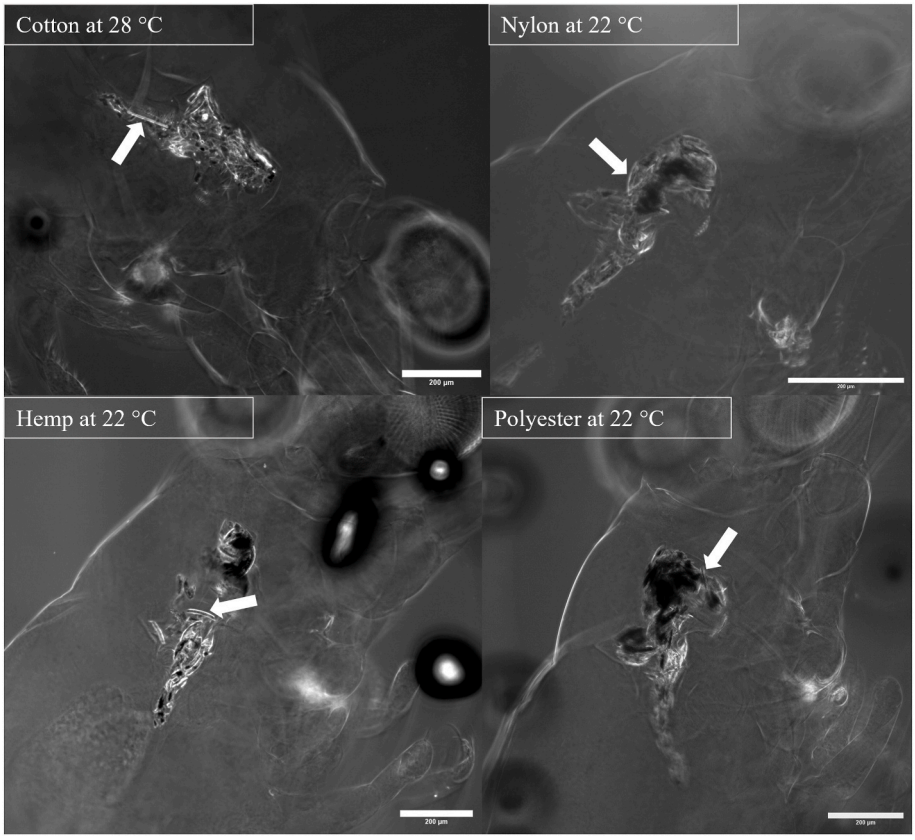


**Fig. 2. Examples of Lightsheet microscopy imaging of the stomach region of *Americamysis bahia*** following a 7-day exposure to fibers (cotton, nylon, polyester, hemp) at different water temperatures. Fibers ingested into the stomach are indicated with an arrow.

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

Adult brood stock was housed and spawned at the Oregon State University Hatfield Marine Science Center under the Animal Care and Use Program (ACUP) protocol #4999.

## 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123906.

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